Probiotics for Human Nutrition in Health and Disease



Edited by
Evandro Leite de Souza
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PROBIOTICS FOR HUMAN NUTRITION IN HEALTH AND DISEASE

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Preface

There has been increasing evidence that probiotic consumption can exert important activity for human health in different age groups linked to the ability of these microorganisms to affect positively the host metabolism, physiology, nutrition, and immune functions. Several probiotic strains have been considered safe and commercially available for human use through delivery by foods, beverages, and supplements. There is encouraging available clinical evidence that distinct probiotic strains are effective in the management and prevention of a variety of metabolic disorders and illnesses in humans, characterizing probiotics as promising innovative therapeutic options for their prevention and treatment. However, current gaps to optimize the use of probiotics for human nutrition and health include the understanding of the mechanisms by which probiotics can affect different physiological functions and optimum dose, frequency, and duration of treatment for distinct probiotic strains. The book "Probiotics for human nutrition in health and diseases" provides a comprehensive resource of information about the traditional and emerging health concepts, development, and application evolution of probiotics and their role in the prevention and treatment of human metabolic disorders and illnesses. Key issues related to "General aspects of probiotics," "Probiotics in human nutrition," and "Probiotics in human health promotion and disease treatment" are described and discussed in three sections comprising 25 chapters constructed regarding the most significant knowledge, mechanistic bases, uses, clinical perceptions, case studies, and perspectives about probiotics for humans considering possibilities and limitations in the light of the appropriate available reference materials. Authors are highly qualified researchers with vast experience in the field who summarized in each chapter a massive quantity of information citing a source from literature, in addition to presenting and interpreting critically the most significant evidence applying their own practical experience from investigations with probiotics. This book is a valuable and comprehensive resource for academics, students, consultants, medical professionals, industry professionals, and other interested in this subject.

SECTION I

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CHAPTER

1

Probiotics: Concepts, evolution, and applications

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1.1 History and concepts

During the past century, the definitions, perceptions, scientific understandings, and practical expectations concerning the concept of probiotics have undergone changes. It is important to review events and varied steps that occurred during the century-long journey from Metchnikoff to the current acceptable concept of probiotics. Before the discovery of microorganisms and the current definition of probiotics, the use of fermented dairy foods already existed. Ancient civilizations, such as Greeks and Romans, developed recipes for fermented milk and the Bible mentions sour milk a few times (Hosono, 1992). During this time, almost every civilization regularly ingested fermented dairy products because of their taste and health benefits. Credited with saying "All disease begins in the gut" the Greek physician Hippocrates considered fermented milk as both a food product and medicine with the potential to cure intestinal disorders. Plinius, the Roman historian, stated that fermented milk could be used in the therapy of various gastrointestinal infections (Suvarna & Boby, 2005).

The researcher Theodor Escherich's (1885) marked a turning point in the understanding of the concept of human gut flora. This researcher was the first to recognize the importance of examining bacteria from feces and intestinal tract of healthy individuals, and, consequently, of understanding the physiology of digestion, as well as the pathology and therapy of intestinal diseases of microbial origin. Theodor Escherich described, for the first time, the existence of *Bacterium coli* commune (posthumously named *Escherichia coli*) in large numbers in the feces of healthy babies (Escherich, 1885). Escherich detailed the bacterial composition of the intestinal tract of infants and the transformation of this flora from birth onwards, the role of these bacteria in food decomposition, and clinical implications of gut microflora (Escherich, 1886).

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After Escherich's discoveries, in 1900 two microbiologists, Tissier and Moro, reported the discovery of a new element for Escherich's studies of babies' feces, which was the "anaerobic culture." Tissier found that most of the intestinal flora of healthy children was not made up of the *Bacterium coli* commune as previously described. Tissier reported that children with diarrhea had in their stools a low number of bacteria characterized by a peculiar Y-shaped morphology, named *Bacillus bifidus communis* (Tissier, 1899). It is now recognized as the genus *Bifidobacterium*, which is one of the most widely used probiotics (Farré-Maduell & Casals-Pascual, 2019). Likewise, Moro (1900) postulated that the isolate, which was named *Bacillus acidophilus* due to its unusual acid tolerance, was derived from mother breast and usually resided in the newborn oral cavity and intestinal contents. Later and Tissier (1907) showed that *Bacillus bifidus* was the predominant microorganism in feces of breastfed babies three days after birth, as opposed to milk-based formula-fed babies, which had *B. acidophilus* as predominant microorganism in their feces (Moro, 1905).

Although there is a reference to sour milk or fermented cultures as far back as the Bible, Elie Metchnikoff is regarded as the grandfather of modern probiotics. Metchnikoff proposed that long-term intestinal disharmony was a major contributor to senility and shortened life span. He reported that regular consumption of lactic acid bacteria in fermented dairy products, such as kefir, was associated with enhanced health and longevity in Bulgarian peasant populations (Anukam & Reid, 2007).

Metchnikoff, in 1907, was the first researcher to propose that drinking sour milk on daily basis ingestion could reduce putrefaction in the intestine through lactic acid production by bacteria, such as *Bulgarian bacillus*. These bacteria were previously isolated by Grigoroff (1905), who cultivated them from "podkvassa" used as a starter for the production of Bulgarian "kiselo mleko" ("sour milk" or "yahourth"). In the process, Grigoroff also identified another organism, *Streptococcus thermophilus*, which received no attention since it was considered a pathogen at that time (Vasiljevic & Shah, 2008).

In addition to lactic acid, Metchnikoff theorized that other bacterial by-products could be account for beneficial effects exerted by lactic acid bacteria (LAB). He considered the role of LAB as a beginning point on the road to intestinal health and suggested that "the dependence of intestinal microbes on food makes it possible to adopt measures to modify the flora in human bodies and to replace the harmful microbes by useful microbes" (Metchnikoff, 1908). To test the hypothesis on the health benefits of consuming LAB, Metchnikoff drank sour milk every day up to his demise at the ripe age of 71 years in 1916 (Anukam & Reid, 2007).

Metchnikoff postulated that "systematic investigations should be made on the relation of intestinal microbes to precocious old age and on the influence of diets which prevent intestinal putrefaction in prolonging life and maintaining the forces of the body" (Metchnikoff, 1907), as well as "there are many useful microbes, amongst which the lactic bacilli have an honorable place" (Metchnikoff, 1907). This proclamation reported more than a century ago is widely accepted as the origin of the "probiotic concept" (Bibel, 1988; Fuller, 1992; Parker, 1974).

Besides the Metchnikoff investigations with *Lactobacillus bulgaricus*, researchers continued to investigate the possible benefits of bacteria to human health. *Lactobacillus acidophilus* strains were isolated and found to be capable of colonizing the human digestive tract where they induced beneficial physiological activities. Rettger and Horton (1914) and Rettger and

Cheplin (1920a; 1920b) reported that feeding of milk or lactose to rats or humans led to a reshape of intestinal microflora resulting in the predominance of acidophilus and bifidus type culture. These findings stimulated commercial interest in products fermented by *L. acidophilus* (Burke, 1938). Other researchers recognized the importance of preventive medicine and modulation of gastrointestinal microflora and succeeded in isolating and culturing a *Lactobacillus* strain capable of surviving the passage through the gastrointestinal tract. The culture identified as *Lactobacillus casei* strain Shirota was successfully used for the production of the fermented dairy product called "Yakult," which initiated the foundation of the same company in 1935 (Yakult, 1998).

The word "probiotics" was initially used as an antonym of "antibiotic." It is derived from Greek words προ and βιοτοσ and translated as "for life" (Hamilton-Miller, Gibson, & Bruck, 2003). The origin of the first use can be traced back to Kollath (1953), who used it to describe organic and inorganic supplements necessary to restore the health of malnourished patients. One year later, the German researcher Ferdinand Vergin (1954) suggested that microbial imbalance in the body caused by antibiotic treatment could have been restored by a proper consumption of probiotics, including fermentation products, and proposed the term *probiotika* to describe "active substances that are essential for a healthy development of life." Similarly, Kolb (1955) recognized the detrimental effects of antibiotic therapy and proposed the prevention by probiotics.

In 1965, the term probiotics was used by Lilly and Stillwell in a different context to represent growth-promoting factors produced by microorganisms in which substances secreted by one organism could stimulate the growth of another (Lilly & Stillwell, 1965), which was contrasted with term "*antibiotic*."

Possibly, due to this general definition, the term probiotic was subsequently applied to other subjects and gained a more general meaning. Fujii and Cook (1973) described it as "compounds that build resistance to infection in the host but do not inhibit the growth of microorganisms *in vitro*," referring to synthetic chemicals that protected mice against infection with *Staphylococcus aureus*.

Different definitions were proposed up to 1974 with Parker, who was the first to use the term *probiotic* to describe the interactions of microorganisms with the host. He described probiotics as "organisms and substances which contribute to intestinal microbial balance" (Parker, 1974). However, this definition included the term "substances" and gave it a wide connotation in which would include antibiotics. In an attempt to improve Parker's definition, Fuller (1989) deleted "other substances" from the definition and redefined probiotics as "live microbial supplement which beneficially affects the host animal by improving its intestinal microbial balance" (Fuller, 1989). This revised definition emphasized the requirement of viability for probiotics and introduced the aspect of a beneficial effect on the host (Schrezenmeir & Vrese, 2001). However, according to this definition, probiotics are restricted to feed supplements, animals, and the intestinal tract, so the term probiotic could not be used for live microorganisms administered in any other way than in food or feed, for application in humans, or for locations other than the gut (Havenaar & Huis in't Veld, 1992).

In 1992, Havenaar and Huis In't Veld extended the definition of probiotics for "a viable mono- or mixed culture of microorganisms which applied to animals or humans beneficially affects the host by improving the properties of indigenous microflora." This proposed definition implies that the term probiotic is restricted to products which (1) contain live

microorganisms, for example, as freeze-dried cells or in a fermented product; (2) improve the health status of humans or animals; and (3) can have their effects in the mouth or gastrointestinal tract (e.g., applied in food or capsules), upper respiratory tract (aerosol) or urogenital tract (local application) (Havenaar & Huis in't Veld, 1992).

Havenaar and Huis in't Veld (1992) proposed that: "If we want to get rid of the mysticism surrounding probiotics, fundamental research is necessary to collect information on how probiotics act." This involves the development of adequate methods to quantify, localize and identify the changes in the intestinal microflora to establish basic criteria for the selection of bacterial strains, and to perform well-controlled animal experiments, field trials, and studies in humans. In this context, the most important characteristics of probiotics are survival under the environmental conditions on the location where it must be active; proliferation and/or colonization on the site where it is active; no immune reaction against the probiotic strain; no pathogenic, toxic, allergic, mutagenic, or carcinogenic activities by the probiotic strain, genetically stable, no plasmid transfer, easy and reproducible production, and viable during processing and storage (Havenaar & Huis in't Veld, 1992).

Guarner and Schaafsma (1998) and Salminen et al. (1998) broadened the probiotic definition limiting the proposed health effects to influence the indigenous microflora. According to Salminen, a probiotic is "a live microbial culture or cultured dairy product (foods or dietary supplements) which beneficially influences the health and nutrition of host." According to Guarner and Schaafsma, "oral probiotics are living microorganisms which upon ingestion in certain numbers, exert health benefits beyond inherent basic nutrition." It is important to highlight that this definition restricted probiotics to products containing live microorganisms and pointed out the need for providing an adequate dose of probiotic bacteria in order to exert the desirable effects (FAO/WHO, 2002). Although all cited authors agreed that probiotics include live microorganisms, Salminen, Ouwehand, Benno, and Lee (1999) approached in their concept that probiotics do not necessarily need to be viable and proposed that "probiotics are microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being of the host".

In 2001, an Expert Consultation of international scientists of the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) established a consensus on the definition of probiotics to the following: "Live microorganisms which when administered in adequate amounts confer a health benefit on the host." This definition has become the most widely adopted and accepted concept worldwide. The Expert Consultation was held in a period that marked the rise of interest in probiotics for scientific communities. The clinical and scientific evidence have progressed quickly, as the development of a number of robust probiotic products had occurred (Hill et al., 2014). The FAO also developed the "Guidelines for the Evaluation of Probiotics in Food" in order to identify and define what data need to be available to accurately substantiate health claims (see more detail in Section II).

After the FAO statements, the new perspectives about host-microbe interactions prompted efforts to redefine the term probiotics and to promote a better understanding of their specific metabolic and immunological functions as microbiome components (Dobrogosz, Peacock, & Hassan, 2010). Clancy (2003) proposed that "the term "probiotic" had served the useful generic function of drawing attention to health-promoting bacteria at mucosal surfaces" but to continue to use "the term probiotic for those bacteria, which promote health by

immunomodulatory means, would appear outmoded, and potentially confusing when communicating about immune-regulating bacterial species." He suggested the term immunobiotics identifies "those bacteria that promote health through activation of the mucosal immune apparatus" and that "recognition of bacteria that promote mucosal T cell function as immunobiotics moves probiotic biology forward by focusing on a mechanism of the outcome, that is, immunomodulation at distant mucosal sites. It does not, however, diminish the need to attend longstanding concerns regarding the biology and accreditation of these bacteria as health-enhancing agents." O'Hara and Shanahan (2006, 2007) recommended the more generic term pharmabiotics "to encompass any form of therapeutic exploitation of the commensal flora, including the use of live probiotic bacteria, probiotic-derived biologically active metabolites, prebiotics, symbiotic, or genetically modified commensal bacteria."

Researches on probiotics have progressed considerably in parallel to the increasing development of probiotic products. Unfortunately, the use of the term probiotic has also become a major issue with many products exploiting the term without meeting the requisite criteria (Hill et al., 2014). Because of that, in 2014, the International Scientific Association for Probiotics and Prebiotics (ISAPP) refined the probiotic definition with a minor grammatical correction to "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002; Hill et al., 2014). Hill et al. (2014) proposed also that probiotics must have "defined contents, appropriate viable count at end of shelf life and suitable evidence for health benefits" and further stated that all probiotics must be "safe for their intended use." A summary of the evolution of the probiotic study is shown in Fig. 1.1.

1.2 Safety aspects, physiological, and technological characteristics

The advance in studies and standardization of the definitions of probiotics also led to the reach of the criteria for the selection of new probiotic strains. Prior to 2002, there was no international regulation to affirm the efficacy and safety of probiotic strains. Thus, the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) published the "Guidelines for Evaluation of Probiotics in Food", which established safety and effectiveness standards for probiotics. The development of these guidelines was intended to streamline the evaluation process and ensure not only the safety of probiotics but also the authenticity of the health benefit claims associated with each microorganism. These reports provided scientific advice concerning the safety assessment of probiotics, general guidance for their evaluation, and specific questions in relation to their pathogenicity, toxigenicity, and allergenicity, as well as for their functional and nutritional properties (FAO/WHO, 2002).

For use in foods, probiotic strains should not only be capable of surviving the passage through the digestive tract but also have the capability of proliferating in the gut. It means these organisms must be resistant to gastric juices and be able to grow in presence of bile under intestinal conditions or be consumed in a food vehicle that allows them to survive the passage through the stomach and exposure to bile (FAO/WHO, 2002). Probiotics must be able to exert their health benefits for the host through growth and/or activity in the human body (Collins, Thornton, & Sullivan, 1998; Morelli, 2000). However, the specificity of the action and not the source of the microorganism are important. The use of probiotics for humans must indicate the dosage regimen and use duration according to the recommendation of the

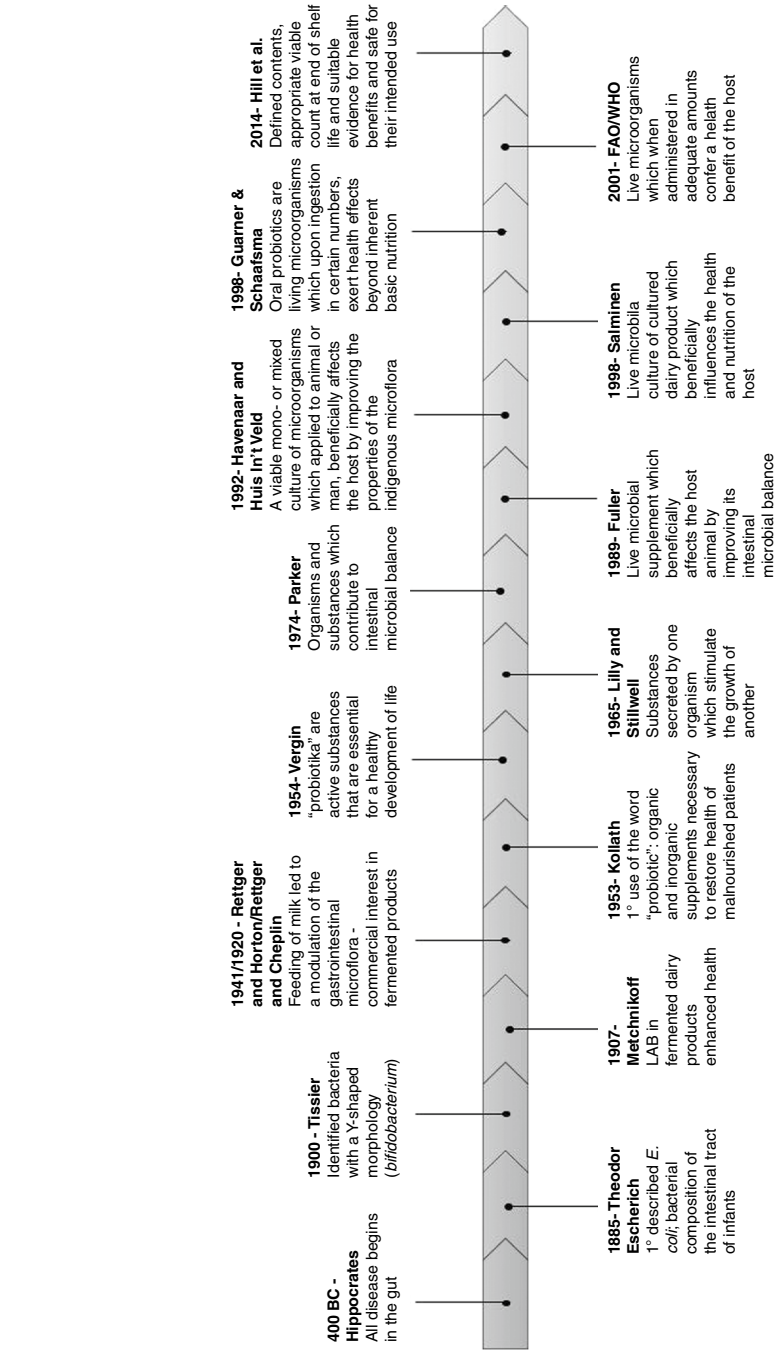


FIG. 1.1 **Evolution of the probiotic study.**

manufacturer of each individual strain or product based on strong scientific evidence and should be regulated and approved in the country of sale. The Consultation strongly recommended that each product should indicate the minimum daily amount required to confer specific health benefit(s). Such evidence should result from *in vitro* and *in vivo* animal (where appropriate) and human studies (FAO/WHO, 2002).

Regarding the classification and identification of individual strains, the Consultation recommended that probiotics must be named according to the International Code of Nomenclature to ensure understanding on an international basis and probiotic strains need to be deposited in an internationally recognized culture collection for full disclosure. Since probiotic properties are strain-related, it is suggested that strain identification (genetic typing) should be done with well-validated methodologies, such as pulse-field gel electrophoresis. It is recommended that phenotypic tests should be done first, followed by genetic identification using DNA/DNA hybridization, 16S RNA sequencing, or other internationally recognized methods. RDP (ribosomal database project) should be used to confirm identity (www.cme.msu.edu/RDP/) (FAO/WHO, 2002).

In terms of the safety of probiotics, the Consultation believes that a set of general principles and practical criteria need to be generated to provide guidelines driving how any given potential probiotic microorganism can be tested and proven to have a low risk of inducing or being associated with etiology of disease, besides conferring a significant health benefit when administered for humans. These guidelines should recognize that some species may require more rigorous assessment than other. In this respect, the evaluation of safety must require at least some studies to be performed in humans, besides addressing aspects of the proposed end use of the probiotic strain. Most of the LAB, such as lactobacilli, lactococci, and bifidobacteria, are historically considered to be safe (Pradhan, Mallappa, & Grover, 2020). However, the Consultation emphasizes that plasmids exist in lactobacilli and bifidobacteria, especially in strains isolated from the intestine, which have genes encoding antibiotic resistance. It implies that bacteria with transmissible drug resistance genes should not be used in foods (FAO/WHO, 2002).

In recognition of the importance of assuring the safety, even among a group of bacteria Generally Recognized as Safe (GRAS), the FAO has recommended that probiotic strains must be characterized minimally with the following tests: 1. Determination of antibiotic resistance pattern; 2. Assessment of certain metabolic activities (e.g., D-lactate production and bile salt deconjugation); 3. Assessment of side-effects during human studies; 4. Epidemiological surveillance of adverse incidents in consumers (post-market); 5. If the strain under evaluation belongs to a species known as mammalian toxin producer, it must be tested for toxin production. One possible scheme for testing toxin production has been recommended by the EU Scientific Committee on Animal Nutrition (SCAN, 2000); and 6. If the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required. Assessment of lack of infectivity by a probiotic strain in immunocompromised animals would add a measure of confidence on the safety of the probiotic.

Significant emphasis has been placed on the investigation of the safety of novel species and genera with potential probiotic aptitudes. Many commonly exploited and commercially available probiotic strains benefit from a generally recognized as safe (GRAS) status in the USA or belong to species with qualified presumption of safety (QPS) status with the European Food Safety Authority (EFSA), although it is not yet the case for other candidate novel

probiotic species with no previous history for use to this end. Submission through GRAS, QPS, and novel food frameworks should enable a path for commercialization and pharmaceutical applications. However, novel regulatory frameworks have been emerging, such as the live biotherapeutic products category defined by Food and Drug Administration (FDA) and European Directorate for Quality of Medicines (EFSA, 2020; Cunningham et al., 2021).

The QPS system of EFSA involves four steps for safety assessment of microorganisms: (1) definition of the taxonomy of the microbe; (2) collection of sufficient information providing the basis for QPS status, including any scientific literature, history of use, industrial applications, ecological data, and human intervention data; (3) exclusion of pathogenicity; and (4) definition of the end-use. If there are no safety concerns for a certain taxonomic group or if any safety concerns have been alleviated (qualification), the QPS status may be granted. Subsequently, a microbial strain categorically assigned to a QPS group would be excused from further safety assessment, other than satisfying any specified qualifications. Microbes not considered suitable for QPS would remain subject to a full safety assessment. Members of LAB, which include some species of probiotic bacteria, will be among the first groups to be evaluated. The introduction of this system appears to be favorably received and considered more flexible than GRAS system used in the United States taking into account new emerging safety risks, such as the acquisition of antibiotic resistance and virulence determinants (Chesson et al., 2002; Wassenaar & Klein, 2008).

A European Union-funded project "Biosafety Evaluation of Probiotic Lactic Acid Bacteria for Human Consumption" (PROSAFE) had the objective to propose recommendations towards an evidence-based safety assessment of probiotics for human use. In 2006, the PROSAFE (Vankerckhoven et al., 2007; Vankerckhoven et al., 2008) presented the following recommendations: (1) Molecular methods, such as ribosomal DNA sequencing, conducted by an expert laboratory, should be used to taxonomically classify LAB (Huys et al., 2005), and the probiotic strain should be deposited in a public culture collection, although with restricted access; (2) Microbes not belonging to the wild-type distributions of resistance levels to relevant antimicrobials should not be developed as future products for consumption by humans or animals in the food chain without proper risk assessment (Klare et al., 2007); and (3) Use of *Enterococcus* probiotics or other LAB harboring known and confirmed virulence genes should be avoided. The presence of other properties including bile acid deconjugation, adhesion to cell lines, and extracellular matrix proteins were insufficiently associated with virulence and along with the survival of probiotics in *in vitro* colon models, were considered irrelevant for biosafety assessment; (4) In addition to the European guidelines for *in vivo* safety assessment, human colonization should be studied in a randomized, placebo-controlled, double-blind design but details of such a study were not specified. (5) The potential use of animal models to measure safety was very controversial. However, the rat model of experimental endocarditis was suggested as the most reliable animal model (Moreillon & Que, 2004; Vankerckhoven et al., 2007). As one of the important conclusions from the PROSAFE project, the finding of acquired resistance genes in isolates intended for probiotic or nutritional use highlights the importance of antimicrobial susceptibility testing in documenting the safety of commercial LAB (Sanders et al., 2010).

In addition to security tests, *in vitro* tests are useful to gain knowledge of strains and the mechanism of probiotic effect. However, available tests could not fully adequate to predict the functionality of probiotic microorganisms in the human body. It was also noted that

in vitro data available for particular strains are not sufficient for describing them as probiotic. The use of probiotics for humans requires substantiation of efficacy with human trials. Appropriate target-specific *in vitro* tests that correlate with *in vivo* results are recommended. The main *in vitro* tests for the study of probiotic strains have been: resistance to gastric acidity, bile acid resistance, adherence to mucus and/or human epithelial cells and cell lines, antimicrobial activity against potentially pathogenic bacteria, ability to reduce pathogen adhesion to surfaces, bile salt hydrolase activity, and resistance to spermicides (applicable to probiotics for vaginal use) (FAO/WHO, 2002).

The FAO Guidelines have also recommended *in vivo* studies using animals and humans. In some cases, animal models exist for the validation of *in vitro* effects and the determination of probiotic mechanisms. Where appropriate, the FAO Working Group encouraged the use of these assays prior to human trials. The principal outcome of efficacy studies on probiotics should be proven benefits in human trials, such as statistically and biologically significant improvement in condition, symptoms, signs, well-being or quality of life, reduced risk of disease or longer time to next occurrence, or faster recovery from illness. Each should have a proven correlation with the probiotic tested. Standard methods for clinical evaluations are comprised of phase 1 (safety), phase 2 (efficacy), phase 3 (effectiveness), and phase 4 (surveillance). Phase 1 studies focused on safety, and phase 2 studies, generally in the form of randomized, double-blind, placebo-controlled design, measure the efficacy compared with placebo. In addition, phase 2 studies measure the adverse effects. No adverse effects related to probiotic administration should be experienced when food is considered. Adverse effects should be monitored and incidents reported (FAO/WHO, 2002).

The selection of probiotic microorganisms requires a systematic approach in most cases, the large number of isolated strains leads to the need to use a "step-by-step approach," consisting of a sequence of tests to progressively reduce the number of probiotic candidates. At the end of this procedure, the strains with the highest number of functional properties and, concomitantly, without any negative traits, must be selected (Pereira, Coelho, Júnior, Thomaz-Soccol, & Soccol, 2018). Besides the FAO/WHO Guidelines, alternatives requirements could be used for screening selection of probiotics (Fig. 1.2).

1.2.1 Microorganisms and derived products used as probiotics

Traditionally, *Lactobacillus*, *Bifidobacterium*, and yeasts (e.g., *Saccharomyces*) genera are among the best-known probiotics (Pradhan et al., 2020). The genus *Lactobacillus* comprising more than 100 different species, belonging to the group of lactic acid-producing bacteria (LAB). This genus is dominant in animal and human gastrointestinal and digestive systems, possessing proven action in the maintenance and recovery of health and recognized species. *Lactobacillus* has been also applied in several industrial processes as those used to produce preservatives, acidulants, drugs, and cosmetics. These bacteria were the earliest discovered probiotics and due to a long history of safe use, they have GRAS status (O'sullivan, Thornton, O'sullivan, & Collins, 1992; Hammes & Hertel, 2006). Other LAB genera with proven probiotic aptitudes include *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, and *Leuconostoc* (Pradhan et al., 2020).

Up to 2020, the genus *Lactobacillus* had 261 species differing greatly from each other in genotypic, phenotypic, and ecological aspects. Therefore, to keep grouped species with more

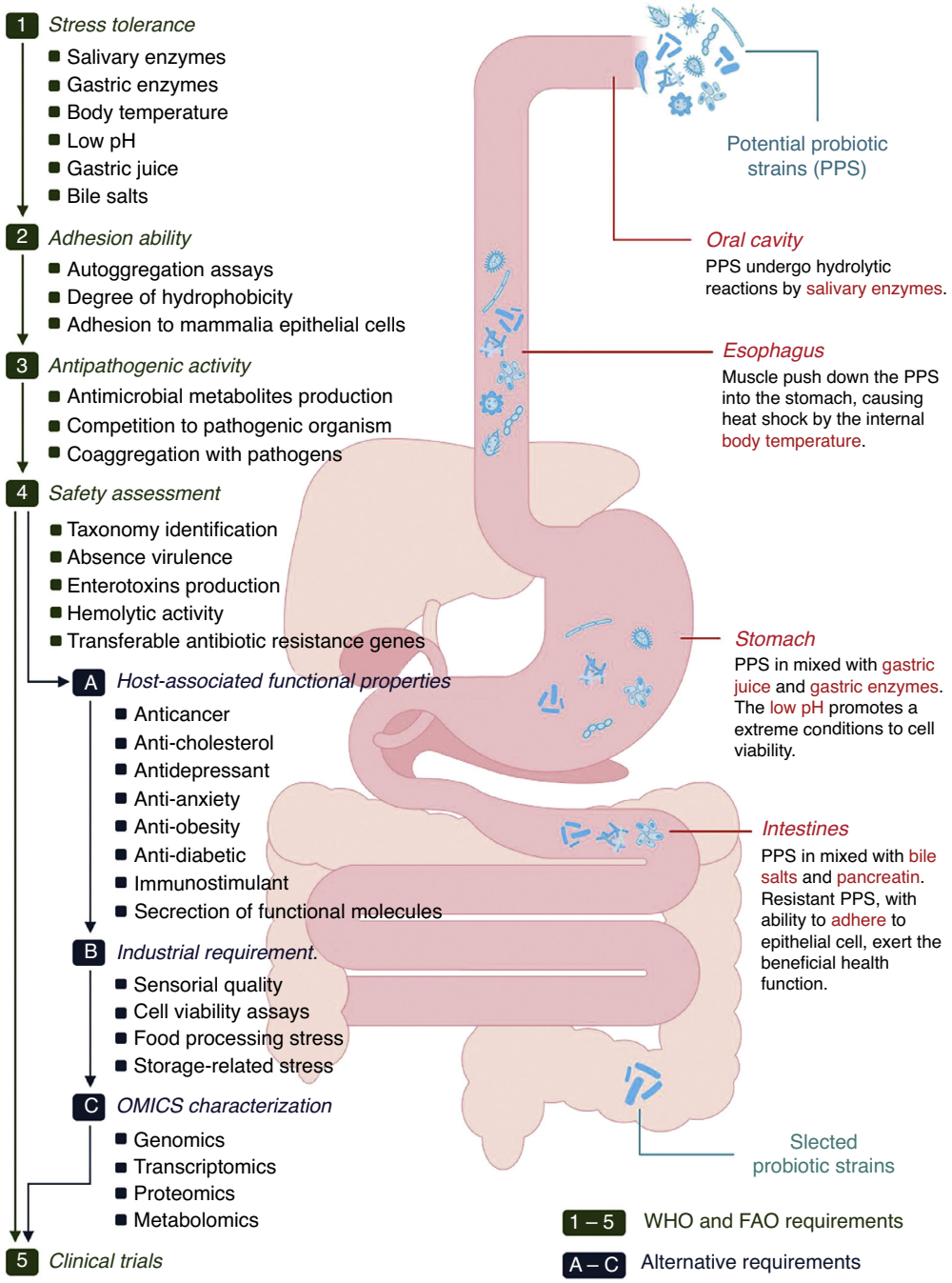


FIG. 1.2 Screening approaches used for characterization of probiotic strains according to WHO/FAO and alternative requirements (Pereira et al., 2018).

affinity with each other, Zheng et al. (2020) proposed a new taxonomic organization of these species. The proposed reclassification of the genus *Lactobacillus* into 25 genera including the emended genus *Lactobacillus*, which comprises host-adapted organisms that have been referred to as the *Lactobacillus delbrueckii* group, *Paralactobacillus* and 23 novel genera for which the nomenclature *Lacticaseibacillus*, *Latilactobacillus*, *Furfurilactobacillus*, *Limosilactobacillus*, *Fructilactobacillus*, *Acetilactobacillus*, *Lentilactobacillus* and other are proposed. It was also proposed to amend the description of the family *Lactobacillaceae* to include all genera previously encompassed in families *Lactobacillaceae* and *Leuconostocaceae*. The generic term 'lactobacilli' will remain useful to designate all organisms classified as *Lactobacillaceae* up to 2020. Previously designated *Lactobacillus* species will be reassigned to the new species and both the old and new names will be retained in QPS list (EFSA, 2020).

Bacillus species (e.g., *B. clausii* and *B. subtilis*) are attractive for use as probiotics in food and pharmaceutical industries due to their ability to produce spores. Their heat stability and ability to survive under the low pH of the gastric barrier should be an advantage over other probiotic microorganisms (Liu, Wu, Chu, & Wu, 2018; Mingmongkolchai & Panbangred, 2018). The main feature of this Gram-positive aerobic bacteria is the formation of endospores (Cutting, 2011) allowing viability maintenance for long periods. The use of viable spores of *Bacillus* as a probiotic supplement raised a number of questions, including their safety. The knowledge gained from their use, as an animal feed supplement, suggests that, for some species, their safety could be assured by QPS approach (EFSA, 2007). Bifidobacteria have been considered promising probiotics (Azad, Kalam, Sarker, Li, & Yin, 2018). The presence of these bacteria in high numbers in the intestinal tract of animals and humans has been associated with the good health status of the host. Bifidobacteria have been frequently used in food and pharmaceutical preparations and their application in feeding has been increasing. Due to the long history of safe use, many *Bifidobacterium* species are proposed for QPS status (Gaggìa, Mattarelli, & Biavati, 2010).

Despite most probiotic microorganisms in human microbiota belong to the group of LAB, some yeast strains existing in dairy and fermented products have been classified as probiotics (Burns & Rowland, 2000). Efforts have been made to find yeast strains with probiotic aptitudes because of the high content of various immune-stimulant compounds, such as proteases, β-glucans and mannan oligosaccharides (Arévalo-Villena, Fernandez-Pacheco, Castillo, Bevilacqua, & Pérez, 2018; Fadda, Mossa, Deplano, Pisano, & Cosentino, 2017; García-Hernández et al., 2012; Gil-Rodríguez, Carrascosa, & Requena, 2015). Yeasts also have the advantages of nonsusceptibility to some antibiotics and good tolerance for industrial processing conditions (e.g., lyophilization and high temperatures). Yeasts are also part of the residual microbial system of intestinal microbiota (Abdel-Rahman, Tashiro, & Sonomoto, 2013; Fleet, 2011; Joshi & Thorat, 2011; Morgunov, Kamzolova, & Lunina, 2013).

Saccharomyces cerevisiae has been extensively studied and used in industries due to their safe status and technological applications, such as in bakery and brewery industries. *S. boulardii* is a widely available probiotic yeast (McFarland, 2010; Shamekhi, Lotfi, Abdolalizadeh, Bonabi, & Zarghami, 2020). The use of *S. boulardii* as a therapeutic probiotic is supported by its mechanisms of action, pharmacokinetics, and efficacy from animal models and clinical studies. *S. boulardii*, according to The European Food Safety Authority (EFSA), has a QPS status, being the yeast most used and studied as a probiotic (Hudson et al., 2016).

There has been an expanding need for new probiotic strains effective in conferring a health benefit on humans and animals. Such microorganisms can be acquired from different natural ecologic niches still few exploited. According to WHO/FAO, the isolation of new probiotics bacterial strains is carried out from disparate natural sources, such as soil, plants, animals, gastrointestinal tract, and dairy products (Alard et al., 2018; Larsen et al., 2014; Sandes et al., 2017; Verso, Lessard, Talbot, Fernandez, & Fliss, 2018). It should be noted that the isolation of complex microflora from traditional dairy products, as a primary probiotic source, is a challenging issue, including a time-consuming process and its need to pass for some fermentation steps (Khangah & Ganbarov, 2014).

The *Lactobacillus* strains traditionally used as probiotics are often of human or animal origin because these strains should be better adapted to conditions found in the human gastrointestinal tract (Monteagudo-Mera et al., 2012; Toscano, De Grandi, Miniello, Mattina, & Drago, 2017). It has been shown that origin source of a probiotic strain is one of the important factors to be considered. Microbial species found in intestinal microflora may have a better performance to survive in their native environment and tolerate harsh gastrointestinal conditions (Vasiljevic & Shah, 2008). It is important to note that probiotic strains from food origin or gut have distinct tolerance to external stresses. For instance, the probiotic strains isolated from foods generally have a higher tolerance to changes of temperature and pH during food processing, but a lower survival rate passing through the gastrointestinal tract compared with probiotic strains isolated from the gut (Klein, Pack, Bonaparte, & Reuter, 1998).

Although a reasonable number of probiotic strains is already well-characterized, these microorganisms have been isolated from a variety of environments in order to search for new strains with probiotic potential (Kumar & Kumar, 2015; Shehata, El Sohaimy, El-Sahn, & Youssef, 2016). Thus, in recent years, potential probiotics have been isolated from raw camel milk (Edalati et al., 2019), healthy equines (Pei et al., 2021), Lebanese Baladi goat milk (Saliba et al., 2021), fruit processing by-products (de Albuquerque et al., 2018; Garcia et al., 2016), sausages (Corbo et al., 2017), and cactus fruit (Verón, Di Risio, Isla, & Torres, 2017).

As the knowledge of the breadth of the human microbiome and its functions have been expanded, the future holds a range of potential new discovery approaches, as well as new potential probiotic taxa (Cunningham et al., 2021). Developments in affordable complete genome sequencing and powerful cultivation methods have allowed the isolation and characterization of a new range of microorganisms from human microbiomes with potential health benefits and opportunities to be developed as next-generation probiotics (NGP) (O'Toole, Marchesi, & Hill, 2017). For the moment, the term NGP can serve as a useful descriptor for these "non-traditional" microorganisms. NGP also fit well within the US Food and Drug Administration (FDA) definition of a live therapeutic product: "a biological product that: (1) contains live organisms, such as bacteria; (2) is applicable to the prevention, treatment, or cure of a disease, or condition of human beings; and (3) is not a vaccine" (O'Toole et al., 2017).

The NGP or Live Therapeutic Products can represent microbial genera and species that have never before been used in the food industry. Currently, candidates have been searched amongst the gut bacteria that are associated with health, including strains from genera *Bacteroides, Clostridium, Faecalibacterium*, and *Akkermansia*, as well as genetically modified strains (typically lactococci carrying a new health benefiting trait) (Foligne, Daniel, & Pot, 2013;

O'Toole et al., 2017; Gómez-Gallego, Pohl, Salminen, De Vos, & Kneifel, 2016). Although the interest in broadening the spectrum of probiotic bacteria is welcome, it also sets new challenges for industry and researchers. The main questions with NGP relate to their efficacy, safety and technological robustness. Most gut bacteria considered as potential NGP are nutritionally fussy and very sensitive to oxygen. These organisms could be difficult to produce economically on a large scale and maintain alive during processing and potential product formulation (Foligne et al., 2013; O'Toole et al., 2017; Khan, van Dijl, & Harmsen, 2014).

Adjacent to probiotics, it has been developed derivatives as postbiotics, also known as either metabiotics, biogenics, or simply metabolites/cell-free supernatants, which refer to soluble factors (products or metabolic by-products) secreted by live bacteria or released after bacterial lysis. These by-products offer physiological benefits to the host by providing additional bioactivities (Cicenia et al., 2014; Konstantinov, Kuipers, & Peppelenbosch, 2013; Tsilingiri & Rescigno, 2013). Another concept that has been studied is paraprobiotic, also known as "non-viable probiotics," "inactivated probiotics" or "ghost probiotics", referring to inactivated (non-viable) microbial cells, which, when administered in sufficient amounts, confer benefits to consumers (Taverniti & Guglielmetti, 2011; Tsilingiri & Rescigno, 2013). Results from *in vitro* and *in vivo* studies have shown that some postbiotics and paraprobiotics have several bioactivities, such as anti-inflammatory, immunomodulatory, anti-proliferative, antioxidant, and antimicrobial. These bioactivities could be involved in health-promoting effects reported in clinical trials, but despite the scientific evidence available, the mechanisms of action and signaling pathways involved have not yet been fully elucidated. Paraprobiotics and postbiotics definitely have the potential for the development of biotechnological products with functional ingredients for the nutraceutical industry. Important safety and regulatory aspects still need to be addressed for these bioengineered paraprobiotics and postbiotics (Cuevas-González, Liceaga, & Aguilar-Toalá, 2020).

1.3 Evolution of probiotic applications in foods

The global probiotic market has attracted the attention of the food industry to produce new products containing probiotics and researchers to study specific characteristics of probiotics, and their functionality on human health (Behera & Panda, 2020; Tangyu, Muller, Bolten, & Wittmann, 2019). The market of probiotics has been estimated at USD 69.3 billion by 2023 (https://www.marketsandmarkets.com/PressReleases/probiotics.asp). Probiotics can be consumed in the form of nutraceuticals, added to foods as part of a fermentation process, or by supplementation (Aspri, Papademas, & Tsaltas, 2020; Behera & Panda, 2020; Tangyu et al., 2019). Probiotics are often sold as dietary supplements, where the microorganisms can be dried and packaged into pills, capsules, tablets, or liquids (FAO/WHO, 2002).

For decades, most of the current probiotic food market focused on dairy-based products (yogurt and other fermented products); however, the increase in lactose intolerant and/or hypercholesterolemic individuals have demanded changes in this scenario (Nguyen et al., 2019). An increasing number of non-dairy food matrices (e.g., fruit, vegetables, cereals, meat products, and soy) have been suggested as potential probiotic vehicles at high viable counts, being a promising alternative to provide benefits without milk proteins and lactose (Min, Bunt, Mason, & Hussain, 2019; Pimentel, da Costa, Barão, Rosset, & Magnani, 2021).

Vegetables are rich in phytochemicals and phytonutrients, and, because of the absence of lactose, these probiotic products are well suited for lactose intolerant individuals. Vegetable cabbage, carrot, tomato, beet, onion, ginger, and peanuts have been used to formulate probiotic products. The LAB, such as *L. acidophilus*, *L. plantarum*, *L. casei*, and *B. longum*, have been used for vegetable-based probiotic foods (Panghal et al., 2018). Cereals and legumes have been used in probiotic food products prepared with sorghum, millet flour, maize, millet malt, wheat flour, finger millet, soya beans, rice, and oat. Fruit, vegetables, cereals, and legume products are promising carriers for probiotics with good nutraceutical components. However, there are some limitations that could preclude non-dairy probiotics production at the industrial level, such as sensory characteristics, overall acceptance, and, most importantly, the probiotic survival during storage (Panghal et al., 2018; Perricone, Bevilacqua, Altieri, Sinigaglia, & Corbo, 2015).

Probiotics can play an important role in fermentation processes, as they typically provide greater viability for probiotic cultures (Min et al., 2019). Traditionally, probiotics have been added to yogurts, with a high number of products containing LAB available in the market worldwide, including sour cream, buttermilk, frozen desserts, and probiotic drinks (Kerry et al., 2018). In this context, lactobacilli or bifidobacteria are the most used probiotic cultures in yogurt and fermented milk (Smith, Rigassio-Radler, Denmark, Haley, & Touger-Decker, 2013).

Factors associated with technological and sensory aspects of probiotic food production are of importance since only by satisfying the demands of consumers can the food industry succeed in promoting the consumption of functional probiotic products (Saarela, Mogensen, Fondén, Mättö, & Mattila-Sandholm, 2000). The incorporation of probiotic microorganisms into dairy products can induce unique flavor profiles and textures. Therefore, the use of probiotic bacteria individually or in combination also needs to be carefully considered since the amounts and types of metabolites after fermentation could be variable and affect the final product quality (Champagne, Tompkins, Buckley, & Green-Johnson, 2010). Some studies have reported that the addition of probiotic cultures did not influence the sensory acceptance of formulated products (Malik, Bora, & Sharma, 2019; Mantzourani et al., 2018; Miranda et al., 2020; Vivek, Mishra, Pradhan, & Jayabalan, 2019).

The use of a specific microorganism in a specific product is based on its probiotic and health-promoting properties. The therapeutic value of any probiotic food typically depends on the viability of these bacteria in the matrix (Sharma & Mishra, 2013). There is no agreed rule by American or European Agencies about the number of probiotics to be viable in a functional food product to reach their probiotic action and to confer generic health claims; however, the probiotic intake of around 10^6–10^8 CFU/g (or mL) or 10^8–10^{10} CFU/day (CFU, colony-forming units) have shown to be efficacious (Champagne, Ross, Saarela, Hansen, & Charalampopoulos, 2011).

The utilization of probiotic bacteria in food has many challenges related to their growth, survival, viability, stability, and functionality in food processing, storage, and consumption, as well as changes in sensory characteristics of probiotic foods (Min et al., 2019) (Fig. 1.3). The environment within or adjacent to probiotics in a food matrix exerts a significant factor in this process. Living cells may adapt to suit a new environment that may be created during food production. For example, the release of citric acid from pomegranate juice promote the proliferation of *L. acidophilus* and *L. paracasei* (Mousavi, Mousavi, Razavi, Emam-Djomeh & Kiani, 2011).

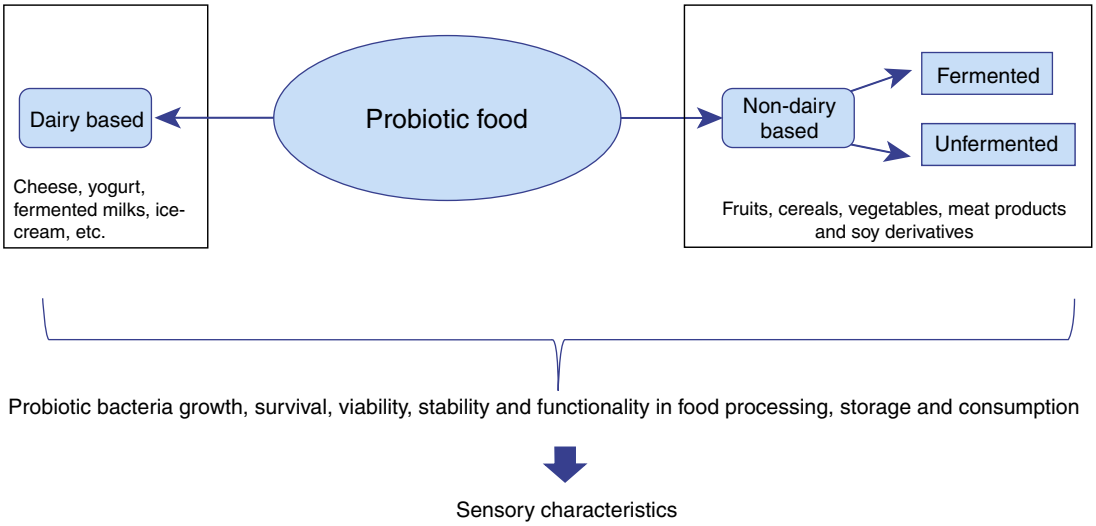


FIG. 1.3 Applications of probiotics in foods.

Exposure to stresses during processing, as well as other factors, such as culture preparation and preservation, food matrices, dietary habits, host age, and health, can also affect the performance of probiotics (Marco & Tachon, 2013). Importantly, strain-dependent properties of bacteria can raise the complexity of research into the interactions between probiotics and foods. The metabolic capabilities of probiotic microorganisms, such as *Bifidobacterium* spp. that utilize a wide range of mono-, di-, and oligosaccharides via "bifidus pathway", may influence the probiotic performance (Kun, Rezessy-Szabó, Nguyen, & Hoschke, 2008). Different nutritional needs for probiotics survival may partly explain the different consumption rates of some media substrates, such as different sugars between different probiotic bacteria (Crittenden et al., 2002).

The efficiency and development of probiotic products can be improved by manipulating the food delivery matrix, including the synergy among phytochemicals and probiotic strains (Boricha, Shekh, Pithva, Ambalam, & Vyas, 2019). The survival of probiotics is powerfully influenced by the physicochemical characteristics of foods, which are partially driven by ingredients used in food formulations (Silva et al., 2017). Technological characteristics of probiotics are also related to their ability to survive during food processing and storage to offer distinct nutritional, physicochemical and sensory properties to the final product (Corbo et al., 2017; Salvucci, LeBlanc, & Pérez, 2016). For years, it was believed that the presence of phenolic compounds, among other constituents, in the plant matrix would inhibit probiotics; However, studies have shown that the presence of phenolic compounds could enhance the survival of probiotics in different food matrices (de Souza et al., 2019). Still, the metabolism of probiotics in fruit-derived matrices could enhance the bioaccessibility of phenolic compounds and increase their functionality (Morais, Borges, dos Santos Lima, Martín-Belloso, & Magnani, 2019).

Several approaches are undertaken to improve and sustain microbial cell viability in food products, such as the appropriate selection of probiotics and suitable food matrices. The

development of symbiotic combinations is an approach to stimulate the growth of probiotics (Sengupta, Koley, Dutta, & Bhowal, 2019). Studies with technologies, such as fermentation and encapsulation, have been developed and successfully applied to protect some probiotics from environmental stresses associated with various non-dairy food matrices, but there are still some technological challenges to produce and preserve probiotic foods to be solved. Indeed, with probiotics as living microorganisms present in a food product, retention of sufficient viable bacteria is a significant quality component (Min et al., 2019).

Microencapsulation of probiotic cells has been suggested as a solution to the problem of viability loss. However, many technical issues need to be resolved when applying this technology to produce new probiotics foods. Selection of appropriate encapsulation technique, safe and effective encapsulating material, and bacterial strain can affect the efficiency of encapsulation or result in decreased cell viability (Huq, Khan, Khan, Riedl, & Lacroix, 2013). The microencapsulation process can also help to maintain the sensory acceptance of probiotic products, which could be impacted by the concentration of probiotic culture (Afzaal et al., 2020).

1.4 Final remarks

Over the past century, several researchers have contributed to advance in knowledge about the probiotic concept, which has evolved from substances to living microorganisms. Nowadays, there are guidelines for screening strains with probiotic aptitudes that should be further confirmed as suitable candidates for use to this end. These guidelines must be applied to avoid the commercialization of strains with claimed health-related properties for which veracity cannot be scientifically justified. The protocols for probiotic research have moved to reach more sophisticated approaches. The use of probiotics in different product formulations has been challenging, especially concerning the growth, survival, viability, stability, and functionality of these microorganisms during processing and storage. Indeed, probiotics represent a valuable market niche for the food and beverage industry.

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CHAPTER

2

Legislation of probiotic foods and supplements

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2.1 Introduction

Laws and regulations that govern food making and marketing are fundamental to provide consumers with foods meeting established safety and quality criteria and, as regards to probiotics, defined by FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization) as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014), to ensure their efficacy as well. On the other hand, since food laws and regulations can differ among countries, they can represent boundaries for international trading opportunities if not based on the same common definitions and principles.

With regard to probiotics, in 2017 the International Probiotics Association (IPA) highlighted a lack of harmonization not only in the industry practice but also within the legislation of probiotics, and proposed, during a Codex Alimentarius (CA) session (39th session of the Codex Committee on Nutrition and Foods for Special Dietary Uses, 4-8 December 2017, Berlin, Germany), to initiate working on harmonized probiotic guidelines for foods and dietary supplements. The end goal of the IPA proposal is the establishment of eligibility criteria for probiotics that could ensure consistency at national and international levels and "provide a guideline for global regulatory agencies to build probiotic-focused regulations" (IPA, 2017). In line with IPA's view, the International Scientific Association for Probiotics and Prebiotics (ISAPP) completed, in 2018, a "position statement on minimum criteria for harmonizing global regulatory approaches for probiotics in foods and supplements" with the aim to submit it to CA, thus contributing in the construction of the harmonized probiotic guidelines (ISAPP, 2018).

Discussion took place during the 2019 CA session (41st session of the Codex Committee on Nutrition and Foods for Special Dietary Uses, 24-29 November 2019, Dusseldorf, Germany) where i) the definition of probiotic; ii) the minimum criteria for the characterization and the

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safety of the probiotic strain; iii) the safety of the food; iv) the food labeling (that should include the name of the microorganism at the strain level; the amount of total probiotic viable cells; the name of the food; serving size; storage conditions) and v) the reference methods for analysis and sampling, were outlined as the main aspects to be covered in the prospective Codex harmonized probiotic guidelines for foods and dietary supplements (CCNFSDU, 2019).

Although the way toward this harmonization started to be paved, the global scenario on probiotics is to date still fragmented, also considering the slowdown due to the COVID-19 pandemic that postponed the meeting agenda of the CA. In this chapter, we provide the reader with an overview of the main regulatory frameworks that govern the use of probiotics in Europe, America, and Asia Pacific.

2.2 Legislation in Europe

In the EU, probiotics, which mainly comprise fermented milk and supplements, fall under the regulation of the General Food Law. As such, communication on the nutritional and health effects of probiotics is regulated by the Nutrition & Health Claims Regulation (NHCR) (EC) No. 1924/2006 (European Commission, 2006). In order to label a food product as probiotic, operators have to apply for a prior-authorization to the EU-Commission for the health claim approval.

The Regulation applies to the use of claims that are "any message or representation, which is not mandatory under Community or national legislation." Consequently, claims (i.e. messages made on a voluntary basis) should be distinguished from the mandatory labeling indications that are required by other Community or national legislation. It is stated that a health claim is such if there is a description or indication of a functionality or an implied effect on health in the naming of the substance or category of substances. The expression "contains probiotics" is given as an example of a health claim, thus this label must imply a health benefit (the reference to probiotic/prebiotic implies a health benefit). In the EU, all health claims are assessed by the European Food Safety Authority (EFSA).

To date, only one product, over more than 400 health claim applications, was authorized pursuant to Article 13(1) of Regulation (EC) No 1924/2006 (European Commission, 2006): claim on live yoghurt and improved lactose digestion for the starter microorganisms *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (minimum 10^8 Colony Forming Unit (CFU)/g) as specified by Codex Alimentarius Standard No. 243/2003 (EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA), 2010).

All the other requested probiotic health claims have been rejected or withdrawn mainly due to insufficient characterization. According to Regulation (EC) No 1924/2006 (European Commission, 2006), the scientific assessment by EFSA for the health claim authorization must be of the highest possible standard. The lack of harmonization and the consequent confusion for the consumers were the main topic of the debate occurred in the meeting between EFSA and the Industrial Probiotic Association (IPA) held on 18 January 2019, in Parma (Italy), which was focused on the scientific substantiation of health claims made on foods. This should follow 3 basic principles: the generally accepted scientific evidence, the totality of the available scientific data, and the weighing of the evidence. Despite the Community Regulation, a few EU Member States have established their own regulation on probiotics. This is the case in Italy, Czech Republic, France, Portugal, Belgium, and Spain.

2.2.1 Italy

In Italy, National Guidelines on Probiotics and Prebiotics have been updated in March 2018 by the Ministero della Salute, Commissione unica per la nutrizione e la dietetica (Ministero della salute, 2018), defining the requirements of probiotics to be used in food or food supplements. According to the Italian regulation, microorganisms permitted for use in food and food supplements must meet these requirements: be traditionally used to supplement gut microbiota, have safety requirements for food use, have a viable administered dose (1×10^9 CFU per day) to be active and vital in the gut.

The safety assessment for their use in food includes a correct taxonomic classification at species level, that must be achieved through Pulsed-field Gel Electrophoresis (PFGE), Multilocus Sequence Typing (MLST), Random Amplification of Polymorphic DNA (RAPD), Amplified fragment length polymorphism (AFLP) or Whole Genome Sequencing (WGS), or by worldwide recognized and accepted genotyping methods (not indicated). Furthermore, the antibiotic resistance profile of each microbial strain must be provided.

2.2.2 Czech Republic

As in Italy, Czech Republic allows the use of the term "contains probiotics" in labels as a nutrition claim, subjected to the fulfillment of nutrition claims defined in the NHCR. The regulation of dietary supplements falls within the powers of the Ministry of Health of the Czech Republic (MZCR) and it is governed by Act No 110/1997 Coll., on Foodstuffs and Tobacco Products (MZCR, 1997) and on Amendments to Some Related Acts, as amended No. 224/2008 Coll. (MZCR, 2008a) and Decree No 225/2008 Coll. (MZCR, 2008b), on the requirements for dietary supplements and fortification of foodstuffs (hereinafter referred to as the implementing legal regulation).

Manufacturers and importers must notify the MZCR of the text in the Czech language, which will be presented on the label of the product prior to the first marketing of the dietary supplement, and the products may be marketed only after a prior approval based an expert position issued by the National Institute of Public Health (NIPH) on the health safety of the product.

2.2.3 France, Belgium, and Portugal

In France, Portugal and Belgium, the term "probiotic" is considered a non-specific health claim, and it is permitted only when accompanied by a specific and authorized health claim related to the probiotic action.

2.2.3.1 France

In France, as reported in the Ministère de l'Économie des Finances et de la Relance (MEF) Q&A on nutrition and health claims "Questions/Réponses concernant l'utilisation des allégations nutritionnelles et de santé" (Règlement (CE) n°1924/2006) (MEF, 2006) all the requests for health claims related to microorganisms, such as probiotics, have been rejected.

2.2.3.2 Belgium

As indicated in the Belgian Q&A on nutrition and health claims by the Belgian Service Publique Fédéral Santé publique, Sécurité de la Chaîne alimentaire et Environnement

(SPF SPSCAE, 2020), to date there are no such authorized health claim related to the probiotic action, so the use of the term probiotic is not permitted.

2.2.3.3 *Portugal*

Pursuant to the Dec-Lei n° 136/2003 (República Portuguesa, 2003), amended with the Dec-Lei n° 296/2007, de 22.08 (República Portuguesa, 2007) and with Dec-Lei n° 118/2015, de 23.06 (República Portuguesa, 2015), manufacturers and importers have to notify the commercialization of any food supplement to the Direcção-Geral de Veterinária with the label used for the product. Supplements must be compliant with Art. 9 of DL 136/2003 and its amendments.

2.2.4 Spain

In 2020, the Spanish Agency for Food Safety and Nutrition (AESAN) has announced the approval of the use of the term "probiotic" on the labels of foods and food supplements produced and commercialized in the country (AESAN, 2020). According to AESAN, the conditions of labeling a product with the term "probiotics" is that they must meet standard food safety requirements stipulated in Regulation (EC) No 178/2002 (European Regulation, 2002). They should not be accompanied by any health claim unless they have specific EU authorization.

Recognizing that there is numerous food supplements labeled with the term "probiotics" coming from different EU countries, Spanish authorities decided to apply the principle of mutual recognition, thus authorizing the marketing in Spain.

2.3 Legislation in America

2.3.1 United States of America

In the United States (U.S.), the market of probiotic products can be considered in its early stage of life where "probiotics" do not have a statutory definition and are not defined as a regulatory product category (Schmelz, 2020; U.S. FDA, 2018a). Consequently, they may be considered as foods, dietary supplements, drugs, or even cosmetics (the latter, falling outside the purpose of the book, will be no further addressed in this chapter), mainly depending on their intended use, which, in turn, largely depend on the claims they bear.

The above product categories are basically governed by the U.S. Food, Drug, and Cosmetic Act (Act), under the authority of the U.S. Food and Drug Administration (U.S. FDA). However, also the Federal Trade Commission (FTC), according to The Federal Trade Commission Act (FTC Act), plays an important role as regards issues related to product marketing and advertising (Hoffmann et al., 2014; Schmelz, 2020).

As drugs, probiotics could be considered as live biotherapeutic products (LBP) that are biological products, different from vaccines and not intended to be administered by injection, that contain live organisms used for "the prevention, treatment, or cure of a disease or condition of human beings" (U.S. FDA, 2016a). Drugs are highly regulated products in the U.S., subjected to a strict pre-market approval process and it is worth noting that U.S. FDA has

not yet approved any probiotic as a live biotherapeutic product, as reported in a recent U.S. FDA's statement (U.S. FDA, 2018b).

Unlike drugs, foods are among the least regulated product categories in the U.S. (Hoffmann et al., 2014) and manufacturers must be compliant with relevant U.S laws and regulations to ensure the food safety but do not need an U.S. FDA approval before marketing (Schmelz, 2020). Basically, probiotics can be added to foods if they have been approved as food additives by U.S. FDA (even after a specific request by the food manufacturer), or if they have been qualified as GRAS (Generally Recognized as Safe) substances, that is they have been widely recognized as safe under the conditions of their intended use by qualified scientists, or considered safe according to a self-affirmation of the GRAS status presented by the food manufacturer (Wasserman, 2016a).

As regards the dietary supplements, they are products intended to be ingested, that contain one or more "dietary ingredients" (such as vitamins, minerals, herbs and other dietary substances) with the function to supplement the diet, usually presented in a non-conventional food form (such as capsules, powders, tablets, gels and liquids, even if other forms could be permissible) (U.S.FDA, 2015) and regulated under the Dietary Supplement Health and Education Act (DSHEA) that was introduced in 1994 amending the Act. According to the DSHEA, a dietary supplement does not need a pre-market approval, thus U.S. FDA could essentially take action after the product is on the market if it recognizes issues regarding consumer safety (Hoffmann et al., 2014; U.S. FDA 2015). A probiotic microorganism used in a dietary supplement formulation could be considered a "new dietary ingredient" (NDI) if it was not marketed as a dietary supplement in the U.S. before 15 October 1994 (that is the date of DSHEA approvement). As such, a NDI notification from the manufacturer to U.S. FDA is requested in order to provide information regarding its safety, unless the NDI and the other dietary ingredients in the dietary supplement "have been present in the food supply as an article used for food in a form in which the food has not been chemically altered" (section 413 of the Act (21 U.S.C. 350b)) (U.S. FDA, 2016b). Such behavior reflects the FDA opinion according to which certain changes in ingredient processing may prompt the need to submit a notification (Wasserman, 2016b).

As regards information on the label, in a final rule published in May 2016 titled "Food Labeling: Revision of the Nutrition and Supplement Facts Label" (81 FR 33742), the U.S. FDA amended labeling regulations for both conventional foods and dietary supplements. However, as highlighted by Merenstein, Guzzi, and Sanders (2019), strain designations and viable cell counts at the end of shelf life are information not yet requested if a probiotic is part of the formulation, although state-level legislations could require additional information. This is the case of the State of California where the recent Bill text N. 1178 specifies to "include the genus, species, and strain of each live microorganism in the dietary supplement on the label of the dietary supplement" sold in the State that contains live microorganisms, commencing January 1, 2022 (CA bill AB1178, emended on 27 March 2019). Nevertheless, in 2018 U.S. FDA issued a draft guidance on the labeling of dietary supplements containing live microbials advising firms on the intent to exercise enforcement discretion in declaring the quantitative amount of live microbial ingredients by CFUs, in addition to the quantitative amount by weight that is, the latter, the current requirement representing the total cellular mass that not necessarily correlates with the number of viable microorganisms present in the dietary supplement (U.S. FDA, 2018a).

A set of acts such as the Nutrition Labeling and Education Act of 1990 (NLEA) that amended the Act, the Food and Drug Administration Modernization Act of 1997 (FDAMA) and the DSHEA, govern the use of claims on the label of conventional foods and dietary supplements. In particular (excluding nutrient content claims that refer to nutrients) products containing probiotics could bear "structure/function claims", "qualified health claims" or "health claims", if the food business operator has scientific evidence for their substantiation (the highest scientific requirements are requested for "health claim" substantiation) and submit a notification to U.S. FDA before placing the product on the market (the notification is not required for structure/function claims made for conventional foods that must be focused on the effects derived from the nutritive value). However, none of the aforementioned claim categories can state to treat, diagnose, cure or prevent any disease and, additionally, for dietary supplements bearing structure/function claims a disclaimer stating that U.S. FDA has not evaluated such claim must be disclosed on the product label (Schmelz, 2020; U.S. FDA, 2018c).

2.3.2 Canada

Food products containing probiotic microorganisms are classified as food. All health claims made for food, including claims about probiotics, are subject to Subsection 5(1) of the Food and Drugs Act (Government of Canada, 1985), where it is declared that all claims and representation on food are required to be truthful and not misleading, or deceptive or likely to create an erroneous impression about the product.

Health Canada (HC) has prepared a guidance document "The Use of Probiotic Microorganisms in Food" (HC, 2009) illustrating the requirements needed for the health claims about probiotics in food. The use of the term "probiotics" on the label is authorized when accompanied by specific and validated statements about the benefits or effects of the probiotic microorganism in the food. If a health claim is reported, the species name of the strain must be indicated as well as the amount of probiotic strains expressed as CFU in a serving dose. This document will serve the Canadian Food Inspection Agency (CFIA) to assess compliance of food products containing microorganisms represented as probiotics with the Food and Drugs Act and Regulations.

Probiotics that are not presented in a food format (e.g. capsules, pills and tablets) are classified as health natural products (www.canada.ca/en/health-canada/services/drugs-health-products/natural-non-prescription.html accessed 1 April 2021) and are regulated by the Natural Health Products Regulations of 2004 (HC, 2004). They have to be authorized after a pre-market analysis and obtain a product license as Natural Product. The Canadian Government has prepared a monograph to help industries in the preparation of Product License Application (HC, 2019).

2.3.3 Latin America

Latin America is a non-uniform region characterized by great economic, political, ethical, cultural, and social differences, including 20 countries, 17 from the Americas, and 3 from the Caribbean region. Brazil accounts for the greatest Probiotic Retail Value Sales, with 3.048 M US$, followed by Argentina and Chile, and a growing market that in Venezuela has increased 143% in the last 5 years (Morán, 2018).

The regulatory status of probiotics in Latin America reflects the fragmentation of its regions, although in the last years a great demand toward harmonization has been raised by several countries. In the great majority of its States, probiotics are regulated as food supplements. Within the MERCOSUR (Mercado Común del Sur) there is no specific regulation on probiotics neither on the health claim in the labeling of food products (De Paula, Vinderola, & Weill, 2018). Brazil, Colombia, and Ecuador have adopted a definition of probiotics that is aligned with the definition proposed by FAO/WHO. In addition, Brazil has a protocol for the evaluation of a probiotic as a food ingredient.

2.3.3.1 *Brazil*

The use of probiotics in food requires a prior evaluation from Brazil's National Health Surveillance Agency (ANVISA), as indicated in RDC No. 241/2018 (ANVISA, 2018): the authorization is based on the unequivocal characterization of the identity of the probiotic microorganism, its safety and beneficial effect.

ANVISA has released in March 2020 the updated guidance on the 2018 dietary supplement regulations outlining the necessary procedures to apply to ANVISA for use of probiotics in food products and any related claims, focused on the design of supporting technical-scientific documentation (ANVISA, 2019).

2.3.3.2 *Argentina*

In Argentina, the National Administration of Drugs, Foods and Medical Devices (Administración Nacional de Medicamentos, Alimentos y Tecnología Médica - ANMAT) regulates food supplements under Chapter XVII of the Argentinian Food Code, where, in 2011, the minimal requirement for the acknowledgment of probiotics were defined (ANMAT, 2011): strains identification achieved by 16S rRNA gene sequencing; *in vitro* and *in vivo* characterization (gut resistance, bile resistance, resistance to lysozyme (optional), *in vivo* and *in vitro* assay demonstrating the probiotic effect; safety evaluation; and viable administered dose (10^6–10^9 CFU/g). Since then, foods with probiotics are classified as dietary foods.

Argentina was the leading country in Latin America for the efforts in harmonizing probiotic regulation worldwide. Indeed, in 2018 Argentina has prepared the Discussion Paper on Harmonized Probiotic Guidelines for use in Foods and Dietary Supplements (Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) held in Berlin, Germany, 26–30 November 2018) presented at the Joint FAO/WHO Food Standards Programme (CCNFSDU, 2018). The aim of the paper was to establish guidance to assist competent national authorities in their evaluation of "probiotics" in order to authorize a strain as a probiotic strain. Within the paper, it was proposed that in the product label, in addition to General Standard for Labeling of Prepackaged Foods (CCFL. CXS 1- 1985), it must be indicated the name of the microorganism(s) (genus, species and strain) mentioned in the list of ingredients, the number of viable cells of each microorganism (CFU/g), the name of the food, the serving size and storage conditions.

2.3.3.3 *Bolivia*

In 2017, with the Resolución Administrativa Senasag N° 0140/2017, Reglamento de etiquetado de alimentos de consumo humano (Estado Plurinational de Bolivia, 2017), the term "probiotic" appeared in the list of the ingredients that could be included in dietary

supplements. The regulation states that the label has not to attribute to Dietary Supplements any health claim or the ability to prevent, treat or cure any human disease, nor does it has to refer in any way to such properties.

2.3.3.4 Chile

This country lacks a specific regulation on probiotics. The Resolución Exenta N° 860 which approved the Norma Técnica n. 191 within the "Directrices Nutricionales para Declarar Propiedades Saludables de los Alimentos" of July 2017 (Republica de Chile, 2017) includes living bacilli into the regulation of the healthy features of foods, and associate *Lactobacillus* spp. and *Bifidobacterium* spp. to beneficial health effects, some of which related to the gastrointestinal tract.

To authorize health claims in the label, products must contain 100% of the viable count declared within the shelf life of the product, that set at the minimum value of 10^7 CFU/g. Bacilli must be resistant to gastric acids or other secretion of the digestive tract.

2.3.3.5 Colombia

Probiotics are defined in the Resolución Número 333 de 2011 (Colombia Regulation, 2011) as a culture of living microorganisms, generally bacteria, which survive the transit through the upper parts of the intestine, and particularly the acidic environment of the stomach, adhering to and colonizing the intestine and favorably modifying the microbial balance. The requirements for the health claim authorization linked to the improvement of digestive function in relation to the probiotic consumption are: the viability of the microorganism; the absence of pathogenic traits; deriving from the gut; being capable of surviving to the intestinal tract, be resistant to gastric juice and bile; adhere to the intestinal mucosa, colonize the intestine and survive throughout the shelf life of the food product; have a number of viable cells equal or greater that the initial dose, with a minimum of 10^6 CFU/g within the shelf life of the product. In addition, the claim has to indicate that the regular consumption of probiotics is not the only way to normalize or improve digestive function.

2.3.3.6 Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua

Probiotics are regulated by the Central America Technical Regulation (2012) which has standardized the nutritional labeling of prepackaged food for human consumers.

The regulation was approved by the Council of Ministers of Economic Integration (COMIECO) Of Central American, by the Resolución N° 277-2011 (COMIECO, 2011). The requirements for a microorganism to be recognized as probiotics are the same of Colombia (Binetti, Burns, Tomei, Reinheimer, & Vinderola, 2019).

2.3.3.7 Uruguay

In Uruguay, the Sanitary Evaluation Division of the Food Department of the Ministry of Public Health utilizes the guidelines provided by Brazil or Canada for the regulation of probiotics, due to the lack of its own regulation in this field. After 2006, MERCOSUL (Mercado Comum do Sul) members followed the harmonizer resolutions adopted by the Common Market Group.

Uruguay has declared reviewing a new version of its draft regulation for food supplements that should be approved as food ingredients. If already approved by ANVISA or EU as new ingredients safety studies should not be required.

In relation to food labeling, the Decree N° 272/018 of Uruguay's Ministry of Health (2018) applies.

2.3.3.8 *Mexico*

In Mexico, there is no specific regulation on probiotics. However, according to the NORMA Oficial Mexicana NOM-131-SSA1-2012 by the Federal Commission for Protection against Sanitary Risk (Comisión Federal para la Protección contra Riesgos Sanitarios) (COFEPRIS, 2012), it is possible to report the presence of probiotics in label only if this is compliant with some articles included in the same regulative document. Labeling is regulated by the NORMA Oficial Mexicana NOM-051-SCFI/SSA1-2010 (COFEPRIS, 2010).

2.3.3.9 *Venezuela*

Probiotics are defined as microbial preparations with a beneficial effect on host health. Their use is permitted by the art. 5.1.21 of the Norma Venezolana General Para Fórmulas Adaptadas Para Lactantes 909 Ministerio Del Poder Popular Para Economía Y Finanzas. *Lactobacillus casei*, *L. paracasei*, *L. reuteri*, *L. acidophilus* and *Bifidobacterium lactis* are listed as authorized microorganisms (Comisión Venezolana de Normas Industriales, COVENIN, 2015).

2.4 Legislation in Asia Pacific

In this section, we focused on the regulations applicable to probiotic products in Australia, Japan and China. As regards the latter two countries, it is noteworthy their relevance in the global market of functional/health foods, in terms of high market share and distribution level, as the case of Japan, or potential as a growing market, as for China (Farid et al., 2019).

2.4.1 Japan

To understand how the regulatory framework developed in Japan, it could be useful to report some historical hints that point out the role of functional and healthy foods in this country. As reported by Hickey (2005), it was in the 1930s that the Japanese Dr. Minoru Shirota isolated the *Lactobacillus casei* strain Shirota and used it in a fermented milk drink believing it could help maintain the health status of the gut. This drink, known as Yakult, started to be sold initially in Japan and then spread in markets around the World. Following awareness achieved on malnutrition problems after World War II and growing consciousness on population aging with the related issue for the public health system (Hickey, 2005), the Ministry of Education, Science and Culture put in place a national scale project to improve knowledge on the functionalities of food. Thanks to this, the term "functional food" was defined for the first time. It was referred to as "food that has physiological functions, including regulation of biorhythms, the nervous system, the immune system, and bodily defense beyond nutrient functions" (Shimizu, 2003). Subsequently, the Japanese Ministry of Health, Labour, and Welfare (MHLW) established in 1991 a regulatory system, enacted under the Nutrition Improvement Law, to regulate foods that are allowed in claiming health benefits, named "Foods for Specified Health Use" (FOSHU) (Shimizu, 2003). Nowadays in Japan, FOSHU are not the only foods that are allowed to claim health benefits; in fact, also "Foods with

Nutrient Function Claims" (FNFC) (that comprise foods containing specific nutrients such as vitamins, minerals and n-3 fatty acid) and "Foods with Function Claims" (FFC), are grouped under the main category of "Foods with Health Claims" (Farid et al., 2019). Below FOSHU and FFC systems are described as they represent the major regulatory frameworks in Japan where the use of probiotics may fall.

2.4.1.1 The "Foods for Specified Health Use" (FOSHU) system

FOSHU products (that include also non-traditional food forms such as tablets or capsules) are not only part of "Foods with Health Claims" but belong also to one of the five categories of "Foods for Special Dietary Uses" (FOSDU) enforced under the Nutrition Improvement Law (then Health Promotion Law) (Shimizu, 2003; Tanaka, Kaneda, Suguro, & Baba, 2004). FOSDU are: i) formulas for pregnant or lactating women; ii) infant formulas; iii) foods for people with dysphagia; iv) foods for medical uses; and, v) FOSHU (https://www.mhlw.go.jp/english/topics/foodsafety/fhc/index.html accessed 21 March 2021). To understand the overall process underlying the approval of a product as FOSHU, it should be noticed that since 1st September 2009 the jurisdiction on Foods with Health Claims passed from MHLW to the Consumer Affairs Agency (CAA), so to obtain the FOSHU approval a food company should present the application to the CAA. It basically requires consultation of the Consumer Commission and the Food Safety Commission that assess efficacy and safety of the candidate FOSHU product, as well as of the MHLW that checks if the proposed labeling violates the Pharmaceutical Affairs Act. In addition, a product sample is analyzed by The National Institute of Biomedical Innovation, Health and Nutrition (or other accredited bodies) before obtaining, or not, the approval for marketing, with the approved health claim and the FOSHU logo that differentiate these products from any other foods (Farid et al., 2019; Sadohara & Martirosyan, 2020) (we invite the reader to refer to Sadohara and Martirosyan (2020) for a summarized and clear description of the required documents to be submitted for FOSHU approval). Therefore, it should be highlighted that through this evaluation system, the approval is given individually for each FOSHU product by the Secretary-General of the CAA (CAA, 2015a).

This approval flow is a strict, long (up to one year) and costly evaluation process for the food company that must provide evidence of efficacy and safety of the active ingredient (including a description of the mechanism of action underlying the beneficial effect), as well as of the whole candidate product, eventually considering the safe consumption history. *In vitro* and *in vivo* experiments and clinical trials on humans are also important requirements of the FOSHU approval procedure (Domínguez Díaz, Fernández-Ruiz, & Cámara, 2020; Sadohara & Martirosyan, 2020).

In addition, FOSHU products must not contain excessive amounts of certain ingredients such as salt, fat or sugar, must guarantee compatibility with product specifications at the time of consumption and undergo established quality control methods (https://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html accessed 21 March 2021).

Different categories of FOSHU exist, and "regular FOSHU" or "qualified FOSHU" represent the categories likely more suitable for FOSHU containing probiotic microorganisms, being the others applicable to products that contain certain ingredients, that is, dietary fibers ("standardized FOSHU") or calcium and folate (risk-reduction FOSHU). "Regular" and "qualified" FOSHU basically differ on the strength of the scientific evidence

supporting the health claim that, in "qualified FOSHU", must state that exist a "possibility" in exerting the health effect because the evidence is not necessarily proved (Sadohara & Martirosyan, 2020).

In FOSHU products health claims can state that the product is suitable to maintain or promote a certain physiological function or health status but they cannot state or intend that the product can treat or prevent any kind of disease or clinical condition (Domínguez Díaz et al., 2020; Farid et al., 2019; Sadohara & Martirosyan, 2020). Considering the FOSHU products on the market, the approved health claims fall in the following categories: gastrointestinal health; teeth health; mineral absorption; osteogenesis; blood sugar level; blood pressure; triglycerides; blood cholesterol level; cholesterol and gastrointestinal health; cholesterol and triglyceride levels (Domínguez Díaz et al., 2020; Iwatani & Yamamoto, 2019).

Probiotics are principally employed in FOSHU products that bear health claims related to gastrointestinal conditions. The latter is about 50% of the total FOSHU products where bacteria such as lactobacilli and bifidobacteria are employed especially in products like yogurts and fermented milk (Iwatani & Yamamoto, 2019). Anyway, also other substances such as oligosaccharides and dietary fibers are also used as active ingredients in such intestine targeted FOSHU products (https://www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html accessed 21 March 2021).

In general, at least 16 different probiotic strains have been used in FOSHU products since their establishment, belonging to the genus *Lactobacillus* (e.g., *L. rhamnosus* GG; *L. delbrueckii* subsp. *bulgaricus* 2038; *L. casei* Shirota; *L. acidophilus* CK92; *L. helveticus* CK60; *L. casei* SBR1202; *L. gasseri* SP; *L. casei* NY1301; *L. johnsonii* La1 (LC1)), *Bifidobacterium* (e.g., *B. longum* BB536; *B. breve* Yakult; *B. lactis* FK120; *B. lactis* LKM512; *B.* SP; *B. lactis* Bb-12), and *Streptococcus* (*S. salivarius* subsp. *thermophilus* 1131) (Amagase, 2008). As regards claims, statements focused on the microorganism ability to increase the number of beneficial bacteria lowering the unfavorable ones, regulate the gut condition and ameliorate the gut environment, are explanatory examples of approved health claims reported on the labels of intestine-targeted FOSHU products that contain probiotics (Hickey, 2005).

Lastly, as regard labeling, the following information is required for FOSHU products: the logo and the statement "Food for Specified Health Uses"; the name and the category of the commodity; approved health claim; net weight; unit(s) of recommended daily intake; instructions for use; ingredients; nutrition information; expiration date; warnings (e.g., related to en excessive dosage); advice to maintain a balanced diet; food business operator contact details. Moreover, if a dietary reference intake for the active ingredient has been specified by the Japanese government, the proportion of the total daily intake is to be reported on the label (Sadohara & Martirosyan, 2020).

2.4.1.2 The "Foods with Function Claims" (FFC) system

The FFC system, introduced in 2015, shares underlying principles with the Dietary Supplement Health and Education Act (DSHEA) established in 1994 in the U.S. to regulate dietary supplements (Iwatani & Yamamoto, 2019). In particular, under the FFC system, food business operators can market products with function claims under their own responsibility as for both the effectiveness and safety. As reported by CAA (2015a, 2015b), differently from the FOSHU system, food business operators have to submit a notification to the Secretary-General of the CAA before the product is placed on the market, providing the evidence of

efficacy and safety for the consumers basing on the scientific evidence they collected, but without undergoing an individually approving governmental process. So the food business operator is responsible for the content of the claim as well as the content and interpretation of the provided scientific data overall.

To substantiate the product safety, tests on the finished products, or its functional substance(s), can be performed or, alternatively, the history of safe human consumption or information retrieved from databases can be also evaluated. Additionally, a quality-control system must be described along with an analytical method used for the quantitative assessment of the functional substance(s) used in the FFC product (CAA, 2015a).

Another important difference with respect to the FOSHU system is related to the substantiation of the effectiveness implied in the function claim. In particular, the scientific evidence can be provided or by clinical trial(s) testing the finished product or, alternatively, by performing a systematic literature review, searching published data on the finished product or its functional substance(s), fulfilling some established reviewing criteria (CAA, 2015a; Kamioka et al., 2019). As with FOSHU products, claims can state that the product is suitable to promote or maintain the health avoiding terms such as "diagnose," "prevent," or "cure" and cannot be specifically addressed to sick people, pregnant, and lactating women as well as minors (CAA 2015a; b).

Products containing probiotics represent a relatively good portion of the total FFC products present on the market, whose claims are principally focused on the gastrointestinal tract. Moreover, body fat reduction claims have been reported on the labels of FFC products that use probiotics as active ingredients (Iwatani & Yamamoto, 2019) and it is noteworthy that within this system new and multiple claims can appear based on the scientific evidence the food business operator may use to substantiate the effectiveness of the employed probiotic microorganism (Koe, 2020).

As detailed by CAA in two guidances for industry and consumers (CAA, 2015a;b), on the label of the FFC products, following the Food Labeling Standards pursuant to the Food Labeling Act, and the Guidelines on Notification of Foods with Function Claims, the information listed below has to be displayed: the statement "Foods with Function Claims"; the notification number that identifies the product and that can be used to retrieve product safety and efficacy information through the CAA website; name of the commodity (with the exception of FOSDU, FNFC, alcoholic beverages, food products containing excessive amounts of certain substances such as salt, fat or sugar, or containing functional substance(s) with a Dietary Reference Intake established by the Japanese government, any food product can apply to the FFC system, including fresh produce); proposed claim (in particular statements reporting that the product i) "has the function to…" or ii) "has been reported to have the function to…" can be claimed but they depend on the method used for their substantiation, that is, clinical trials testing the finished product for the first type of statements or systematic literature reviews for the second type); quantity of the functional ingredient and unit(s) of recommended daily intake; instructions for use; ingredients; nutrition information; expiration date; warnings; advice to maintain a balanced diet; food business operator contact details.

Moreover, a statement explaining that the responsibility lies with the food business operator, and that the product has not been subjected to individual evaluation by the Secretary-General of the CAA has to be reported (CAA, 2015a; b).

2.4.2 China

In 2015, the new Food Safety Law was approved by the National People's Congress in China (Binns, Lee, & Lee, 2018). A category of foods allowed to bear health claims are the so-called health foods, also known as functional foods since 1996 when the Ministry of Health promulgated "The provision for functional foods administration", and subsequently defined as health foods in "The guideline of registration for functional foods" released in 2005 by the State Food and Drugs Administration (SFDA) (Yang, 2008). To date, several regulations and technical standards regulate health foods in China, including, among others, GB 16740-2014 National Food Safety Standards for Health Food, Administrative Measures for Registration and Filing of Health Food (2016), Health Food Raw Materials Directory (1st Batch) (2017), List of additives for Health Food Filing and Terms of Use (Trial) (2017), Directory of Health Function Available to Claim for Health Food (The 1st Batch) (2017), and Provisions for Functional Food Labeling (1996) (Tao, 2020).

According to GB 16740-2014 National Food Safety Standards for Health Food, health foods are foods that claim to have specific health functions or provide minerals and vitamins. They are suitable for specific groups of people, have beneficial effects on the human body without the purpose to treat or cure any disease and they will not cause any acute, subacute or chronic harm to the human body as well.

Twenty-seven health claims are approved for health foods in China (Lahteenmäki-Uutela, 2010; Tao, 2020; Yang, 2008) that can be grouped in the following categories based on their targeted claimed functions:

- Targeted to blood parameters: i) "assist in blood lipids reduction" ii) "improve nutritional anemia" iii) "assist in blood sugar reduction" iv) "assist in blood pressure reduction";
- Targeted to skin condition: i) "eliminate acne" ii) "eliminate skin chloasma" (also known as melasma) iii) "improve skin water content" iv) "improve skin oil content";
- Targeted to gastrointestinal condition: i) "regulate gastrointestinal tract flora" ii) "facilitate digestion" iii) "facilitate feces excretion" iv) "assist in protecting against gastric mucosa damage";
- Targeted to skeletal condition: i) "increase bone density";
- Targeted to liver condition: i) "assist in protecting against chemical injury to the liver";
- Targeted to eye condition: i) "alleviate eye fatigue";
- Targeted to throat: i) "moisten and clean throat";
- Targeted to a physiological condition or function: i) "enhance immunity" ii) "antioxidative" iii) "facilitate lead excretion" iv) "improve sleep" v) "facilitate milk secretion" vi) "improve child growth and development" vii) "enhance anoxia endurance" viii) "assist in irradiation hazard protection" ix) "alleviate physical fatigue" x) "assist in memory improvement" xi) "weight loss" (Yang, 2008).

The safety and efficacy of health foods must be evaluated, and directories that include authorized ingredients are established. *In vivo* experiments on animals and clinical trials on humans are also required for their whole assessment (Lahteenmäki-Uutela, 2010).

The Probiotic Health Food Review Regulation, enacted in 2005, regulates the category of health foods containing probiotic microorganisms and its remarkable principles are the

following: a probiotic health food is defined as a micro-ecological formulation that promotes the balance of the gut flora, exerts beneficial effects to the human body and is safe. The probiotic strains must belong to the normal human microbiota and must have clear and stable biological, genetic and functional characteristics. A list of authorized probiotics is provided by the State Food and Drug Administration (SFDA) but new strain(s) can be added by submitting an application to the SFDA. It is also specified that the use of both live and dead bacteria, as well as their metabolites, is allowed in probiotic health foods formulations (Koe, 2019; Lahteenmäki-Uutela, 2010).

By April 2020, as reported by CIRS (2020), probiotics used in the approved probiotic health foods belonged to three genera, that is, *Bifidobacterium* (*B. bifidum*, *B. infantis*, *B. longum*, *B. breve*, *B. adolescentis*, *B. animalis* subsp. *lactis*), *Lactobacillus* (*L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, *L. casei*, *L. reuteri*, *L. rhamnosus*, *L. plantarum*, *L. paracasei*, *L. helveticus*, *L. fermentum*), and *Streptococcus* (*S. thermophilus*). They were principally commercialized as powder, granules and electuaries but also capsules, tablets, liquids, and milk-based beverages were present on the market. Anyway, probiotic health foods still represent a relatively small fraction of the total of health foods registered in China (Koe, 2019).

It should be emphasized that China underwent an extensive government restructuring in 2018 that lead to the establishment of the State Administration for Market Regulation (SAMR) that undertook responsibilities from other Chinese competent bodies, including SFDA (that was reformed as the new State Drug Administration (SDA) operating under SAMR) and became responsible for comprehensive market regulation in China (Ropes and Gray LLP, 2018). In 2019, SAMR issued the Provisions for Declaration and Review of Probiotic Health Food (Draft) that is expected to introduce important changes. In particular, a clearer definition of probiotics, that must be living microorganisms, is reported in Article 2 that reads: "Probiotics refer to living microorganisms, which, when ingested in sufficient quantities, are beneficial to the health of the host." In the same article, it is also specified that "Probiotic health foods refer to microbial products that contain probiotics as the main functional ingredients and add necessary excipients, and when ingested in sufficient quantities, they have a beneficial effect on human health. Probiotic health foods must be safe to eat and must not cause acute, subacute or chronic harm to the human body", consequently health foods that contain dead bacteria or microbial metabolites would no longer be considered as probiotic health foods (CIRS, 2019). It should also be noted the remark outlined in Article 8: "the number of viable bacteria of each type of probiotic health food during its shelf life shall not be less than 10^6 CFU/mL(g)."

Moreover, the labeling of health food is also expected to undergo many changes. According to the Provisions for Functional Food Labeling issued in 1996 and later amended, i) the product name, ii) the health food logo (represented by a blue hat plus the words "health food product"), iii) the product code number, iv) the net weight, v) ingredients, vi) functional components, vii) health claim, viii) category of target consumers, ix) provisions for consumption and x) manufacturer contact details are to be displayed on the label of health food products (Yang, 2008), but due to the draft "Measures for Supervision and Administration of Food Labeling" released in 2019 by SAMR along with a series of other regulations, such as "Guidance for Warning Labels of Health Food" and "Guidance for Naming of Health Food", labeling features of health foods such as claims, naming, content and label

format are expected to be affected in the next future in China (Keller and Heckman LLP, 2020).

2.5 Australia

In Australia, probiotic products are considered either functional foods or complementary medicine. Probiotic foods are regulated by Food Standards Australia New Zealand (FSANZ). The term "probiotic" on a food label can be considered as a health claim since it implies a relationship between food and health. Health claims have to be compliant with the food regulatory standard defined in Standard 1.2.7 updated in 2018 (FSANZ, 2018).

If a probiotic is not covered by a food standard or is not traditionally used as food, it could be considered as therapeutic goods, and regulated by the Therapeutic Goods Administration (TGA), where it is accompanied by a therapeutic claim, statement, or instruction for use. The TGA has released in 2020 the Australian Regulatory Guidelines for Advertising Therapeutic Goods (ARGATG) (TGA, 2020a), guidance for advertisers which applies to the Therapeutic Goods Advertising Code (No.2) (Australian Government, 2018) and guidance material: Complying with the Therapeutic Goods Advertising Code (No. 2) 2018 (TGA, 2020b).

Probiotics are contemplated in the novel food category (FSANZ, 2017) as food, which requires authorization based on their historical use and safety information. As stated by the Part 3 Application guidelines subsection 22(2) of the Food Standards Australia New Zealand Act 1991 (FSANZ, 1991) the application for a novel food, which is a microorganism (including probiotics), must contain: information on potential pathogenicity; information on the effects of the microorganism on gut microflora, which must be demonstrated to be not adverse; information on the use of this microorganism in food or as a food in other countries; and information on human toleration studies: clinical evaluation of potential probiotics must use double-blind, placebo-controlled human trials, with detailed reporting of adverse side effects, which can be used to confirm the results observed in animal tests or in vitro studies.

2.6 Conclusion

As arises from this book chapter, the global scenario on the legislation of probiotic foods and supplements is fragmented, being different in the various countries all over the world based on their legal or statutory position. Only a limited number of probiotic preparations are sold as pharmaceuticals or drugs, which are rigorously regulated, whereas most of them are differently categorized all over the world as natural health products, foods or dietary supplements and foods for specified health use, in Canada, in the U.S. and Europe, and in Japan, respectively, to give some examples.

The request to policy makers for a worldwide harmonized regulation rises from producers, traders and consumer's associations, and it is foreseen to impact the global probiotic world at the economic, health and social level. Great efforts are being made but further work is needed to harmonize the regulatory frameworks that govern probiotics.

Authors' note

Recently a reclassification of the genus *Lactobacillus* into twenty-five genera has been proposed by Zheng et al. (2020). In this chapter, examples of microorganisms used in the probiotic products are reported referring to the traditional classification of *Lactobacillus* as a unique genus in order to facilitate the readers' comprehension, also considering that the classification issue does not affect the information herein provided.

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CHAPTER

3

Authenticity of probiotic foods and supplements: Up-to-date situation and methods to assess it

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3.1 Introduction

The global probiotic market has increased significantly in the last decade and is projected to pass from an estimated size of 48.88 billion USD (United States Dollar) in 2019 to 94.48 billion USD by 2027, exhibiting a CAGR (compound and annual growth rate) of 7.9% during the forecast period (https://www.fortunebusinessinsights.com/industry-reports/probiotics-market-100083, accessed 27 July 2021).

This trend is due to several factors, such as the increasing health consciousness among people, growing penetration of e-commerce, increasing healthcare spending worldwide and increasing consumption of healthy dietary supplements. Their intrinsic nature of being "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014) makes challenging the design, development, scale-up, production, marketing and life cycle management of probiotics. Indeed, probiotics are available on the market in different forms, such as foods, pills, granulates and chewable bars, but in each of these products the dose of viable probiotic cells must be at least equal to that used in efficacy trials up to the end of shelf-life (Fusco, Fanelli, & Chieffi, 2021) despite the stresses probiotics undergo during their production and storage (Fiocco et al., 2020; Gueimonde & Sánchez, 2012). The beneficial effects of probiotics depend not only on the dose but also on the specific microorganism, which indeed has to be identified at genus, species and strain level (Huys et al., 2013). Thus, it is of outmost importance to verify not only the dose, which should be the one claimed on the label of the probiotic product, but also the identity of the probiotic strain, which should be consistent with the label, that, in turn, should have the correct and up-to-date nomenclature. Several methods are to date available to perform such analyses. Herein, an overview of the current situation of the authenticity of probiotic foods and supplements and of the methods available to assess will be provided.

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3.2 Labels of probiotic foods and supplements and implications of incompliance

3.2.1 What should be written on a label of probiotic foods and supplements?

Authentic is any probiotic product that has a content compliant with its label. Considering the guidelines proposed for probiotic foods and supplements, such as those of the Council for Responsible Nutrition and International Probiotics Association (2017), as well as those of the Food and Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO, 2006), the label of probiotic products may display the following information useful for consumers:

- **(i)** Ingredients/allergens: all active and inert ingredients are listed in descending order by weight (it is mandatory for any allergens to be included in this list);
- **(ii)** Genus, species and strain of the probiotic;
- **(iii)** Colony forming units (CFUs), that represent, the number of live probiotics in the product. CFUs are usually expressed as total count although count for each strain is preferred. CFUs listed on the label should equal the amount shown to be beneficial in human studies;
- **(iv)** Daily dosage: the amount that needs to be consumed daily, which should be at or above the daily dose tested in human studies;
- **(v)** Claim/recommended use: it provides information about how to use the product and what benefit can be expected from the product. Any claim must be scientifically substantiated and then evaluated and approved by a national or international food safety authority, such as the European Food Safety Authority (EFSA);
- **(vi)** Storage information: how to storage the product to maintain the probiotic potency;
- **(vii)** Best before date: it provides information on how long the probiotic product will contain adequate CFUs of probiotics to deliver any claimed benefit. Products are formulated to have the indicated CFUs up to the best before date;
- **(viii)** Company name/contact information: consumers should always be able to contact the company with questions to get more information or to report any adverse effect.

3.2.2 What happens if a probiotic food or supplement is not authentic?

A probiotic food or supplement may be not authentic if the information on the label is not correct or false (for example, when the probiotic is incorrectly named using inaccurate, not updated or completely wrong nomenclature) and if the actual content of the product is not consistent with that claimed on the label (for example, if the CFUs of the probiotic in the product are lower than those claimed on the label). Mislabeling can be both unintentional and fraudulent (Fusco et al., 2021). Frauds of probiotic products may include the replacement of a less stable probiotic strain with a more stable one without claiming it on the label. In multistrain probiotic products, another frequent implemented fraud consists in producing formulations that contain the labeled total viable count but that do not reflect the individual strain viable counts. Unintentional mislabeling is mainly due to the lack of proper quality control measures in the food or supplement chain and include: (i) strain misidentification due to the use of inappropriate identification and typing methods; (ii) strain contamination or strain mix-ups which may

lead to the presence of not claimed strains in the product; and (iii) probiotic instability due to improper production and storage conditions.

Such fraudulent and unintentional noncompliances may impact not only the quality (if the probiotic product contains, in addition or in place of the claimed probiotic(s), alternative microorganisms or microorganisms that confer a taste and texture different than expected) and the ability to exert the claimed beneficial effect (if it actually contains less CFUs of probiotic than claimed on the label), but even the safety of the product (if it contains, in addition or in place of the probiotic claimed on the label (opportunistic) pathogenic microorganisms) (Fusco et al., 2021). In any case, it may erode consumer confidence in probiotic products (Jackson et al., 2019).

3.3 Characterization and authenticity of probiotics: From culture-dependent methods to multi-omic approaches

3.3.1 What are the methods available to assess the authenticity of a probiotic product?

Enumeration of probiotics is essential to provide information on the microbial cell content present in a food or supplement, focusing in particular on the loads of probiotic viable cells that are able to exert beneficial effects to the host. The plate count method is a commonly used method that enumerates probiotics as CFUs, which are the number of colonies grown on a solid media inoculated with serially diluted aliquots of the sample to be analyzed for the probiotic content, and reported as CFUs/g or mL (Davis, 2014). Although microorganisms with probiotic features may belong to *Streptococcus*, *Lactococcus*, *Enterococcus*, *Pediococcus*, *Bacillus*, *Propionibacterium*, *Escherichia* and *Saccharomyces* genera; *Lactobacillus* and *Bifidobacterium* represent the genera including to date most of the probiotics used in human formulations (Davis, 2014; Huys et al., 2013; Vinderola, Reinheimer, & Salminen, 2019). In order to allow the selective growth of a certain genus or species to be enumerated, selective media have been evaluated, with the MRS (deMan, Rogosa & Sharpe) medium, amended by adding supplements or changing ingredients, being one of the possible suitable choices. For example, MRS supplemented with neomycin, paromomycin, nalidixic acid and lithium chloride (NPNL) could be useful for the selective enumeration of bifidobacteria and MRS supplemented with clindamycin could be useful for the enumeration of *Lactobacillus acidophilus* (Ashraf & Shah, 2011) while, recently, Di Lena et al. (2015) developed a Maltose-MRS-Vancomycin (MMV) medium for the quantitative detection of the *Lactobacillus casei*-group. As highlighted by Vinderola et al. (2019), at the present no single or combined culture media are available to enumerate all the probiotics in all possible food matrices where they can be present, even together with other microorganisms such as the starter cultures. Thus, the enumeration of probiotics in a particular product needs to be "tailor-made." Vinderola et al. (2019) proposed a flow diagram to facilitate the choice of a set of culture media and culture conditions, based on the available literature information, for the selective/differential enumeration of probiotics in a given product. Nevertheless, few standardized protocols, developed and published by the International Organization for Standardization (ISO), are suitable for the enumeration of certain probiotics in specific food matrices, such as ISO 29981:2010 [IDF 220:2010], last reviewed and confirmed in 2015, and ISO 20128:2006 [IDF 192:2006], last reviewed and confirmed in 2018. Both these protocols are applicable to milk products (such

as fermented and non-fermented milk, milk powders and infant formulae) allowing the enumeration of presumptive bifidobacteria and presumptive *Lactobacillus acidophilus*, respectively (International Organization for Standardization, 2006, 2010).

It should be emphasized that the plate count method, besides being relatively time-consuming (considering the time for media preparation and the subsequent incubation time), excludes from the count microorganisms in the viable but nonculturable (VBNC) status. VBNC microorganims are unable to form colonies on solid media likely after their exposure to inauspicious environmental conditions (Fusco & Quero, 2014), such as those that could occur during manufacturing, distribution and storage of the probiotic product (Fusco et al., 2021). However, it was reported that the supplementation of 0.05% (w/v) cysteine to the agar media along with incubation under anaerobic conditions may allow the resuscitation of many VBNC probiotic cells (Vinderola et al., 2019). Moreover, as for the plating techniques used for pathogenic microorganisms, such as the Thin Agar Layer (TAL) method (Fusco, Riccardi, & Quero, 2012; Quero, Santovito, Visconti, & Fusco, 2014a), the enumeration of the viable and culturable cells together with the stressed (or damaged) VBNC cells on solid MRS-based media, can be combined with the enumeration of the sole intact viable and culturable cells on agar media such as MRS added with NaCl, to allow the enumeration of probiotics distinguishing among viable and VBNC cells (Di Lena et al., 2015).

To overcome the plate count drawbacks, culture-independent methods have been proposed to enumerate viable probiotics in foods, such as fluorescent in situ hybridization (FISH) (Babot, Hidalgo, Argañaraz-Martínez, Apella, & Perez Chaia, 2011) and flow cytometry (Wilkinson, 2018). In particular, the International Organization for Standardization (2015) with the ISO 19344:2015 [IDF 232:2015], last reviewed and confirmed in 2021, specified a standardized method, by means of flow cytometry, for the quantification of active and/or total lactic acid bacteria and probiotic strains used in dairy products. This method is also applicable to fermented milk products, such as yogurts containing primarily lactic acid bacteria. Although this valuable approach is able to discriminate among live and dead bacteria (Chiron, Tompkins, & Burguiere, 2018; Foglia, Allesina, Amoruso, Prisco, & Pane, 2020), it is not able to differentiate cells based on genotype (Davis et al., 2014). For this purpose, other techniques based on polymerase chain reaction (PCR) may be suitable. This is the case of the reverse transcription quantitative real-time PCR (RT-qPCR) and the so-called viability-PCR (vPCR), including the propidium monoazide (PMA) quantitative real-time PCR (PMA-qPCR) (Davis, 2014; Fusco & Quero, 2014), as well as the recently proposed digital droplet PCR (ddPCR) (Hansen et al., 2020), which overcome the issue related to the use of DNA targeted PCR protocols. Indeed the latter cannot discriminate between viable and dead cells (PCR techniques will be better addressed later in this section).

Along with enumeration, another key aspect related to the authenticity of a probiotic product is the correct taxonomic assignment of the contained probiotic microorganism(s) at the genus, species and strain level, since it links to the particular abilities of the probiotic microorganism. To date, reference method(s) for probiotic identification have not been established, as also emerges from a Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) discussion paper (FAO/WHO, 2019). Moreover, as pointed out by some authors, the inappropriate use of the identification methods is the major cause for the incorrect taxonomic designation of the probiotic microorganism and the mislabeling of the probiotic product (Huys et al., 2013; Kolaček et al., 2017). In light of this,

an overview of methods for probiotic identification and strain typing will be given, highlighting the advantages and drawbacks.

Biochemical identification methods essentially rely on the ability of microorganisms to metabolize, or not, some chemical substances, providing as first instance useful information on their phenotypic metabolic features. Examples of commercially available systems for biochemical identification are analytical profile index (API) kits or multi-well microplates containing prearranged chemical substrates coupled to a spectrophotometric reading system. However, these systems are biased by their intrinsically cumbersome and time-consuming nature, and, especially, by their frequent failure in properly identifying microbes at the species level (Fusco & Quero, 2014), mainly due to the variability and instability of phenotypic characters and the subjectivity in the interpretation of results (Fusco et al., 2015). Indeed, inconsistencies in microorganism identification using such methods compared to DNA-based identification techniques were reported in several studies (Barakat et al., 2011; Boyd, Antonio, & Hillier, 2005; Brolazo et al., 2011; Moraes, Perin, Júnior, & Nero, 2013; Yeung, Sanders, Kitts, Cano, & Tong, 2002). Apart from accuracy and reproducibility issues (Miller, Biddle, Quenzer, & McLaughlin, 1993; Moraes et al., 2013), the high intraspecific variability in the phenotypic traits observed in many bacterial species (Huys et al., 2013) limits the suitability of these biochemical methods, which also need extensive and updated databases including a wide range of possible detectable species (Huys et al., 2013; Yeung et al., 2002).

The matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), basically based on the analysis of microbial proteins, is another method that, more than the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), is increasingly used for microbial identification (Singhal, Kumar, Kanaujia, & Virdi, 2015). Also in this method, the identification of microorganisms relies on the presence of a reference database to which the so-called peptide mass fingerprint (PMF), or the masses of the analyzed biomarkers, are matched (Singhal et al., 2015). Therefore, the availability of extensive databases is a crucial factor for the species, or even strain, identification (Seol et al., 2019; Singhal et al., 2015). Additionally, reproducibility issues have been raised especially between laboratories using different MALDI-TOF equipment, and the creation of a multiple laboratory PMF database has been proposed as a solution (Singhal et al., 2015; Wunschel et al., 2005). This method has been applied for the identification of species belonging to genera of interest in the probiotic field, and in particular to *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus* (Angelakis, Million, Henry, & Raoult, 2011; Huang & Huang, 2018; Ledina et al., 2018; Nguyen et al., 2013).

A large portion of the identification methods is represented by methods that target nucleic acids, and most of them rely on the use of polymerase chain reaction (PCR) and DNA sequencing. DNA-DNA hybridization has been considered the traditional "gold standard" for microorganism identification but it is not suitable for practical routine identification purposes (Huys et al., 2013). On the contrary, PCR amplification of 16S rDNA and its subsequent sequencing is a widely used and traditionally recognized tool for bacterial species identification (Fusco et al., 2021) that is also applied to probiotics (Huys et al., 2013; Tannock, 1999; Yeung et al., 2002). As highlighted by Huys et al. (2013), to allow an accurate identification, complete 16S rDNA sequences of multiple reference strains per taxon should be chosen for comparison to the unknown 16S rDNA sequence. Sequences for comparison are available in

public databases, such as GenBank, but for final validation, additional curated databases, such as PATRIC, should also be used (Binda et al., 2020; Wattam et al., 2017).

The accuracy in sequencing can be influenced by the various sequencing platforms capable of short or long sequencing (Seol et al., 2019). However, it should be stressed that for genetically closely related species, the 16S rDNA analysis may show insufficient resolution due to the high similarity of this gene sequence and be not suitable for species identification (Huang, Li, Huang, & Watanabe, 2018; Huys et al., 2013). It is the case, for example, of species within the *Lactobacillus casei*-group, the *L. acidophilus*-group and *L. plantarum*-group (Huang et al., 2018; Huys et al., 2013; You & Kim, 2020). To overcome this issue, species-specific PCRs, targeting specific genes, are valuable and rapid tools not requiring sequencing. Multiplex species-specific PCRs able to detect *L. gasseri*, *L. acidophilus*, *L. helveticus*, *L. jensenii*, *L. crispatus*, and *L. gallinarum* within the *L. acidophilus*-group (You & Kim, 2020) and *L. casei*, *L. paracasei*, *L. rhamnosus*, and *L. chiayiensis* within the *L. casei*-group (Kim, Yang, Cho, & Kim, 2020a) represent examples of recently developed assays. Additionally, strain-specific PCRs allow identification at the strain level, as done by Galanis, Kourkoutas, Tassou, and Chorianopoulos (2015) to identify *L. plantarum* 2035 and *L. plantarum* ACA-DC 2640, two potential probiotic strains isolated from feta cheese that could be also monitored during yogurt production. It is worth mentioning that quantitative real-time PCR (qPCR) is a variant of PCR able to quantify the detected microorganism. Moreover, the RT-qPCR that detect RNA (through its reverse transcription into complementary DNA) and the PMA-qPCR, in which PMA binds DNA of membrane-damaged cells preventing its PCR amplification (Davis, 2014), may represent valuable tools for the detection and quantification of viable microorganisms.

Such methods applicable to probiotics have been developed by several authors (Dias, Scariot, de Mello Castanho Amboni, Arisi, & A., 2020; Postollec, Falentin, Pavan, Combrisson, & Sohier, 2011; Reimann, Grattepanche, Rezzonico, & Lacroix, 2010; Scariot, Venturelli, Schwinden Prudêncio, Arisi, & A., 2018; Shao, Wang, Bao, & Zhang, 2016; Villarreal et al., 2013). However, a recently proposed viability-PCR (vPCR), that is a ddPCR, was reported as an alternative method for viable probiotic strain identification and enumeration (Hansen et al., 2020), being demonstrated to be faster and less variable than both plate count and flow cytometry, allowing, in addition, strain-specific counts.

Besides the described methods, the DNA fingerprinting techniques represent suitable tools used either for species/subspecies identification or, to a greater extent, for strain typing, displaying a pattern of DNA bands usually separated by electrophoresis on an agarose or polyacrylamide gel. Among these, PCR-denaturing gradient gel electrophoresis (DGGE) has frequently been used as a culture-independent method for the analysis of microbial communities in various environments (Ercolini, 2004), including food products containing probiotics (Chen et al., 2017; Fasoli et al., 2003; Theunissen, Britz, Torriani, & Witthuhn, 2005). Typically, after DNA extraction from the food sample, hypervariable regions of 16S rDNA (18S rDNA or 26S rDNA for eukaryotes such as *Saccharomyces*) (Ercolini, 2004) are amplified by PCR and separated by electrophoresis in a polyacrylamide gel with a denaturing gradient. The microorganism identification is achieved by band position comparison to a reference, band sequencing or band hybridization (Herbel, Vahjen, Wieler, & Guenther, 2013; Huys et al., 2013). However, it should be taken into account that, targeting the variable V3 region of the 16S rDNA, the identification of some species or subspecies may be hindered due to several drawbacks, including preferential PCRs and identical band positions. This is the case

of certain species and subspecies belonging to *Pediococcus*, *Enterococcus* and *Lactococcus* genera (Aponte, Fusco, Andolfi, & Coppola, 2008; Ercolini, Moschetti, Blaiotta, & Coppola, 2001; Quero et al., 2014b).

Restriction Endonuclease Analysis (REA)-Pulsed Field Gel Electrophoresis (PFGE), a PCR-independent technique that employs restriction enzymes to fragment intact genomic DNA (previously extracted and purified *in situ* in low melting agarose) and then uses PFGE to separate the obtained DNA fragments, is able to discriminate probiotic strains due to its high sensitivity and accuracy (Donelli, Vuotto, & Mastromarino, 2013). PFGE fingerprinting has been used for characterizing or monitoring probiotic strains belonging to *Bifidobacterium* (Grand, Küffer, & Baumgartner, 2003; Masco, Huys, De Brandt, Temmerman, & Swings, 2005; Xu et al., 2018), *Lactobacillus* (Coudeyras, Marchandin, Fajon, & Forestier, 2008; Wind, Tolboom, Klare, Huys, & Knol, 2010), and *Bacillus* (Klein, 2011a) genera, and its use in combination with biochemical tests, such as carbohydrate fermentation, may enhance its discriminatory power for strain differentiation (Gueimonde et al., 2004a).

Lastly, amplified fragment length polymorphism (AFLP) (combining restriction of genomic DNA and selective PCR amplification of the restricted fragments) and randomly amplified polymorphic DNA (RAPD) (using arbitrary primer that allows PCR amplification of random DNA segments) are other examples of strain typing techniques used for the characterization or monitoring of probiotic strains (Collado, Moreno, Cobo, & Hernández, 2006; Di Lena et al., 2015; Santos et al., 2016). Fluorescent AFLP has also been applied to select species-specific fragments used to design PCR assays specific for *L. brevis* and *Weissella confusa* (Fusco, Quero, Chieffi, & Franz, 2016; Fusco, Quero, Stea, Morea, & Visconti, 2011). Although AFLP is a labor-intensive method, it exhibits a much higher reproducibility than RAPD (Paun & Schönswetter, 2012), and, therefore, it may represent, along with PFGE, a preferable choice.

3.3.2 The importance of omics technologies

In the last decades, omics technologies have revolutionized all domains of modern science. This impact was tremendous also in the probiotics field (Baugher & Klaenhammer, 2011; Johsnon and Klaenhammer, 2014). Omics analysis was fundamental not only to describe the composition of gut microbiota and increase the knowledge on its activity, but also to demonstrate how the dysbiosis of the intestinal microbiota underlies several diseases and that probiotic consumption can be used to restore microbial balance (Apanna, 2018; de Oliveira, Leite, Higuchi, Gonzaga, & Mariano, 2017). Based on this evidence, probiotics implementation in nutrition and healthcare has been proposed to improve health in several diseases, conditions or processes, including cardiovascular disease (Oniszczuk, Oniszczuk, Gancarz, & Szymańska, 2021), dental health (Mahasneh et al., 2017; Rosier, Moya-Gonzalvez, Corell-Escuin, & Mira, 2020), metabolic syndrome (He & Shi, 2017), neurologic diseases (Umbrello & Esposito, 2016), multiple sclerosis (Kohl, Castillo, & Ochoa-Repáraz, 2020), neurodegenerative diseases (Peterson et al., 2020), viral infections (Harper et al., 2021), gastrointestinal cancer (Kaźmierczak-Siedlecka et al., 2020), Covid 19 (Sundararaman, Ray, Ravindra, & Halami, 2020), and aging (Ale & Binetti, 2021; Tsai et al., 2021).

The use of omics technologies has also been crucial for the identification and characterization of novel species, the selection of novel potential probiotics and the development of personalized probiotic-based therapies (Cunningham et al., 2021; Luo et al.,

2019). The characterization of genetic elements conferring a health benefit to the host, defined by Ventura et al. (2009) with the term probiogenomics, started in 2001 with the publication of the first complete genome of a probiotic strain of *Lactococcus lactis* subsp. *lactis* (Bolotin et al., 2001).

In the last decade, probiogenomics also revealed the diversity and evolution of probiotic microorganisms by reconstructing the genetic signature of individual strains and by providing insights on genetic determinants responsible for advantageous features such as: the modulation of the immune response (Yan & Polk, 2011), the antagonism towards pathogens, the production of lactic and short-chain fatty acids and antimicrobial compounds (Rezaei Javan, van Tonder, King, Harrold, & Brueggemann, 2018), the production of bacteriocins (Collins, O'Connor, & O'Sullivan, 2017), the regulation of the intestinal transit and the strengthening of the intestinal barrier.

Pan-genome analysis has highlighted that common features of human intestinal probiotic genomes (reviewed by Salminen, Nurmi, & Gueimonde, 2005) include the presence of genes involved in the sensing, colonization and adaptation to the upper intestinal tract (Kankainen, Paulin, Tynkkynen, von Ossowski, & Reunanen, 2009), achieved by the action of cell surface proteins, permeases, hydrolases and transporters genes with specific enzymatic activities, such as glycolases and peptidases for the uptake and metabolization of sugars and amino acids (Buron-Moles, Chailyan, & Dolejs, 2019), as well as transcriptional regulators (Lebeer, Vanderleyden, & De Keersmaecker, 2008), which reflect the flexibility of these microorganisms. These features are commonly used as biomarkers for the selection of probiotic strains (de Melo Pereira, de Oliveira Coelho, Magalhães Júnior, Thomaz-Soccol, & Soccol, 2018; Dunne et al., 2001; Tuomola, Crittenden, Playne, Isolauri, & Salminen, 2001).

In combination with functional metagenomics (Verma, Ahmed, & Gupta, 2018), probiogenomics can lead to the identification of novel traits and properties, and to the description of the effects of variants in modified or improved strains with technological or functional roles.

Multi-omics approaches and predictive modeling have contributed, on one hand, to reveal and describe the cross-talk and the connection within the human microbiome and the regulation of the gut ecosystem dynamics (Nyholm et al., 2020), and, on the other hand, to support the development and exploitation of the biotechnological potential of probiotic strains. These methodologies are indeed vital not only to optimize the cultivation conditions for industrial applications, but also to maximize the functionality and adaptation of probiotics to the gut environment by genomic manipulation, shaping their activity as therapeutics in chronic or acute diseases (Allen, Martinez, Gregorio, & Dans, 2010; Nazir, Hussain, Hamid, & Song, 2018).

The effectiveness of a probiotic strain is essential as its safety, and omics technologies are also fundamental to perform a preliminary evaluation before proceeding with further *in vivo* investigations (Alayande, Aiyegoro, Nengwekhulu, Katata-Seru, & Ateba, 2020; Castro-López et al., 2021; Senan, Prajapati, & Joshi, 2015). The *in silico* safety assessment aims to determine the absence of undesirable genetic determinants, such as antibiotic resistance or virulence factors genes (Klein, 2011b). Safety assessment is an essential requisite for the preselection and utilization of strains in industrial formulation, ruled by a fragmented regulatory framework. Several reviews cover this aspect (Gueimonde, Ouwehand, & Salminen, 2004b; Lahtinen, Boyle, Margolles, Frias, & Gueimonde, 2009; Zielińska, Sionek, & Kołożyn-Krajewska, 2018; Žuntar, Petric, Bursać Kovačević, & Putnik, 2020); however, where

probiotics are used as therapeutics towards specific diseases or conditions they must be evaluated with reference to the administered dose, duration of administration and the peculiar vulnerability of the patient or consumer (Sanders et al., 2010).

The plasticity of probiotics genomes, due to the presence of mobile genetic elements, has allowed probiotics, through horizontal gene transfer (HGT) mechanisms, to shape their gene repertoire, favoring the adaptation to the gut environment and the exertion of their functions and specific metabolic activities, such as the metabolization of complex sugars derived from the diet and the host (Siezen & Wilson, 2010; van Reenen & Dicks, 2011). Some of these activities are plasmid-encoded, as in domesticated *Lactococcus lactis* strains, where enzymes for the lactose utilization and citrate metabolism are located in the dispensable genome (Kelleher, Murphy, Mahony, & van Sinderen, 2015; van Hylckama Vlieg et al., 2006). According to Passerini et al. (2010) these strains also show variability in the genome size, an increase of pseudogenes number, extra gene cluster for galactose and lactose metabolism, as well as casein hydrolyzation. These extra clusters appear to be responsible for the *L. lactis* domestication. The acquisition of these elements from *L. lactis* through HGT is similarly correlated with the *Streptococcus gallolyticus* milk adaptation (Papadimitriou et al., 2014). *Bifidobacterium longum* ssp. *infantis*, which is the first colonizer of the sterile gastrointestinal tract of human infants, has a genome of 2.83 Mb, the largest among sequenced bifidobacteria (Duar et al., 2020). Within this genome, it is present a novel 43 kb gene cluster encoding a system for the import and degradation of human milk oligosaccharides and complete pathways for the synthesis of some vitamins (Siezen & Wilson, 2010).

The industrial-scale production of probiotics by long-term fermentation can significantly impact the genomic stability of probiotic strains: it may lead to the accumulation of point mutations which, althought seem not to highly impact the safety (Sanders et al., 2014), can tremendously affect the functionality and adaptability of these microorganisms.

Bifidobacterium longum DJO10A, an intestinal isolate, after 1,000 generations, was shown to lose functionality by gene loss after prolonged pure culture (Lee et al., 2008), due to two large deletions and a significant reshaping of its chromosome, which significantly reduced its competitive ability against *Clostridium difficile* and *Escherichia coli*. *L. plantarum* ATCC 14917 lost the ability to ferment the milk but retrieved probiotic traits (Feng et al., 2020). On the contrary, Stage et al. (2020) demonstrated the genomic and phenotypic stability in an industrial production process of *Lactobacillus rhamnosus* GG, one of the most widely marketed and studied probiotic strains. Whole-genome sequencing (WGS) can thus be used for quality control during probiotics production (Jackson et al., 2019) to verify potential genetic drift.

If a product contains a recognized probiotic, despite the fragmented scenario of probiotics regulation regarding their use as food supplement or drug, its label must contain a clear indication of the species of the strain used. 16S rDNA sequencing-based taxonomy, despite its historical use as a phylogenetic marker (Almeida, Mitchell, Tarkowska, & Finn, 2018; Woese and Fox, 1997) and the existence of curated reference databases (Quast et al., 2013) as mentioned above, in the last decade has lost the role of benchmark as a marker of species definition. Indeed, the conservative nature of this gene did not show enough resolution (Richter et al., 2009), although the full-length gene (1500 bp) seems to partially overcome this limitation (Johnson, Spakowicz, & Hong, 2019).

In addition, in a single genome multiple and heterogeneous copies of this gene might be present (Ibal, Pham, Park, & Shin, 2019), as in species of *Bifidobacterium* and *Lactobacillus* genera (Milani et al., 2014; Sun et al., 2015). A higher resolution has been achieved by using other markers, such as *rpo*B (Dahllöf, Baillie, & Kjelleberg, 2000), MLST (Glaeser & Kämpfer, 2015; Sharma, Lee, & Park, 2020) or genome-based phylogeny inferred by using large concatenated protein datasets (Parks et al., 2018; Uelze, Grützke, & Borowiak, 2020; Zhu et al., 2019).

Taxogenomics based on whole-genome sequence comparison allows the reconstruction of a more robust taxonomic framework. Chun et al. (2018) have defined minimal standards for the use of genome data for the taxonomy of prokaryotes based on the overall genome-related indexes (OGRI), such as the average nucleotide identity (ANI) and the digital DNA-DNA hybridization (dDDH) (Chun & Rainey, 2014): threshold values of 95~96 and 70%, respectively, are indicated for the proposal of novel species. With the availability of complete genomes of probiotic strains, the correct taxonomic placement of broadly used commercial strain has been clarified (Briczinski et al., 2009; Holzapfel and Wood, 2014; Loquasto et al., 2013; Lugli et al., 2014; Makarova et al., 2006; Milani et al., 2014).

Sequencing technologies have significantly contributed to the development of novel combined approaches in which the high-throughput sequencing by metagenomics has offered rapidity and high sensitivity (Lugli et al., 2019; Mora et al., 2019; Patro et al., 2015, 2016) to define the microbial formulation of complex blends of multiple probiotics, thus validating their authenticity. A 16S rDNA sequencing metagenomic approach named "Genetic Identity Card (GIC)" was developed by Lugli et al. (2019) to describe the microbiota composition of probiotic supplements. Mora et al. (2019) used a shotgun metagenomics protocol to characterize the commercial VSL#3 product, combining single-cell analysis by flow cytometry to evaluate the cell viability. Lots of these products were also analyzed by a metaproteomic approach with advanced mass spectrometry, identifying also the proteome present in the formulation. This was one of the first efforts toward the definition of combined high throughput-based protocols aimed to describe the quality of complex probiotic formulations.

Culture-independent approaches described by Mora et al. (2019), Patro et al. (2015), and Morovic, Hibberd, Zabel, Barrangou, and Stahl (2016) pave the way to the introduction of omics protocols for the surveillance of the authenticity of probiotic compositions and quality monitoring processes in the industrial environment.

3.4 Up-to-date studies assessing the authenticity of probiotic foods and supplements

Many studies have been carried out to assess the authenticity of probiotic foods and supplements. The earliest was published on 1977 by Gilliland and Speck (1977). It enumerated bile resistant lactobacilli in 15 commercial products sold in the United States offered as sources of *Lactobacillus acidophilus*. Only 5 out of the 15 products analyzed revealed counts of lactobacilli (on MRS agar) higher than 10^8 CFU/g, whereas the counts of bile resistant lactobacilli varied among these products. In 3 products, the counts of lactobacilli were lower than 10^2 CFU/g on selective and nonselective media. Moreover, the phenotypic identification of lactobacilli revealed that only 3 out of the 10 products analyzed contained the *L. acidophilus*

claimed on the label, whereas the remaining product contained either a strain of a different species or additional microorganisms (Gilliland & Speck, 1977).

Thereafter, Hamilton-Miller, Shah, and Smith (1996) assessed the number and the type of microorganisms contained in capsules, powder, and tablets sold in Great Britain as probiotics, finding that only 2 out of the 13 products where compliant with their labels, whereas the remaining 11 either did not contain *L. acidophilus*, contained other species, or had counts lower than those claimed on the labels. But also in this occasion, the identification of the probiotics was done by phenotypic methods, namely by using the API kit, which may lead to misidentifications. Canganella et al. (1997) used the cultivation and API kits to assess the authenticity of probiotic pharmaceutical products, finding that 80% of the 15 products tested contained lower counts of probiotics than those claimed on the label, and that the expected species was absent, or other species than the labeled was present. Cultivation and API kits were used also by Hamilton-Miller, Shah, and Winkler (1999) who analyzed 52 yogurts, dietary supplements and health products, finding that only 6 (11.5%) of these products were compliant with the species and the loads claimed on the labels.

Even in the 21 century certain researchers have used the sole cultivation, or the cultivation coupled with phenotypical identification of the isolates at species level, to enumerate and verify the dose and identity of the claimed probiotics. Goldstein, Citron, Claros, and Tyrrell (2014) and Begum, Jakaria, Anisuzzaman, Islam, and Mahmud (2015) used the sole cultivation on selective media to enumerate probiotics, finding that only one of the 9 probiotic products analyzed contained an amount of viable cells compliant with the label. Zimmer and Dorea (2019) used the sole cultivation to determine the counts of viable *Escherichia coli* in 2 *E. coli* –claimed probiotic products, also after simulated gastro-intestinal digestion. Viable counts of *E. coli* in both products were 2 orders of magnitudes lower than claimed on the labels and after simulated digestion they were further significantly reduced (Zimmer & Dorea, 2019).

Cultivation and phenotypic identification by the API kits were used by Fredua-Agyeman, Parab, and Gaisford (2016) who found only 3 probiotic products out of 7 to contain the claimed counts of viable cells and the species claimed on the labels; the remaining 4 products contained fewer counts of viable cells than claimed in the labels and missed some claimed species.

Temmerman, Pot, Huys, and Swings (2003a) verified the identity of probiotics isolated from 55 European probiotic products, including 30 dried food supplements and 25 dairy products, by cultivation and whole-cell protein profiling. Only four of the 55 probiotic products (7.3%), specifically 2 of the 30 probiotic supplements and 2 of the 25 dairy products, were authentic in terms of species and dose. In the remaining products, either only some of the claimed species, extra species, or species different from those claimed in the label were found. Moreover, in 4 products the claimed species had an incorrect nomenclature. As for the counts of viable cells, they ranged from 10^5 to 10^9 CFU/mL in dairy products, whereas in the 30 food supplements the counts of viable cells ranged from <1 to 10^6 CFU/g.

Nonetheless, in the 21st century, other researchers started to couple the cultivation with more reliable and specific molecular methods. Hoa et al. (2000) assessed the authenticity of 5 probiotic products by cultivation, electron microscopy, biochemical characterization (API kits) and 16S rRNA gene sequencing, finding that 4 of the 5 products were mislabeled as concerns the *Bacillus subtilis* content they claimed on the labels. Vermeulen, Luijendijk,

van Toledo, van Kaam, and Reiss (2020), by using cultivation and MALDI-TOF MS, investigated the quality of 15 capsules from 5 different strips, originating from 3 different lots of Infloran, finding that only 1 lot contained the species claimed on the label, whereas the remaining lots missed one or both claimed probiotics and contained additional contaminant species. Cultivation, phenotypical identification by API kits, and MALDI-TOF MS were used by Zawistowska-Rojek, Zareba, Mrowka, and Tyski (2016) to assess the loads and identities of probiotics contained in 25 products including 16 dietary supplements, 7 foods for special medical purposes and 2 medicinal products. Twelve products contained the claimed dose of probiotics, whereas the remaining products contained a lower count of viable cells (Zawistowska-Rojek et al., 2016). Moreover, 12 products did not contain some claimed species or contained extra species or species other than those claimed on the labels (Zawistowska-Rojek et al., 2016).

Vecchione et al. (2018) used a culture-dependent approach including cultivation, identification by biochemical tests (Vitek II test cards), and MALDI-TOF MS to assess the authenticity of 10 probiotic products marketed in Italy in form of vials, capsules, bottles, drops and sachets. Mislabeling due to incorrect nomenclature of the strains in 4 products were detected: *Bifidobacterium* BB-12 and *B. lactis* HN019 instead of *B. animalis* subsp. *lactis*; *S. faecium* instead of *E. faecium* and *S. thermophilus* St-21 and *S. thermophilus* BT-01 instead of *S. salivarius* subsp. *thermophiles* (Vecchione et al., 2018). Only 1 out of the 10 products had a count of viable probiotics lower than that claimed on the label, while in 4 products the viable counts were compliant with their label and in the remaining 5 products, they were 1-3 log higher than those claimed on the labels (Vecchione et al., 2018). In 2 products, the claimed *Lactobacillus delbrueckii* subsp. *bulgaricus* as well as three *Bifidobacterium* species were identified only at genus level (Vecchione et al., 2018). However, the MALDI-TOF MS and biochemical identification results overlapped for all identified microorganisms (Vecchione et al., 2018).

The most used approach of the 21st-century couples cultivation with molecular methods based on the amplification of the DNA for the enumeration and identification at species and strain level of probiotics. Szajewska et al. (2004) used cultivation, the API kits for the identification and, only for bifidobacteria, genotypic methods consisting in partial 16S rRNA gene amplification and sequencing, finding that 57 out of 64 (89%) samples of 5 probiotic products licensed for medicinal purposes contained viable probiotics at the dose claimed on the label. Moreover, 3 of the 5 probiotic products contained the claimed species, whereas the two products containing bifidobacteria were found to contain species other than that claimed, using genotypic methods (Szajewska et al., 2004).

Cultivation, species-specific PCR and 16S rRNA gene sequencing, as well as biochemical identification with the API kit (only for *S. boluardii*) were applied by Toscano, de Vecchi, Rodighiero, and Drago (2013) to assess the authenticity of 24 commercial probiotic-based dietary supplements available on the Italian market in 2011. Fourteen of the 24 products (58%) were compliant with their labels as concerned the species and the amount of the claimed probiotic microorganisms, even after the expiry date. The remaining products (42%) did not contain the claimed loads of at least one of the species claimed in the label or contained extra species.

Aureli, Fiore, Scalfaro, Casale, and Franciosa (2010) used a culture dependent approach including selective plate counting with phenotypic and genotypic identification by API kits and genus- and species-specific PCRs of the isolates, respectively, and a culture-independent

approach consisting in species-specific and genus-specific PCRs of the DNA directly isolated from the probiotic samples to assess the authenticity of 72 probiotic samples from 29 processing plants (41 from processing plants and 31 samples of the same brand from retailers collected at timed intervals): 21/41 species were incorrectly named or had fictitious microbial names, and 9/41 had obsolete names; furthermore, the culture-independent approach revealed that only 22 probiotic preparation out of the 41 tested contained the species claimed on the labels, while the remaining 19 food supplements did not contain one or more of the labeled species. The culture-dependent approach revealed that only 5 of the 41 tested supplements contained viable cells of the species claimed in the labels, whereas 25 of the 41 samples were compliant with the labels as concerns the number of microorganisms. Although according with their labels, 25 supplements should contain *Bifidobacterium bifidum*, this microorganism was detected seldom and as dead cells (i.e., by culture independent approach). Although claimed with the improper name of *Lactobacillus sporogenes* in the labels of numerous supplements analysed, viable *Bacillus coagulans* was not found (Aureli et al., 2010). Instead, other spore-forming species, such as *B. cereus* and *B. subtilis* were found in 1 and 3 probiotic preparations, respectively. After 3 months, 7/24 samples had the same composition as those from the manufacturer, while after 8 and 13 months only 2 and 1, respectively, had the same composition (Aureli et al., 2010).

Vanhee, Goeme, Nelis, and Coenye (2010) used cultivation and solid phase cytometry (SPC) to enumerate 15 probiotic products containing lyophilized *Saccharomyces boulardii*, which was confirmed by microsatellite typing in all products, whereas the dose of viable cells ranged from 9.15 to 10.57 log CFU/g in the different products. In particular, its amount was higher than that claimed on the label in 7 products and lower in 5 products, as determined by SPC, whereas it was higher only in 3 products and lower in 9 products as determined by plating method (Vanhee et al., 2010). However, the SPC method allowed to detect significantly higher cell counts than by plating, as it allowed the enumeration of VBNC cells that do not grow on the selective media utilized for the plate counting (Vanhee et al., 2010). Moreover, <1% of *S. boulardii* cells survived after 120 minutes in simulated gastric conditions and storage for 3 months at 40°C with 75% relative humidity (Vanhee et al., 2010).

Di Lena et al. (2015) developed a selective medium for the enumeration and isolation of lactobacilli of the *casei* group and combined it with a multiplex species-specific PCR and with REP-PCR and RAPD-PCR for the genotypic identification and typing of the isolates. By this PCR-culture-dependent approach, these researchers were able to assess the authenticity of 8 probiotic products available on the market, including 2 probiotic cheeses and 6 probiotic drinks. Four of the 8 products contained one species different than those claimed on the labels (*L. paracasei* in place of *L. casei*) (Di Lena et al., 2015). The loads of viable probiotics in the 2 probiotic cheeses were lower than those claimed in the label (Di Lena et al., 2015).

Nine commercial probiotic products, 5 oral and 4 vaginal, marketed in Italy were analyzed by Blandino et al. (2016) to assess the compliance with the label of the probiotic lactobacilli contained in the products by a culture dependent approach including the enumeration and isolation on selective media, the identification by phenotypic methods (API kits) and multiplex species-specific PCRs. Six of the 9 products were compliant with the label as concerns the probiotic species found in the product (Blandino et al., 2016). The remaining 3 products contained a species other than that claimed on the label (*L. paracasei* instead of the claimed *L. casei*) or contained additional *Lactobacillus* species

(Blandino et al., 2016). Seventy-eight percent of the products (7/9) were compliant with the label as concerns the dose, while in the labels of the 2 remaining vaginal products the dose was not specified (Blandino et al., 2016).

Di Pierro, Polzonetti, Patrone, and Morelli (2019) assessed the authenticity of 9 probiotic products claimed to contain *L. crispatus*, including 7 nutritional supplements and 2 medical devices, collected from the Italian and French markets by cultivation, 16S rRNA gene sequencing and analysis, and RAPD. Only 2 out of the 9 products contained viable *L. crispatus* cells with a daily dose of at least 1×10^9 CFU/g and with an acceptable correspondence with their labels. Moreover, between these 2 probiotic products, only 1 was found to contain a strain (M247) that has been scientifically documented (Di Pierro et al., 2019).

Cultivation and species-specific PCR were used to assess the authenticity of 10 commercial probiotic products by Korona-Glowniak et al. (2019), finding that 5 of the 10 products had loads of probiotic bacteria lower than those claimed on the labels and that 3 of the tested products did not contain the claimed species.

Ansari, Colasacco, Emmanouil, Kohlhepp, and Harriott (2019) assessed the authenticity of 21 commercial probiotic supplements and beverages by cultivation, 16S rRNA gene sequencing, MALDI-TOF MS and phenotypical typing (Biolog). Eighty-two percent of the species identified by 16S rRNA gene sequence analysis matched those claimed on the labels, with 60% of the isolates identified correctly by the Biolog and 71% identified by MALDI-TOF MS (Ansari et al., 2019).

Ten pharmaceutical probiotic products were analysed by Celandroni et al. (2019) using cultivation, BCL Vitek2, MALDI-TOF MS and 16S rRNA gene sequencing. The load of viable probiotics was lower than those claimed on the label in 5 of the 10 products, and higher than claimed on the label in 3 products. Five products were compliant with the label as concerned the microbial content, whereas the remaining contained extra species in addition to those claimed on the label.

Marinova, Rasheva, Kizheva, Dermenzhieva, and Hristova (2019) assessed the authenticity of 26 dietary supplements (16 from the market and 10 from a local manufacturer) by cultivation, genus- and species-specific PCRs. Only 5 out of the 26 products were compliant with the label as concerns the amount of viable cells, whereas in the remaining 21 products, no viable cells or loads of viable cells lower than those claimed on the labels were found (Marinova et al., 2019). Only 17 out of 44 isolates belonged to the species claimed on the labels. Numerous products contained extra species. Moreover, most products, according to their labels should have contained *L. delbrueckii* subsp. *bulgaricus* but this species was found only in 3 of the 26 probiotic products (Marinova et al., 2019).

Apart from the culture-dependent approach, in the 21st century several culture-independent approaches are being used to detect the microorganisms contained in the probiotic foods and supplements. Kim et al. (2020b) assessed the microbial composition of 19 probiotic products (10 pharmaceuticals in capsules and 9 powdered supplements available on the market) by total genomic DNA extraction and species-specific PCRs targeting 37 *Lactobacillus* species, finding compliance with the label as concerned the microbial species only in 11 products (58%). Seven products contained a species other than that claimed on the labels and in 1 product extra species were detected (Kim et al., 2020b). Another culture-independent approach consisting in total genomic DNA extraction from the probiotic products followed by Terminal Restriction Fragment Length Polymorphism Analysis (T-RFLP) has been used

by Marcobal, Underwood, and Mills (2008) to assess the bacterial composition of 14 commercial probiotic products. Only 2 products were compliant with their labels based on the microbial content. The remaining products missed one or two claimed species (5 products) and/or contained extra species (12 products). (Marcobal et al., 2008). Results were successfully validated by species-specific PCRs.

Bifidobacterial T-RFLP and *Bifidobacterium logum/infanctis* ratio analysis (BLIR) were used by Lewis et al. (2016) to assess the conformity of the species present in 16 probiotic products to the label claims. To validate this approach, isolation combined with MALDI Biotyper and BLIR, which was further confirmed by genomic sequencing and analysis of two isolates, were used. Fifteen out of the 16 products were not compliant with the labels as concerns the species content. In particular, they missed the labeled species or contained species other than bifidobacteria. Only 1 matched the bifidobacterial species claimed in the label. Differences among pills of the same lot and different lots were found.

Seven studies used direct DNA extraction from the products followed by PCR-DGGE (Chen et al., 2017; Elliot & Taversham, 2004; Fasoli et al., 2003; Masco et al., 2005; Patrone, Molinari, & Morelli, 2016; Temmerman, Scheirlinck, Huys, & Swings, 2003b; Theunissen et al., 2005). In particular, Fasoli et al. (2003) found compliance with the label in 7/7 commercial probiotic yogurts as concerns the microorganisms present but the loads determined by plate count were lower than 10 CFU/g in 3 (43%) products and anyway $\leq 8 \times 10^7$ CFU/g in the 7 yogurts. The cultivation combined with the PCR-DGGE allowed Fasoli et al. (2003) to find that two out of 6 commercial probiotic lyophilized products did not contain the expected species and had counts <10 CFU/g, whereas the remaining contained extra species or species other than those expected with viable counts ranging from $<10^3$ to 1.5×10^{10} CFU/g.

Elliot and Taversham (2004) investigated the compliance with the label of species and number of probiotics in 9 probiotic products available in Africa, by cultivation and PCR-DGGE, finding that 6 of the 9 products did not contain the bacteria claimed in the label or contained extra species. Moreover, 4 of the 9 products did not contain the required amount of viable probiotic bacteria (Elliot & Taversham, 2004). Theunissen et al. (2005) analyzed 20 different commercially available South African probiotic products, including 11 different yogurt products, 8 probiotic lyophilized preparations (in tablets and capsules) and 1 baby milk formula, by PCR-DGGE and species-specific PCR. Only 54.5% and 33.3% of the probiotic yogurts and lyophilized probiotic products, respectively, contained the claimed species. Moreover, in certain products, the *Bifidobacterium* species were incorrectly identified and extra species were found. Sequence analyses confirmed the presence of *Streptococcus* spp. other than *Streptococcus thermophilus* in some products and in some cases label information was vague and non-scientific. Patrone et al. (2016) assessed the authenticity of 5 probiotic products by cultivation, 16S rRNA gene sequence analysis and PCR-DGGE, finding that only 1 of the 5 products was compliant with its label as concerns the amount and the species claimed on their labels. Chen et al. (2017) assessed the authenticity of probiotic yogurts and supplements available on the Chinese market, by culture-dependent (plating) and independent (PCR-DGGE) approaches, finding that 5 of the 20 (25%) probiotic yogurts and 2 of the 8 (25%) probiotic supplements did not contain viable probiotics, whereas in the remaining 15 yogurts and 6 supplements the loads of viable cells were more than 10^6 CFU/g (minimum load required by the Chinese National Standards for yogurt),

but only 5 yogurts showed loads ≥10^9 CFU/g. Four of the 20 yogurts (20%) were compliant with the label declaration to contain lactic acid bacteria. The remaining 16 (89%) yogurts plus the 8 (100%) supplements were not compliant with the label as they contained species other than that declared or additional species or did not contain the species claimed on the label (Chen et al., 2017).

However, due to the well-known drawbacks in the PCR-DGGE assay (Aponte et al., 2008; Quero et al., 2014b), the actual microbial composition of the probiotic products could have been underestimated, due to co-migration of bands of different species, or overestimated, due to the PCR amplification of DNA from dead cells. Temmerman et al. (2003b) analyzed 10 probiotic products available on the market, including 5 freeze-dried products, 1 fruit drink and 4 dairy products, by culture-independent PCR-DGGE and by culture-dependent approach based on cultivation and identification of the isolates by SDS-PAGE of whole-cell proteins. From 10^5 to 10^7 CFU/g viable cells were found in freeze-dried products, whereas from 10^7 to 10^9 CFU/mL were found in dairy products (Temmerman et al., 2003b). Based on the culture-dependent approach, only 3 out of the 10 products were compliant with the label as concerns the claimed species, while the remaining products either missed one or more claimed species, or contained extra species or contained a species different than the one claimed on the label. The culture-independent approach overlapped with that of the culture-dependent approach for 5 products but for the remaining 5 products were detected more claimed species than recovered by the culture-dependent approach (Temmerman et al., 2003b).

The culture-independent PCR-DGGE approach does not allow identifying the microorganisms at the strain level. Progress in this sense has been made by Masco et al. (2005) who assessed the authenticity of 58 *Bifidobacterium*-claiming probiotic products including 28 food supplements, 22 yogurts, 5 dairy fruit drinks and 3 pharmaceuticals by culture-dependent (cultivation combined with BOX-PCR and PFGE) and culture-independent (PCR-DGGE) approaches. From 10^3 to 10^8 CFU/mL, from 10^4 to 10^6 CFU/mL, from 10^2 to 10^9 and 10^7 CFU/g of viable cells were found in yogurts, dairy fruit drinks, food supplements and pharmaceutical preparations, respectively. The culture-dependent approach allowed the detection of bifidobacteria in 70.7% of the products, whereas the latter were detected in 96.5% of the products by a culture-independent approach. For 39 products, results of the culture-dependent approach overlapped with those achieved by the culture-independent approach, and the same species of bifidobacteria were detected. In the remaining products, PCR-DGGE detected more species than those recovered by the culture-dependent approach (Masco et al., 2005). Anyway, in 5 products no viable cells were found at all, while in 11 products no bifidobacteria were identified by the culture-dependent approach. Moreover, 33 of the 58 products were not authentic due to one of the following cases: (i) the species contained in the product was different than the one claimed in the label, (ii) the species on the label was incorrectly named; and (iii) the product contained extra species (Masco et al., 2005).

Another approach that is being used in the last decades is based on metagenomics combined or not with culture-dependent methods. Fifty-two samples of commercial probiotic dietary products were analyzed by Morovic et al. (2016) using a polyphasic approach that combined plating with targeted genotyping and high-throughput sequencing (HTS) of the 16S rRNA gene sequence. Loads of viable probiotics were lower than that claimed on the labels in 17 out of the 52 samples analyzed. Multiplex-PCRs demonstrated compliance with

the labeled species of 30 out of 52 samples (58%), whereas in the remaining products the species were incorrectly named, the species were absent or extra species were found. The HTS approach found that the most abundant species in the blended products were *Lb. acidophilus* and *Bifidobacterium animalis* subsp. *lactis*.

Mora et al. (2019) used metaproteomics and shot-gun metagenomics by deep Illumina sequencing on the DNA isolated from commercial VSL#3, a probiotic multi-strain product used as a model, to assess its strain identity, composition and safety. Species-specific quantitative real-time PCR was used to investigate loads of each species, whereas flow cytometry was used to quantify live, damaged and dead cells of probiotics. The cell viability was evaluated also by assessing *in situ* the beta-galactosidase and urease activities. The metagenomics allowed to detect the 8 strains that compose the VSL#3 and to estimate the relative abundance of the individual strains, although this latter has the drawback of including in the estimation also the number of dead cells. Flow cytometry, biochemical and metaproteomic analyses allowed to confirm the compliance of the product with its label as concerns the number of viable probiotics and to find high levels of S-layer proteins of *L. acidophilus* and *L. helveticus*. Moreover, only 54 of the 1659 proteins detected by the metaproteomic approach were differentially expressed among the two different lots thus revealing a standardized fermentation process used for the production of the strains composing the VSL#3.

Patro et al. (2016) used cultivation and species-specific PCR and a culture-independent approach based on metagenomics and custom-designed Affymetrix microarray to assess the authenticity of 10 online available commercial probiotic dietary products. The culture-dependent and independent approaches allowed to reveal that only 1 of the products contained loads of probiotics lower than those claimed on the label. The expected species was not present in different lots of 1 product which contained extra species. High relative abundance of *Enterococcus faecium* was found in one lot of one product by metagenomics and confirmed by isolation on selective medium, followed by genetic identification. This latter approach confirmed the presence of *E. faecium* in another lot of the same product where the metagenomics approach did not reveal it.

Lugli et al. (2019) combined a PCR-culture-based approach including, culturomics, genus-specific PCRs, partial 16S rRNA sequencing and analysis and quantitative real time PCRs, with a culture-independent approach including MiSeq sequencing of 16S rRNA gene-based amplicons, ITS microbial and fungal profiling and shot-gun metagenomics. Based on the culture-independent approach, 8 of the 10 products were compliant with their labels as concerns the species composition, while the remaining 2 contained extra species. These results were confirmed by the culture-dependent approach. The quantitative real-time PCR also revealed low levels of some probiotics.

A polyphasic approach combining cultivation and species-specific PCRs, and high-throughput 16S rRNA gene sequencing of the pool of DNA isolated from the colonies obtained by cultivation, with a culture-independent approach based on high-throughput 16S rRNA gene sequencing of total DNA isolated directly from the products was applied by Ullah, Raza, Ye, and Yu (2019) to assess the authenticity of 19 probiotic products from local and multinational healthcare industries. One product did not contain viable cells, and in another one product, no viable cells of 6 out of the 15 probiotics claimed were present. However, the culture-independent metagenomics approach revealed the presence of all the claimed species, leading to hypothesize that those not found by the culture-dependent

approach were dead or VBNC. Three products had loads of probiotics lower than those claimed on their labels, while the remaining 14 products contained loads of probiotics compliant with their labels. Two products were excluded from the metagenomics analyses since they provided low-quality reads. Three of the remaining 17 products were found to miss claimed species or to contain extra species.

Ten probiotic products intended for children's consumption were analyzed by cultivation, 16S rRNA gene sequencing and analysis, and metagenomic analysis of the 16S rRNA V3-V4 region (Dioso et al., 2020), finding that only 1 of the 10 products contained loads of probiotics lower than claimed on the label, whereas 4 of the 10 products contained strains of *Enterococcus* as extra species. Metagenomic analysis revealed that 1 of the products did not contain most of the microorganisms claimed on its label.

Shehata and Newmaster (2020) analyzed 182 probiotic products collected from various manufacturers in the United States and Canada, using cultivation, species- and/or strain-specific PCRs, and culture-independent high-throughput sequencing of V3 and V4 region of 16S rRNA gene. Five of the 132 single-strain products did not contain the claimed species but contained different species, whereas the remaining were compliant with the labeled species. Forty-five of the 50 multi-strain products were compliant with their labels as concerns the species content; whereas in the remaining 5 products, 6 claimed species were missing. Seventy-two samples were subjected to cultivation to assess loads of viable probiotics, finding that in 67 samples (93%) the viable counts were compliant or higher than those claimed on the labels. In 1 product no viable cells were found while in the remaining 4 samples the loads were lower than claimed on the labels. The HTS approach allowed to detection of undeclared species and their relative abundances in 14 out of 132 single-strain products and in 1 of the 50 multi-strain products.

Kesavelu, Rohit, Karunasagar, and Karunasagar (2020) assessed the authenticity of 20 probiotic products including 8 single strain and 12 multi-strain products by cultivation, metagenomic analysis of V3-V4 regions of the 16S rRNA gene, and species-specific PCR and sequence analysis of the ITS for the confirmation of *S. cerevisiae* subsp. *boluardii*. Only 2 products had loads of probiotics higher than those claimed on the labels, while the remaining had lower loads, whereas in 1 product claimed to contain heat-killed cells, no viable cells were found. The metagenomics approach provided no amplicons for the product containing heat-killed cells, as well as for the other 2 products that according to their labels contained only *S. boluardii* strains. Ten products were compliant with their claimed species whereas seven products missed the claimed species, contained extra species or species different from the one claimed on the label. ITS analyses confirmed the identity of all *S. cerevisiae* cultures. The PCR species-specific for *S. cerevisiae* confirmed the presence of this species in the products labeled with this species.

According to all the above-mentioned studies, the rate of mislabeling detected in probiotic products available on the market varies with the number and type of probiotic products analyzed as well as with the methods used to detect it. Most studies have targeted a few number of probiotic foods and/or supplements ranging from 4 to 25 products. Few studies assessed the authenticity of a more representative number of probiotic products, finding mislabeling in 42.3% 56.9%, 83%, 88%, 93%, 3.78% and 10% of the 58, 58, 72, 52, 55, 132 (single strain) and 50 (multi-strain) products analyzed (Aureli et al., 2010; Morovic et al., 2016; Shehata & Newmaster, 2020; Temmerman et al., 2003a). Single strain products seem to be more compliant with the label as concerns the species claimed. But

even the study by Sehata and Newmaster (2020) assessing the authenticity of 182 probiotic products (132 single strain and 50 multi-strain), was not complete as it did not assess the authenticity of all the claimed probiotic strains. Indeed, strain-specific PCRs were not used for all the probiotic microorganisms. Moreover, in this study, only 72 products were analyzed for the number of viable probiotic cells and anyway only one sample per probiotic product was analyzed when other studies have demonstrated that variations in the number of CFU or in the presence of the claimed species or of extra species among the different lots or among different samples of the same lot can occur (Lewis et al., 2016; Vermeulen et al., 2020).

In some cases "fantastic" names or incorrect names of the species were found on the labels, while in other cases the CFU of the probiotic microorganism were not specified (Aureli et al., 2010; Di Pierro et al., 2019; Masco et al., 2005; Morovic et al., 2016; Temmerman et al., 2003a; Vecchione et al., 2018; Weese, 2002; 2003). It will be not surprising if this kind of mislabeling will increase in the next future, especially due to the fact that the taxonomy of the *Lactobacillus* genus has been emended (Zheng et al., 2020, see the note for the reader at the end of the chapter) and, within this genus, the nomenclature or the species belonging to the *Lactobacillus casei*-group is controversial (Di Lena et al., 2015). Several products contained loads of viable probiotics higher than those claimed on the label. "Overfilling" of probiotic products with viable microbial cells in excess to the claimed number is a practice usually adopted by the producers as a mean to replace those that inevitably may die during the shelf-life of the products due to physical, biological and chemical stresses (Terpou et al., 2019). As a consequence, an amount of CFU not declared in the label may be found, which of course, alters the dose and, potentially, the health effects of the products (Fiore, Arioli, & Guglielmetti, 2020). The effects can further increase if postbiotics (also called ghost probiotics, paraprobiotics, metabiotics, parapsychobiotics, bacterial lysates, and tyndallized probiotic), which have been recently defined by the International Scientific Association of Probiotics and Prebiotics (ISAPP) as "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021), are present. Overall, this might be an issue, especially in vulnerable people, whose consumption of probiotic products may theoretically be linked to four specific types of side effects, including gene transfer, systemic infections, excessive immune stimulation in susceptible individuals, and deleterious metabolic activities (Kothari, Patel, & Kim, 2019).

On the other hand, a high number of probiotic products have been demonstrated to have a lower amount of viable probiotic cells than claimed on their labels. But the inability to detect VBNC cells of the probiotic microorganisms, which may result from the biotic and abiotic stresses encountered during the production and shelf-life of the products, is the main bottleneck of the enumeration by plating on selective media as mentioned before, which is still the gold and most used method to assess the CFU of viable probiotic cells present in a product. For all these reasons, enumeration approaches able to discriminate among viable, dead and VBNC cells of probiotics should be used.

3.5 Conclusion

The authenticity of probiotic foods and supplements is an emerging issue to be addressed. Numerous methods are available to assess it but each one has its strengths and its

weaknesses. Culturomics combined with molecular identification and typing methods still appear as the gold and most used method, but more appropriate and less time and labor-consuming approaches, such as PMA real-time quantitative PCR and PMA-metagenomics are arising as promising. Although only a few studies have addressed the authenticity of probiotic foods and supplements by assessing all the attributes claimed in the labels and using appropriate methods, a high number of these products results mislabeled and not compliant with their labels for either or both the species and the strain of the claimed probiotic, or the amount of viable probiotic cells. Further adequate studies, assessing a more representative number of samples of higher numbers of probiotic products by methods able to detect the probiotic at species and strain level and to enumerate them discriminating among viable, dead and VBNC cells, are highly demanded.

Note for the reader: Recently Zheng et al. (2020) proposed a reclassification of the genus *Lactobacillus* into 25 genera including the emended genus *Lactobacillus*, *Paralactobacillus* and 23 novel genera that are *Holzapfelia*, *Amylolactobacillus*, *Bombilactobacillus*, *Companilactobacillus*, *Lapidilactobacillus*, *Agrilactobacillus*, *Schleiferilactobacillus*, *Loigolactobacilus*, *Lacticaseibacillus*, *Latilactobacillus*, *Dellaglioa*, *Liquorilactobacillus*, *Ligilactobacillus*, *Lactiplantibacillus*, *Furfurilactobacillus*, *Paucilactobacillus*, *Limosilactobacillus*, *Fructilactobacillus*, *Acetilactobacillus*, *Apilactobacillus*, *Levilactobacillus*, *Secundilactobacillus*, and *Lentilactobacillus*. This is an example of a taxonomic amendment that is expected to impact the labeling of probiotic foods and supplements in which the contained probiotic microorganism(s) should be reported following the current and validly published nomenclature.

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CHAPTER

4

Indigenous probiotic microorganisms in fermented foods

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4.1 Introduction

In the last decades, consumers' interest in healthy foods is growing worldwide. Probiotic foods are among the most demanded functional foods due to their beneficial effects on consumers' health. Probiotic microorganisms isolated from humans are believed to be more resistant to the physicochemical stresses encountered along the gastrointestinal tract (Saarela, Mogensen, Fonden, Matto, & Mattila-Sandholm, 2000), but as highlighted by the FAO/WHO (2001) the specificity of their action rather than their source makes microorganisms probiotics. Several researchers have demonstrated that fermented foods may be a reservoir of probiotic microorganisms (Beena Divya, Kulangara Varsha, Madhavan Nampoothiri, Ismail, & Pandey, 2012; De Filippis, Pasolli, & Ercolini, 2020; Franz et al., 2014; Heller, 2001; Marco et al., 2017; Nuraida, 2015; Ogunremi, Sanni, & Agrawal, 2015; Rezac, Kok, Heermann, & Hutkins, 2018; Syal & Vohra, 2013; Tamang, Watanabe, & Holzapfel, 2016a). The interest in this category of food as reservoir and carrier of probiotics has grown notably in the last century but inconsistencies in the use of the term "fermented" led the International Scientific Association for Probiotics and Prebiotics (ISAPP) to draft a consensus statement on fermented foods (Marco et al., 2021). In particular, an expert panel of this association has defined fermented foods as "foods made through desired microbial growth and enzymatic conversions of food components" (Marco et al., 2021). This book chapter provides an overview of the isolation, characterization and use of indigenous probiotic microorganisms for the production of probiotic supplements and fermented foods.

Note for the reader: Among the potential probiotic microorganisms in fermented foods, some candidates belong to the genus *Lactobacillus* for which a reclassification into 25 genera has been recently proposed. In this chapter, examples reporting the old and the new nomenclature are provided, and the reader is invited to refer also to Zheng et al. (2020) for further detailed information on the proposed emended taxonomy.

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4.2 Fermented milk and dairy products

Milk is a nutritious food not only for humans but also for both desired and undesired microorganisms (Fusco & Quero, 2014; Fusco et al., 2020). As a mean to counteract the spoilage of milk, fermentation is one of the most ancient methods used to preserve it. Fermented milks and dairy products may be obtained by either natural fermentation or back-slopping of the milk previously thermized or boiled, or by fermentation due to the adjunct of starter or selected autochthonous microorganisms. The typical sensorial, textural, functional/probiotic properties of the final products are the result of the metabolic activity of a complex and heterogeneous microbiota, which may vary with the type of raw materials, the process, the equipment and the environment of production (Fusco, Oguntoyinbo, & Franz, 2017; Leroy & De Vuyst, 2004; Tofalo et al., 2020). Lactic acid bacteria are the main responsible for the transformation of milk in (probiotic/symbiotic) yogurt, fermented milk and dairy products but certain yeasts seem to play a functional and technological role in several fermented milk and dairy product fermentations (Tofalo et al., 2020).

Fermented milks, which are the most popular probiotic food carrier (Khorshidian, Yousefi, & Mortazavian, 2020), are among the most traditional sources of probiotic microorganisms (Tamang et al., 2016a, 2016b; 2020); however, numerous potentially probiotic microorganisms have been isolated also from traditional cheeses and other fermented dairy products.

4.2.1 Characterization and selection of indigenous probiotics from fermented milk and dairy products

A copious number of studies assessed the probiotic potential of microorganisms, mainly lactic acid bacteria, isolated from fermented milk and dairy products. Apart from the safety assessment, in vivo tests are required to demonstrate the probiotic features of the isolated microorganisms. Nevertheless, most studies focused solely on the *in vitro* tests (Table 4.1). A safety assessment, including susceptibility to antimicrobials, toxin production, metabolic activity, hemolytic activity, infectivity in immunocompromised animal models, side effects in humans, and adverse outcomes in consumers is needed (Huys et al., 2013). Nonetheless, numerous studies dealing with the probiotic potential of microorganisms did not perform the safety assessment or limited it to the antibiotic susceptibility assessment (Table 4.1).

Among the potentially probiotic microorganisms isolated from fermented milk and dairy products, lactic acid bacteria, mainly belonging to the *Lactobacillus* genus have been reported, with strains of *Lb. plantarum*, *Lb. fermentum*, and *casei*-group lactobacilli most frequently showing probiotic features (Table 4.1). Lactobacilli isolated from kefir belonging to *Lb. delbruekii* and *Lb. buchneri* groups described by Salvetti, Harris, Felis, and O'Toole (2018) mainly include *Lb. kefiranofaciens* and *Lb. kefiri* (now *Lentilactobacillus kefiri*) strains (Zheng et al., 2020) with demonstrated probiotic potential (Table 4.1). As arises also from Table 4.1, certain species of the *Pediococcus* genus, mainly *P. pentosaceus* and *P. acidilactici* and probiotic substances they produce, such as pediocins, are attracting attention for the probiotic features of the relevant strains (Jiang, Cai, Lv, & Li, 2021; Mehta, Arya, Goyal, Singh, & Sharma, 2013; Porto, Kuniyoshi, Azevedo, Vitolo, & Oliveira, 2017).

Within the potentially probiotic lactic acid bacteria, a separate discussion deserves enterococci. As reported in Table 4.1, certain strains of the *Enterococcus* genus, mainly belonging

TABLE 4.1 Potential probiotic microorganisms isolated from fermented milks and dairy products.

Strain	Species	Source	Tests	References
1QB77	*Lb. plantarum*	Brazilian artisanal cheese	In vitro functional and safety characterization: resistance to low pH, to bile salts, autoaggregation, hydrophobicity, adhesion, peristance to simulated gastrointestinal tract stress, biogenic amine genes detection, antagonistic activity, antimicrobial properties of the potential probiotic strain in microscale cheeses	Margalho et al. (2021)
SIM12; SIS16; 1734; Lh43	*Lb. helveticus*	Italian hard cheeses	In vitro functional characterization: ability to produce folate, antioxidant, proteolytic, peptidase and beta galactosidase activities. In vitro assessment of the immunomodulatory properties (cytokine production in culture media and in cheese)	Zago et al. (2021)
894	*Enterococcus faecium*	Turkish Tulum cheese	In vitro functional and safety characterization: tolerance to bile and simulated gastric juice, auto-aggregation, cell surface hydrophobicity, antibacterial activity, cholesterol removal, exopolysaccharide production, beta galactosidase actibity, antibiotic susceptibility, biogenic amine production, hemolytic activity	Özkan, Demirici, and Akin (2021)
SMVDUDB2	*Pediococcus acidilactici*	Kalarei (traditional cheese)	In vitro functional and safety characterization: tolerance to gastrointestinal conditions, hydrophobicity, auto and coaggregation, antibiotic susceptibility, antagonistic activity, exopolysaccharide production	Bhagat et al. (2020)
IS-10506	*Lb. plantarum*	Dadih (Indonesian fermented water buffalo milk)	Intestinal stem cells activation to counter inflammation in a rodent model. Level of blood lipopolysaccharide and immune response in HIV-infected children. Fecal secretory immunoglobulin A level and immune response in children younger than two years. Effect of IS-10506 and zinc supplementation on humoral immune response and zinc status of Indonesian pre-school children. Effect on Scoring Atopic Dermatitis Index (SCORAD) in children with atopic dermatitis. Renal tubular regeneration in pyelonephritic rats	Athiyyah et al. (2019, 2018), Kusumo, Bela, Wibowo, Munasir and Surono (2019), Prakoeswa et al. (2017), Prasetyo et al. (2020), Surono, Martono, Kameo, Suradji and Koyama (2014)
L3C21M6	*Lb. paracasei*	Artisanal Pico cheese	In vitro ability to degrade histamine and cholesterol-lowering activity. In vitro functional and safety characterization: bile salts deconjugation, resistance to gastric acidity, bile acid and pancreatin, adherence to Caco-2cell line, susceptibility to antibiotics, and presence of virulence genes.	Domingos-Lopes, Stanton, Riss, and Silva (2020)

(Continued)

TABLE 4.1 Cont'd

Strain	Species	Source	Tests	References
N16	*Lactobacillus (Lb.)* spp.[a]	Indonesian dadih (fermented buffalo milk)	In vitro functional characterization: resistance to acid and bile salts, adhesion to mucosal surface and antagonism against enteric pathogens	Harnentis et al. (2020)
FS103	*Lb. paracasei*	Sheep cheese	In vitro functional, safety and technological characterization: acidity and bile salt resistance, tolerance to gastric and intestinal juice models, antagonistic activity against pathogens and antibiotic resistance, survival in sheep and cow fermented milk during cold storage	Mangia et al. (2019)
GCC_19M1	*Lb. platarum*	Indian fermented raw milk	In vitro functional and safety characterization: acidity, bile and pancreatine tolerance, tolerance to gastric juice model, cell surface hydrophobicity, cellular auto-aggregation, glucose fermentation, NaCl tolerance, antagonistic activity against pathogenic bacteria, hemolytic activity and antibiotic susceptibility	Nath, Sikidar, Roy, and Deb (2020)
Os4; Kor14	*Lb. plantarum*	Polish regional cheeses	In vitro functional and technological characterization: antibiotic resistance, enzymatic profile, anti-staphylococcal activity, tolerance to the gastro-intestinal conditions, adhesion to caco-2 cells. Co-culture in skim milk and assessment of the anti-staphylococcal activity in this food matrix.	Ołdak et al. (2020)
YS5	*Lb. plantarum*	yogurt	In vitro and In vivo cholesterol assimilation, in functional and safety characterization:survival in acidic and bile salt condition, antimicrobial activity, antibiotic susceptibility, haemolytic activity, bile salt hydrolase activity	Nami, Bakhshayesh, Manafi, and Hejazi (2019)
PE24; PE25; PE44; PE61; PE85; PE86	*Lb. paracasei* (PE24, PE85, PE86); *Lb. rhamnosus* (PE25, PE44, PE61)	Piacentino Ennese PDO cheese	In vitro functional and safety characterization: DNAse, gelatinase, hemolytic and mucin degradation, biogenic amine production, antibiotic susceptimility and MIC determination, lysozyme, acidic and bile salts tolerances, survival during simulated gastrointestinal transit, antimicrobial activity, hydrophobicity, auto aggregation and co aggregation, adhesion to Caco 2 cells, antioxidant activity, anti-inflammatory activity, preliminary identification of metabolites responsible for antagonistic activity against pathogens, detection of genes for virulence and antibiotic resistance	Pino et al. (2019)
MSR101	*Lb. kefiri*	Chinese kefir grains	Characterization and in vitro anti-tumor activity of exopolysaccharide produced by the strain	Rajoka et al. (2019)

Strain	Species	Source	Tests	References
C1Lb21; GiLb5; G4Lb7	*Lb. brevis* (C1Lb21); *Lb. plantarum* (GiLb5); *Lb. pentosus* (G4Lb7)	Serpa Cheese (soft cheese)	In vitro functional and safety characterization: tolerance to simulated gastro intestinal tract (GIT), aggregation activity, cell surface hydrophobicity, growth on prebiotic, short chain fatty acid (SCFA) production, antibiotic susceptibility, biogenic amine production	Ruiz-Moyano et al. (2019)
A6	*Lb. brevis*	Minas artisanal cheese	In vitro functional and safety characterization: tolerance to artificial gastric juice, tolerance to biliary salts, antimicrobial susceptibility test, synthesis of hydrogen peroxide, antagonistic activity	Silva et al. (2019)
B7; D1	*Lb. plantarum* (B7); *Lb. rhamnosus* (D1)	Minas artisanal cheese	In vivo (in mice) and In vitro probiotic potential: tolerance to acidity and bile salts, sensitivity to antimicrobials, inhibitory activity	Valente et al. (2019)
Pro4; Pro7	*Lb. paracasei* (Pro4); *Lb. rhamnosus* (Pro7)	Raw and fermented camel milk	In vivo assessment of the mucosal immune response in mice. In vitro functional characterization: antimicrobial activity, phenol tolerance, tolerance to acidic pH, tolerance to bile salts and sodium chloride salt, hemolytic activity, antibiotic susceptibility.	Elbanna et al. (2018)
DD2	*Lb. kefiranofaciens*	Kefir	In vitro survivability in an experimental oral environment, antimicrobial activity, and anti-biofilm formation activity against *S. mutans* and *S. sobrinus*.	Jeong, Kim, Song, and Seo (2018)
C4	*Lb. plantarum*	Kefir	In vivo immunomodulatory effect in mice. In vitro evaluation of the fermentation properties and potential probiotic activity in batch culture systems. Physicochemical, nutritional, and organoleptic characterization of a skimmed goat milk fermented with C4.	Bergillos-Meca et al. (2014) Bujalance, Moreno, Jimenez-Valera, and Ruiz-Bravo (2007), Moreno-Montoro et al. (2018)
DP3; DP21	*Lb. plantarum* (DP3); *Lb. casei* (DP21)	Iranian artisanal cheeses	In vitro functional, technological and safety characterization: tolerance to acid and bile salts, adhesion to human intestinal cells, cholesterol assimilation, bile salt hydrolase activity, autoaggregation, cell surface hydrophobicity, antagonistic activity, exopolysaccharide production, antibiotic susceptibility hemolytic activity, production of probiotic fermented milk added with DP3 and DP21 and monitoring of the pH and microbial survival	Nami et al. (2018)
L3C1E8	*Lb. plantarum*	Pico cheese	Production of conjugated linoleic acid from free linoleic acid, biofilm formation. In vitro functional characterization: hydrophobicity, auto aggregation, resistance to low pH, bile salts and pancreatin, adhesion capacity to intestinal human cells, ability to inhibit the adhesion of E. coli ATCC 25922 to HT-29 cells.	Ribeiro et al. (2018)

(Continued)

TABLE 4.1 Cont'd

Strain	Species	Source	Tests	References
J20; J23; J24; J25; J27; J28; J32; J37	*Lb. fermentum* (J20, J23, J28, J32); *Lb. pentosus* (J24, J27, J37); *Lb. plantarum* (J25)	Cocido cheese	In vivo study of the immunomodulatory effect of milk fermented with the probiotic strains in vitro functional, technological and safety characterization: survival under pH and enzyme conditions, bile salt tolerance, auto aggregation and hydrophobicity, mucin degradation, haemolytic activity, antibiotic susceptibility, beta galactosidase activity, survival under cold, lyophilizing and freezing conditions, survival at pasteurization temperatures	Santiago Lòpez, Hernàndez-Mendoza, Mata-Haro, Vallejo-Cordoba, and Gonzàlez-Cordova (2018)
KCTC 5075	*Lb. kefiranofaciens*	Kefir	In vivo assessment of the therapeutic effects of extracellular vesicles derived from KCTC 5075 in mice with 2,4,6-trinitrobenzene sulfonic acid-induced inflammatory bowel disease	Seo, Park, Ko, Choi, and Kim (2018)
ACA-DC 2640; ACA-DC 4039; ACA-DC 264; ACA-DC 170	*Lb. plantarum* (ACA-DC 2640, ACA-DC 4039); *Streptococcus (S.) thermophilus* (ACA-DC 26, ACA-DC 170)	Traditional Greek dairy products	In vitro antibacterial activity, adherence ability and anti-inflammatory properties	Zoumpopoulou et al. (2018)
Kp10	*Pediococcus acidilactici*	Milk products	In vitro functional, technological and safety characterization: inhibitory activity, adhesion on mammalian cells and ileal mucosal epithelium, auto and coaggregation, adhesion to solvents, tolerance to phenol, haemolytic activity, enzymatic characterization, acidification and coagulation activities, qualitative proteolytic activity and starch hydrolysis.	Abbasiliasi et al. (2017)
B7	*Lb. plantarum*	Minas artisanal cheese	Protective effects of milk fermented by B7 on a Salmonella enterica serovar Typhimurium infection in BALB/c mice (in vivo)	Acurcio et al. (2017)
26; 27; 55; 56	*Lb. plantarum*	Portuguese raw ewe milk semisoft cheese	Production in milk fermented with the potentially probiotic strains of bioactive peptides with demonstrated (in vitro) antihemolytic, antimutagenic, anti-inflammatory, antioxidant and antimicrobial activities	Aguilar-Toalà et al. (2017)
SJRP30; SJRP145; SJRP146	*Lb. fermentum* (SJRP30); *Lb. casei* (SJRP145, SJRP146)	Water buffalo mozzarella cheese	In vitro functional and safety characterization: tolerance to simulated gastrointestinal conditions, adhesion to Caco-2 cells, presence of genes encoding adhesion, aggregation and colonization factors, antimicrobial activity, beta galactosidase activity, antibiotic susceptibility, mucin degradation, presence of genes encoding virulence factors, antibiotic resistance and biogenic amines	Casarotti et al. (2017)

Strain	Species	Source	Tests	References
M1	*Lb. kefiranofaciens*	Taiwanese kefir grains	In vivo and in vitro anti-allergic, anti-asthmatic, anti-colitis and immunomodulatory effect, in vivo oral toxicity. Effects of heat, cold, acid bile salt adaptations on the stress tolerance and protein expression	Chen and Chen (2013), Chen, Tang, and Chiang (2017), Chen, Wang, and Chen (2008, 2012, 2013a), Owaga, Chen, Chen, Chen, and Hsieh (2014, 2011), Hong, Chen, and Chen (2010)
LP4	*Lb. plantarum*	Minas artisanal cheese	In vivo protection against Salmonella Typhimurium infection in mice and in vitro functional and safety characterization: tolerance to biliary salts and artificial gastric juice, antagonistic activity, production of hydrogen peroxide, antimicrobial sensitivity	de Sant'Anna et al. (2017)
Lp3	*Lb. plantarum*	Fermented Tibetan yak milk	In vivo cholesterol lowering potential (in rats), in vitro cholesterol lowering test. In vitro probiotic characterization: tolerance to simulated gastrointestinal fluids, bile tolerance assay, vell surface hydrophobicity assay.	Ding et al. (2017)
17bKL1; 17bKL2; NS2KL9; NS1KM2; 14KM1; 6688 KM	*Kluyveromyces (K.) lactis* (17bKL1; 17bKL2; NS2KL9) *K. lactis K. marxianus* (NS1KM2; 14KM1; 6688 KM)	Fiore sardo cheese	In vitro functional and safety characterization: resistance to gastro-intestinal conditions, bile salt hydrolase activity, autoaggregation, hydrophobicity, adhesion to Caco-2 cells, antimicrobial activity, hemolytic activity, susceptibility to antifungal agents.	Fadda, Mossa, Deplano, Pisano, and Cosentino (2017)
DN1	*Lb. kefiranofaciens*	Kefir	In vivo modulation of gut microbiota and increase in fecal water content in mice induced by administration of DN1	Jeong et al. (2017)
K5	*Lb. paracasei*	Feta-type cheese	In vitro functional and safety characterization: tolerance to simulated gastro-intestinal conditions, antibiotic susceptibility testing	Plessas et al. (2017)

(Continued)

TABLE 4.1 Cont'd

Strain	Species	Source	Tests	References
XL10	*Lb. kefiranofaciens*	Tibetan kefir grain	In vivo and in vitro functional characterization: acid and bile salt tolerance, cell surface hydrophobicity, cell aggregation, gut microbiota profiling after XL10 oral administration, residual time and retained position of XL10 in the mouse gut by fluorescence imaging, quantitative distribution (by fluocitometry) of XL10 in ileums and colons of the mice after oral administration, colonization of XL10 in the gut (detected by quantitative PCR)	Xing, Tang, Geng, Zheng, and Wang (2017)
SJRP17	*Enterococcus durans*	cheese	In vitro functional and safety characterization: effect of different types of medications on the viability, presence of genes encoding virulence factors, vancomycin resistance and biogenic amine production, susceptimility to antimicrobial agents, tolerance to gastrointestinal tract conditions, tolerance to NaCl, bile salt hydrolase activity, hydrophobicity, auto and coaggregation, mucin degradation	Amaral, Silva, Casarotti, Nascimento, and Penna (2016)
KJ722784	*Lb. plantarum*	Chhurphe (Indian dried cottage cheese)	In vitro functional characterization: acid and bile tolerance, lysozyme tolerance, cholestorl removal, cell surface hydrophobicity, autoaggregation, antibacterial activity, antibiotic resistance, beta galactosidase activity, exopolysaccharide production, haemolytic activity	Angmo, Kumari, Tek, and Bhalla (2016)
KLDS 1.8701	*Lb. helveticus*	Chinese traditional cheese	In vitro functional characterization: tolerance to gastrointestinal conditions, antimicrobial activity	Bian et al. (2016)
KII13	*Lb. helveticus*	Fermented cow milk	In vivo cholesterol lowering activity (in mice) and In vitro functional probiotic characterization: orogastointestinal tolerance, antagonistic activity, adherence assay, bile salt hydrolase activity, cholesterol assimilation.	Damodharan, Palaniyandi, Yang, and Suh (2016)
S1K3 (MTCC5957)	*Lb. rhamnosus*	Indian fermented milk	In vivo assessment of the protective effect against Salmonella enterica in mice after long term (30 days) consumption of S1K3-fermented milk. In vitro characterization: antibacterial activities, adhesion to caco-2 cell line, protective effect against Salmonella enterica.	Kemkang et al. (2016)
1133; 1086-1; 1089; 1138; 1059; 1141; 1197	*Lb casei* (1133, 1089, 1138); *Lb. plantarum* (1086-1, 1141, 1197); *Lb. buchneri* (1059)	Tibetan qula (raw yak milk cheese)	In vitro functional and safety characterization: acid and bile salt tolerance, tolerance to the gastrointestinal conditions, antibacterial activity, sensitivity to antibiotics, cell surface hydrophobicity	Zhang et al. (2016)

Strain	Species	Source	Tests	References
FS10; PM8	*Lb. rhamnosus* (FS10); *Lb. paracasei* (PM8)	Ragusano and Pecorino Sicialiano cheeses	In vitro functional and safety characterization: transit tolerance in simulated upper gastrointestinal tract, bacterial adhesion capacity, hydrophobicity, co-aggregation and auto-aggregation activities, antimicrobial activity, antibiotic resistance, hemolytic and bile salt hydrolase activities	Caggia, De Angelis, Pitino, Pino, and Randazzo (2015)
SJRP55	*Leuconostoc mesenteroides*	Brazilian water buffalo mozzarella cheese	In vitro functional and safety characterization: tolerance to pH, bile and NaCl, bile salts deconjugation, auto and coaggregation, cell surface hydrophobicity, adherence to Caco-2 cells, enzymatic activity, resistance to medications and antibiotics	De Paula et al. (2015)
NS8	*Lb. helveticus*	Mongolian fermented koumiss	In vivo and ex vivo assessment of the anti-inflammatory attributes. Functional characterization: acid and bile salt tolerance, adhesion to Caco-2 cells, aggregation, cell surface hydrophobicity	Rong et al. (2015)
ZIM 2408	*K. lactis*	Traditional cheese from Serbia and Croatia	In vitro functional characterization: survival in GIT conditions, adhesion to intestinal cell lines and proliferation of gut-associated lymphoid tissue cells in the presence of non-viable yeast strain	Živković et al. (2015)
SJRP57	*Lb. delbruiekii* subsp. *bulgaricus*	Water-buffalo mozzarella cheese	In vitro functional and safety characterization: tolerance and effect of matrices on the survival under simulated gastrointestinal conditions, auto and co aggregation, hydrophobicity, beta galactosidase activity, antibiotics and medicines susceptibility, adhesion and virulence genes detection	Jeronymo-Ceneviva et al. (2014)
PRA205; PRA211; PRA172	*Lb. casei* (PRA205); *Lb. rhamnosus* (PRA211, PRA172)	Ripened Parmigiano Reggiano cheese	In vitro functional and safety characterization: Resistance to lysozyme, acid and salt bile, tolerance to simulated gastric and pancreatic juices, bile salts deconjugation, autoaggregation, cell surface hydrophobicity and susceptibility to antibiotics	Solieri, Bianchi, Mottolese, Lemmetti, and Giudici (2014)
LP2	*Lb. fermentum*	Tulum cheese	In vitro functional and safety characterization: survival under gastrointestinal conditions, enzymatic activity, antibiotic susceptibility, antimicrobial activity, cholesterol assimilation, adhesion ability to caco-2 cells.	Tulumoğlu, Kaya, and Şimşek (2014)
ZGPR3-18; BGGO5-47	*Lb. plantarum* (ZGPR3-18); *Lb. rhamnosus* (BGGO5-47)	Croatian fresh soft cheese and Serbian white pickled cheese	In vitro functional and safety characterization: survival to simulated gastrointestinal conditions, antibiotic susceptibility, bile salt hydrolase activity, cholesterol assimilation, assimilation of prebiotic substrates, cell surface hydrophobicity and adhesion to intestinal cell lines, capacity to modulate the immune response on GALT (gut associated lymphoid tissues) from rats.	Uroić et al. (2014)

(Continued)

TABLE 4.1 Cont'd

Strain	Species	Source	Tests	References
AQ71	*Enterococcus faecium*	Azerbaijani Motal cheese	In vitro functional and safety characterization: antimicrobial activity, effects of enzymatic and physicochemical treatments on antimicrobial activity, kinetics of bacteriocin production, partial purification of bacteriocin, detection of bacteriocin genes, tolerance to acid and bile salts, auto and co aggregation, antibiotic susceptibility, detection of virulence genes, enzymatic activity,	Ahmadova et al. (2013)
ZS07; K21	*Lb. plantarum*	Slovak Bryndza cheese	In vitro functional and safety characterization: resistance to simulated gastro intestinal conditions, beta halactosidase activity, bile salt deconjugation, antibiotic susceptibility testing, production of biogenic amines	Belicovà, Mikulasova, and Dusinsky (2013)
L10; L11; L13	*K. marxianus* (L10); *S. cerevisiae* (L11); *K. lactis* (L13)	Autochthonal cheese starters	In vitro functional and technological characterization: survival to simulated gastrointestinal digestion, hydrophobicity, antimicrobial activity, auto aggregation and coaggregation with pathogens.	Binetti, Carrasco, Reinheimer, and Suàrez (2013)
Mb26	*Lb. plantarum*	Farmhouse goat's milk cheese	In vitro functional and safety characterization: DNase activity, biogenic amine production, antibiotic resistance, detection of functional enzymatic activities, adhesion to cellular lines and inhibition of L. monocytogenes CECT 4032, resistance to HCl and oxgall, bile salt hydrolase activity, biofilm formation, production of hydrogen peroxide and bacteriocines, detection of plantaricin genes	Lavilla-Lerma, Pérez-Pulido, Martínez-Bueno, Maqueda, and Valdivia (2013)
Three halotolerant lactobacilli (name of the strains not provided)	*Lb. plantarum, Lb. pentosus, Lb. acidipiscis*	Ripened Mexican tropical cheese (double cream Chiapas cheese)	In vitro functional characterization: tolerance to orogastrointestinal transit, hydrophobicity, adhesion to mucin, antimicrobial activity, beta galactosidase activity, bile salt deconjugation	Melgar-Lalanne, Rivera-Espinoza, Reyes Méndez, and Hernández-Sánchez (2013)
FT259	*Lb. paraplantarum*	Brazilian semi-hard artisanal cheese	In vitro functional and safety characterization: antilisterial activity and protease susceptibility of the antimicrobial compound produced by FT259; antimicrobial spectrum, acid and bile salts tolerance, survival in simulated gastric juice, adhesion to Caco-2 cells, antibiotic susceptibility	Tulini, Winkelströter, and De Martinis (2013)
VJ13	*Pediococcus pentosaceus*	Idly butter (Indian fermented food)	In vitro functional and safety characterization: resistance to acidic, gastrointestinal conditions and bile salt, bile salt hydrolase, cholesterol assimilation, auto-aggregation and co-aggregation, beta galactosidase activity, antagonistic activity, in vitro cell adhesion and inhibition of pathogen adhesion, antibiotic resistance, haemolytic activity	Vidhyasagar and Jeevaratnam (2013)

Strain	Species	Source	Tests	References
SM-B; SM-1	*Lb. brevis* (SM-B); *Lb. plantarum* (SM-1)	Brazilian regional ovine cheese	In vitro functional and safety characterization: acid and bile salts tolerance, bile salt deconjugation autoaggregation, hydrophobicity, beta galactosidase activity, antioxidant activity, antagonistic activity, hemolysis	Meira, Helfer, Velho, Lopes, and Brandelli (2012)
T3L	*Lb. coryniformis* subsp. *Torquens*	Tibetan fermented yak milk	In vitro immunomodulating activity	Tuo et al. (2011)
F6	*Lb. fermentum*	Traditional dairy product	In vitro functional characterization: acidic and gastrointestinal transit tolerance, auto and co-aggregation	Bao et al. (2010)
BFE6058; BFE6059	*Lb. acidophilus*	Maasai femented milk products	In vitro functional and safety characterization: acid tolerance, tolerance to simulated stomach duodenum passage, susceptibility to antibiotics, biogenic amine production, DNAse activity, mucin degradation, haemolytic activity, hydrophobicity, adhesion properties to human cell lines, bile salts deconjugation, cholesterol assimilation, auto aggregation, antigenotoxic properties (ability to protect cells from DNA damage).	Mathara et al. (2008)
CDCA 8348	*Lb. kefiri*	Kefir	In vitro protective action against the invasion of Salmonella enterica serovar Enteritidis (Salmonella enteritidis). In vitro ability to produce antimicrobial compounds. In vitro safety characterization (hemolytic activity; susceptibility to antibiotics) and in vivo proinflammatory cytokines secretion and absence of translocation of microorganisms to blood, spleen, and liver in mice orally administered with and without CDCA 8348. In vivo impact of CDCA 8348 oral administration for 21 days on the mucosal immune response and gut microbiota in mice. In vitro ability to antagonize the cytotoxic effects of clostridial toxins on Vero cells. In vitro adhesion properties.	Carasi et al. (2015, 2014a, and b, 2012), Golowczyc, Mobili, Garrote, Abraham, and de Antoni (2007)
56; D4; D7; H78; SR82; SR41; SR246	*S. cerevisiae*	Danish blue veined cheese, Gorgonzola, Italy Cheese, Europe Spontaneous fermented cow milk	In vitro functional characterization: adhesion to nontumorigenic porcine jejunal epithelial cell line, tolerance to low pH and bile, cytokine assay	van der Aa Kühle, Skovgaard, and Jespersen (2005)

(Continued)

TABLE 4.1 Cont'd

Strain	Species	Source	Tests	References
832	*S. cerevisiae*	Feta cheese	In vitro cholesterol assimilation	Psomas, Fletouris, Litopoulou-Tzanetaki, and Tzanetakis (2003)
CYC 10058	*Lb. kefiranofaciens*	Kefir	In vitro functional characterization:adhesion, resistance to acid and bile salts, antimicrobial profile, inhibition of Salmonella typhimurium adhesion to Caco-2 cells, production, purification and structural characterization of kefiran.	Santos, San Mauro, Sanchez, Torres, and Marquina (2003)

[a]Gram positive bacilli bacteria, catalase and oxidase negative, classified as *Lactobacillus* spp.

to the *E. faecium* and *E. faecalis* species, have been isolated and subjected to probiotic and safety characterization (Table 4.1), but the use of enterococci as probiotics is debated so that it has not the GRAS status and is not recommended for the QPS list (Hanchi, Mottawea, Sebei, & Hammami, 2018).

Apart from lactic acid bacteria, yeasts mainly belonging to the *Saccharomyces cerevisiae* and *Kluyveromyces* (K.) *lactis* and *K. marxianus* species with probiotic potential have been isolated from fermented milk and dairy products but only in vitro functional and safety characterization of these strains have been carried out (Table 4.1). Moreover, *K. marxianus*/*C. kefir* has been recently recognized by EFSA as a significant opportunistic fungus (Tofalo et al., 2020), whereas opportunistic *S. cerevisiae* strains not only of clinical but also of food origin are arising as opportunistic pathogens. EFSA confirmed the QPS status of *S. cerevisiae* intended to be added in the food or feed chain as viable cells imposing the prerequisite of demonstrating the absence of resistance to antimycotics compounds used in human medicine and inability to grow above 37°C (Tofalo et al., 2020).

Apart from the *in vitro* and *in vivo* functional and safety characterization, potential probiotic microorganisms require mandatory analyses to be performed. Identification at species and strain level of the potentially probiotic microorganism is a pre-requisite crucial to characterize a probiotic microorganism and to verify the authenticity of a probiotic food/supplement (Di Lena et al., 2015; Fusco, Fanelli, & Chieffi, 2021). However, changes in the taxonomy of several probiotic species as well as the application of inadequate/improper species- and strain-specific identification methods are challenging this issue. This is the case, for example, of the *Lactobacillus* genus recently emended and reclassified by Zheng et al. (2020).

In order to use in probiotic foods and supplements, the potential probiotic strains should be investigated for their technological features, as well as for their ability to survive in adequate amounts (also exerting the beneficial effects) to the technological stresses encountered during the production of such foods and supplements. As shown in Table 4.1, only a few studies assessed these features (Abbasiliasi et al., 2017; de Souza, Borgonovi, Casarotti, Todorov, & Penna, 2019; Kemkang, kapila, Shanmugam, Reddi, & Kapila, 2016; Mangia, Saliba, & Deiana, 2019; Margalho et al., 2021; Nami et al., 2018; Ołdak, Zielińska, Łepecka, Długosz, & Kołożyn-Krajewska, 2020). In particular, Margalho et al. (2021) manufactured probiotic microcheese also artificially contaminated with *Listeria monocytogenes* and *Staphylococcus aureus* strains in order to assess the antimicrobial properties of the probiotic *L. plantarum* 1QB77, isolated from Brazilian artisanal cheese, against these pathogens in the food matrix, besides verifying the ability of the probiotic strain to survive to stress encountered during manufacturing (low pH and salt content). Once produced, the contaminated probiotic microcheeses were also subjected to in vitro gastrointestinal digestion. The probiotic strain demonstrated high tolerance to the stresses encountered during the microcheese production reaching a load of ca. 9 log CFU/g at the end of the ripening. It was also able to lower loads of *S. aureus* and *L. monocytogenes* of ca. 3.5 log units during the production, ripening and simulated gastrointestinal transit of the contaminated probiotic microcheese (Margalho et al., 2021).

Ołdak et al. (2020) successfully assessed the loads of two potentially probiotic strains isolated from Polish regional cheeses in skim milk stored either at 8°C or at 37°C, as well as their protective activity against *S. aureus* during 120 of storage. Two *L. casei* and *L. fermentum* strains isolated from mozzarella cheese were evaluated for their ability to produce fermented milk in co-culture with a commercial *S. thermophilus* strain (de Souza et al., 2019). Moreover,

the survival of the two strains was assessed in simulated gastrointestinal tract conditions during refrigerated storage of fermented milk (de Souza et al., 2019). The *L. fermentum* strain resulted to need more time to reach the maximum acidification rate in respect to the *L. casei* strain, whereas the other kinetic parameters were similar (de Souza et al., 2019). In the refrigerated fermented milk under gastrointestinal (GIT)-simulated conditions these strains were present in loads of more than 8 log CFU/mL, demonstrating good viability of both strains under the harsh conditions they undergo during the (simulated) GIT transit (de Souza et al., 2019). Mangia et al. (2019) assessed the survival of a potentially probiotic *L. paracasei* strain isolated from sheep cheese in sheep and cow fermented milks during cold storage. The acidifying capacity of the strain was higher in sheep than in cow milk although it was able to acidify both types of milk during 12 hours of fermentation (Mangia et al., 2019). The strain exhibited a high rate of survivability in both types of fermented milk during 21 days of refrigerated storage at 4°C (Mangia et al., 2019). Nami et al. (2018) assessed the effectiveness of potentially probiotic *L. plantarum* DP3 and *L. casei* DP21 isolated from artisanal cheeses in producing fermented milk. Both strains reduced the fermentation time to 10 hours, remaining viable at loads higher than 10^8 cfu/mL (Nami et al., 2018).

Abbasiliasi et al. (2017) assessed the technological potential of the potentially probiotic *P. acidilactici* kp10 strain isolated from a traditional dried curd in terms of enzymatic characterization by using the API ZyM strips (API Identification Sustams, bioMerieux, France), the acidification and coagulation activities in skim milk, proteolytic activity and starch hydrolysis in M17 broth. This strain showed good starter culture properties as it endowed strong peptidase and esterase-lipase activities, discrete amylolactic activity, low level of alkaline phosphatase and absence of proteinase activity (Abbasiliasi et al., 2017). Moreover, it was able to lower the pH to 5.3 and showed a strong coagulating activity (Abbasiliasi et al., 2017). Kemkang et al. (2016) successfully demonstrated the ability of fermented milk with probiotic *L. rhamnosus* S1K3 isolated from the Indian fermented milk called Dahi to protect mice from *Salmonella* by enhancing immune and nonimmune protection mechanisms at intestinal mucosal level.

None of the studies listed in Table 4.1 has focused on clinical trials to assess and measure the effects of the fermented milk/dairy product added with the potentially probiotic indigenous strain on the human health, which are of outmost importance to demonstrate the effectiveness of the probiotic fermented food.

However, it should be mentioned that for several fermented milk and dairy products, from which potentially probiotic strains are being isolated, the beneficial effects have been demonstrated in clinical trials (De Filippis et al., 2020; Ghosh, Beniwal, Semwal, & Navani, 2019; Nuraida, 2015; Sakandar & Zhang, 2021; Şanlier, Gökcen, & Sezgin, 2019; Tamang, Shin, Jung, & Chae, 2016b).

4.3 Indigenous probiotics isolated from non-dairy fermented foods

Potential probiotic microorganisms have been also isolated from fermented food matrices other than milk and dairy products, including fermented fruits and vegetables and fermented products of animal origin other than milk, including fermented meat and fish. The next paragraphs provide an overview of indigenous probiotic microorganisms isolated from these matrices and characterization regarding their functional, technological and safety attributes.

4.3.1 Fermented vegetables and fruits

Fresh vegetables and fruits are extremely perishable food products due to high water activity and nutritive value, and, consequently, they are characterized by their relatively short shelf life, requiring different preservation methods to prolong their stability. In order to meet the demand of the increased world's population on every continent for safe vegetables and fruits, fermentation is the oldest biotechnological and proven natural preservation method (Fleming, Kyung, & Breidt, 1995; Oguntoyinbo et al., 2016) to provide nutritious and probiotic foods, improving the general attractiveness of fruits and vegetables. In this sense, fermented vegetables and fruits are a potential source of nutrients and probiotics with health benefits especially for populations in underdeveloped and developing countries unable to access other expensive preservation methods, such as refrigeration, freezing and modified atmosphere. Furthermore, these products as "unconventional sources" contribute to satisfy demands for probiotics required by a larger segment of world population, such as lactose intolerant individuals, dyslipidemics, vegetarians and vegans, for which usual dairy probiotics are not appropriate (Di Cagno, Coda, De Angelis, & Gobbetti, 2013). These fermented products may enhance livelihoods of millions of people around the world (Battcock & Azam-Ali, 2001; FAO, 1998; Panda, Parmanik, Sharma, Panda, & Ray, 2005).

Fermented vegetables and fruits are associated with social and cultural aspects of different races, thus fermentation is mainly following traditions and relied on spontaneous processes carried out by the autochthonous consortia of microorganisms, such as lactic acid bacteria (LAB), yeasts and fungi, present in the raw material, utensils, containers, condiments and the environment (Franz et al., 2014). Therefore, these products constitute the best and rich sources to explore for new non-dairy and non-intestinal healthy probiotic candidates (Granato, Branco, Nazzaro, Cruz, & Faria, 2010; Prado, Parada, Pandey, & Soccol, 2008) and the differences in the raw materials and ingredients used to make fermented foods are the main factors leading to the different available probiotic species or strains in the food sources. Among fermented vegetables and fruits, fermented root vegetables "FRV" (carrots, radishes and sweet potato), fermented leafy vegetables "FLV" (cabbage, mustard and lettuce), fermented mixed vegetables "FMV", fermented vegetable fruits "FVF" (olives, tomatoes and cucumbers), fermented vegetable juices "FVJ" (carrots, onion and sweet potato) and fermented fruits "FF" (apples, lemons and pears), in which mixed cultures of yeasts, bacteria and fungi may participate in parallel, or act in a sequential manner, have been found. This uncontrolled fermentation carried out by LAB as the main protagonists under environmental conditions shaped the diversity of such products producing typical and unique fermented products (Holzapfel and Wood, 2014). These fermented foods represent an important and valuable cultural heritage in most regions of geographical zones regarding their organoleptic properties (flavor, aroma and texture) and their huge genetic potential of valuable but hitherto undiscovered strains.

4.3.1.1 Characterization and selection of indigenous probiotics from vegetable and fruit fermentation

Traditional fermented fruits and vegetables were highly consumed throughout history, and they are characterized by a diverse microbial community-culturable and non-culturable whose composition depends on the characteristics of the food matrix and geographical origin. Culture-dependent and -independent methods (especially omics) thrown new light on the microbial

diversity of several naturally fermented foods from different regions, such as Europe, Africa, Asia, North and South America. Under favorable conditions, these foods obtained through spontaneous fermentation carried out by indigenous microorganisms exhibit the predominance of LAB with probiotic potential in a changing environment through fermentation process. Due to the great importance of these fermented products as suitable carrier for probiotics, in the last decades several reports were focused on isolation, identification and characterization in vitro of potential indigenous probiotics from raw vegetables and fruits and their by-products, and also from local fermented products (Ranadheera, Baines, & Adams, 2010; Sornplang et al., 2016). Studies of these new healthful ingredients as potential probiotics with the aim to make functional vegetables and fruits were based on the assay of tolerance to gastrointestinal conditions (acids and bile), adhesion to human intestinal cells (Caco Cells), gut colonization, antimicrobial activity against common pathogens and beneficial effect on health (antitumoral effect, modulation of immune system, etc.), as well as safety assessment at strain level as established by the World Health Organization (Abriouel et al., 2012; Martins et al., 2013; Peres, Peres, Hernández-Mendoza, & Malcata, 2012). Furthermore, probiotic of vegetable origin exhibited interesting survival characteristics due to the harsh conditions in their original habitat besides the high amounts of prebiotic compounds present in the plants (oligosaccharides), which stimulate their growth and thus improve their functionality. This "first colonization" of vegetables by lactic acid bacteria (LAB) and inhibition of spoilage and pathogenic microorganisms allows their predominance through fermentation process via acids and antimicrobial compounds production, and thus serves for the subsequent colonization of gut which exhibits similar conditions.

Indigenous probiotic LAB isolated from raw and fermented vegetables and fruits are promising candidates in controlled fermentations of these products (Abriouel et al., 2012; Russo et al., 2017; Szutowska, 2020) due to their inherent stability and high capacity to survive hostile conditions encountered in their habitats. LAB as dominant fermenting microbiota are mainly represented by *Lactobacillus* (or novel genera after emended description of the genus *Lactobacillus;* Beijerinck, 1901; Zheng et al., 2020), *Pediococcus, Leuconostoc, Weissella* and *Enterococcus,* as the most common genera involved in the fermentation of vegetables and fruits, being *Lactobacillus* (or novel genera) the genus most studied due to its genetic heterogeneity, metabolic versatility, role in food fermentation and its use as probiotic. In this sense, the ubiquitous and the metabolic versatile most frequently isolated species was *L. plantarum* (now *Lactiplantibacillus plantarum*).

Unlike other fermented foods (dairy and alcoholic beverages), scarce information is available about the use of starter cultures in fermented vegetables and fruits. In this sense, some fruits and vegetables were fermented/or evaluated for fermentation with autochthonous probiotic LAB improving their safety, organoleptic properties (texture, color and sensory properties) and functional and nutritional value (concentration of vitamin C, antioxidant compounds, oxidative stress, etc.). Thus, several fermented vegetables and fruits have been made as functional foods, such as cactus pear juice fermented with *L. plantarum* S-811 (Verón et al., 2019); carrots, French beans or marrows fermented with *L. plantarum* M1, *Ln. mesenteroides* C1 and *P. pentosaceus* F4 (Di Cagno et al., 2008); tomato juice fermented with *L. plantarum* POM1 and POM35 (Di Cagno et al., 2009); red and green smoothies fermented with *L. plantarum* Pr3, *W. cibaria* B1, B6 and P9, *L. plantarum* K3 and *L. pentosus* P1 (Di Cagno, Minervini, Rizzello, De Angelis, & Gobbetti, 2011a); sweet red cherry fermented with *P. pentosaceus* SWE5 and *L. plantarum* FP3 (Di Cagno et al., 2011b); bitter melon juice fermented with

L. plantarum BET003 (Mazlan, Annuar, & Sharifuddin, 2015); date fruit puree fermented with *L. plantarum* T1.3, T1.15, O1.8, T2.8 and T1.18 (Di Cagno et al., 2017); broccoli juice fermented with *Ln. mesenteroides* B-25 and B4-25 (Xu, Luo, Bao, Liao, & Wu, 2018); Indonesian sauerkraut fermented with *L. plantarum* sa28k (Kusumawati, Jenie, Setyahadi, & Hariyadi, 2003); kimchi fermented with *Ln. mesenteroides* F27and *L. plantarum* C182 (Lee et al., 2016); Aloreña green table olives fermented with *L. pentosus* MP-10 (Abriouel, Benomar, Lucas, & Gálvez, 2011, 2017); leek fermented with *L. plantarum* IMDO 788, *L. sakei* IMDO 1358 and *L. mesenteroides* IMDO 1347 (Wouters et al., 2013).

In this section, the selected autochthonous good starters and potential probiotic LAB isolated from fermented vegetables and fruits rather than raw materials with the aim to make safe and functional fermented foods are highlighted. The advantage of selected LAB from food fermentations is their capacity to predominate the fermentation process and overcome different barriers under environmental conditions and following the technical procedures in a determined region. Thus, a high microbial diversity is displayed by these fermented foods providing new opportunities to search for new strains with health-promoting and technological features that can be used as probiotics or starters in controlled fermentation processes of functional fruits and vegetable foods. Contrary, the native LAB isolated from raw sources exhibited less diversity and uncertainty about the success of the fermentation process. The fermented products subjected to the isolation of indigenous probiotic LAB include fermented root vegetables (FRV), fermented leafy vegetables (FLV), fermented mixed vegetables (FMV), fermented vegetable fruits (FVF), fermented vegetable juices (FVJ), and fermented fruits (FF).

Among the FRV, carrots, radishes, garlic and sweet potato were used as unique or main ingredients in different fermented vegetables depending on the geographical region and the traditional practices employing a variety of condiments to make a typical product. *L. plantarum* was the unique (i.e., garlic or carrots) or the main species isolated from different fermented root vegetables, however other LAB species were isolated and characterized (Table 4.2). *Lactiplantibacillus plantarum* (before *Lactobacillus plantarum*) and *L. fermentum* were isolated from fermented radishes, however additional species were isolated, such as *L. brevis*, *Ln. fallax* and *P. pentosaceus* from Indian product (Tamang, 2009) since *L. pentosus* was isolated from Korean product (Damodharan, Palaniyandi, Yang, & Suh, 2015). Fermented leek showed a diverse LAB profile depending on the region, such as *L. plantarum*, *L. sakei* and *Ln. mesenteroides* from the Belgian product (Wouters et al., 2013), and *W. confusa* and *L. plantarum* from the Korean product (Yang et al., 2014). On the other hand, fermented bamboo shoot products of Arunachal Pradesh exhibited a diverse LAB profile with probiotic potential, such as *L. plantarum* (*Lactiplantibacillus plantarum*), *L. brevis* (*Levilactobacillus brevis*), *L. casei* (*Lacticaseibacillus casei*), *T. halophilus* (Ekung); *L. plantarum, L. fermentum* (*Limosilactobacillus fermentum*, Eup), *L. plantarum,* and *L. lactis* (Hirring) (Tamang & Tamang, 2009).

Similarly, traditionally FLV were reported as a source of probiotic *L. plantarum* and other species, such as *P. pentosaceus* (pickled cabbage or sauerkraut, Goyang), *P. acidilactici* (Inziangsang), *L. mesenteroides* (Portuguese Sauerkraut Kimchi), *L. brevis* (Inziangsang and Goyang), *L. gasseri* in Vietnamese fermented radish leaves, and *E. faecium* and *L. Lactis* in Goyang (Table 4.2).

On the other hand, traditionally fermented olives, tomatoes, cucumbers, eggplants and other local vegetable fruits (FVF) were subjected to probiotic isolation and characterization (Table 4.2).

TABLE 4.2 Traditional and emerging fermented vegetable and fruits and their corresponding indigenous probiotics.

Category of fermented foods	Fermented product	Raw material	Autochthonous probiotic strains	References
Root vegetables (FRV)	Pickled carrots	Carrots	*L. plantarum*	Dallal et al. (2017)
	Sinki	Radishes	*L. fermentum*, *L. brevis*, *L. plantarum*, *Ln. fallax*, *P. pentosaceus*	Tamang (2009) Mir, Raja, and Masoodi (2018)
	Fermented radish	Radishes	*L. plantarum*, *L. pentosus*, *L. fermentum*	Damodharan et al. (2015)
	Pickled garlic	Garlic	*L. plantarum*	Dallal et al. (2017)
	Fermented leek	Leek	*L. plantarum*, *L. sakei* and *Ln. mesenteroides; W. confusa* and *L. plantarum*	Wouters et al. (2013)Yang et al. (2014)
	Ekung	Bamboo shoot	*L. plantarum*, *L. brevis*, *L. casei*, *T. halophilus*	Tamang and Tamang (2009)
	Eup	Bamboo shoot	*L. plantarum*, *L. fermentum*	Tamang and Tamang (2009)
	Hirring	Bamboo shoot	*L. plantarum*, *L. lactis*	Tamang and Tamang (2009)
Leafy vegetables (FLV)	Pickled cabbage	Cabbage	*L. plantarum* and *P. pentosaceus*	Chiu, Tsai, Hsih, and Tsen (2008)
	Portuguese Sauerkraut Kimchi	Cabbage	*Ln. mesenteroides*, *L. plantarum*	Lee et al. (2016)
	Chineese Sauerkraut Kimchi	Cabbage	*P. pentosaceus* and *L. plantarum*	Chiu et al. (2008)
	Croatian Sauerkraut	White cabbage heads	*L. plantarum*, *L. brevis* and *Ln. mesenteroides*	Beganović et al. (2014)
	Inziangsang	Mustard	*L. plantarum*, *L. brevis*, *P. acidilactici*	Yan, Xue, Tan, Zhang, and Chang (2008)
	Goyang	Maganesaag	*L. plantarum*, *L. brevis*, *Lc. Lactis*, *P. pentosaceus*, *E. faecium*	Tamang and Tamang (2007)
	Dua-muoi (Vietnamese fermented radish leaves)	Radish	*L. gasseri*	Ho and Wills (2013)

Category of fermented foods	Fermented product	Raw material	Autochthonous probiotic strains	References
Vegetable fruits (FVF)	Pickled olives	Olives	*L. plantarum*	Dallal et al. (2017)
	Aloreña green olives	Green olives	*L. pentosus*	Abriouel et al. (2012)
	Conservolea and Halkidiki green olives	Green olives	*L. plantarum, L. pentosus, L. paracasei*	Argyri et al. (2013)
	Conservolea and Kalamata	Black olives	*L. pentosus, Ln. mesenteroides*	Doulgeraki et al. (2012)
	Pickled eggplant	Eggplant, local aromatic vegetables	*L. plantarum*	Dallal et al. (2017)
	Khalpi	Cucumbers	*L. plantarum, L. brevis, Ln. fallax*	Tamang (2009)
	Fermented *Cornus officinalis* fruits	*Cornus officinalis* fruits	*L. plantarum* and *L. casei*	Tang et al. (2018)
Mixed vegetables (FMV)	Kimchi	Cabbage, garlic, ginger, radish, red pepper powder	*L. sakei, L. plantarum, Lc. Lactis, Ln. mesenteroides*	Lee et al. (2011, 2015, 2016)
	Pickled cabbage	Cauliflower, cucumber, carrot, celery, garlic	*L. plantarum*	Dallal et al. (2017)
	Pickled cabbage and carrots	Cabbage, carrot	*L. plantarum*	Dallal et al. (2017)
	Pickled cucumbers	Cucumber, garlic, pepper, local aromatic vegetables	*L. plantarum*	Dallal et al. (2017)
	Makdoos	Eggplant, garlic, walnuts, dried pepper	*L. plantarum, L. pentosus, L. brevis, L. rhamnosus, L. salivarius*	Mahasneh and Mahasneh (2017)
	Gundruk	Cabbage, radish, mustard leaves, cauliflower leaves	*Pediococcus* spp., *Lactobacillus* spp.	Dahal, Karki, Swamylingappa, Li, and Gu (2005), Tamang (2009)
	Pickled liteh	Eggplant, cabbage, garlic, pepper	*L. plantarum*	Dallal et al. (2017)
	Fermented mixed vegetables	Carrot, green beans, snake gourd, eggplant, green chili, bitter gourd, turnip, beet root, ladies finger, radish, cabbage	*L. animalis, L. rhamnosus, L. fermentum* and *L. reuteri*	Kazemipoor, Radzi, Begum, and Yaze (2012)

(Continued)

TABLE 4.2 Cont'd

Category of fermented foods	Fermented product	Raw material	Autochthonous probiotic strains	References
Vegetable juices (FVJ)	Shalgam	Black carrots	*L. plantarum, L. brevis, Lactobacillus* spp.	Ekinci et al. (2016)
	Broccoli juice	Broccoli	*Ln. mesenteroides, Weissella* spp., *E. gallinarum* and *Lactococcus* spp.	Xu et al. (2018)
	Cherries juice	Cherries	*P. pentosaceus* and *E. gallinarum*	Xu et al. (2018)
	Ginger juice	Ginger	*Lactobacillus* and *Weissella*	Xu et al. (2018)
	White radish juice	White radish	*W. confusa* and *E. durans*	Xu et al. (2018)
	White-fleshed pitaya juice	White-fleshed pitaya	*Streptococcus* and *Lactococcus*	Xu et al. (2018)
Fruits (FF)	Fermented mango juice	Mango	*Ln. mesenteroides, P. pentosaceus* and *L. brevis*	Liao et al. (2016)
	Bambangan	Wild mango	*L. plantarum*	Ng, Koon, Padam, and Chye (2015)
	Pineapple tepache	Pineapple	*L. pentosus*	Escobar-Ramírez et al. (2020)
	Yan-taozih	Peaches and plums	*Ln. mesenteroides, L. lactis* subsp. *lactis, L. brevis, W. cibaria, W. paramesenteroides, W. minor* and *E. faecalis*	Chen et al. (2013b)
	Red wine	Grapes	*O. oeni, P. parvulus, P. pentosaceus, L. casei* and *L. plantarum*	García-Ruiz et al. (2014)

The most common genera were *Lactobacillus* (now *Lactiplantibacillus; L. plantarum* followed by *Lactiplantibacillus pentosus*) and *Leuconostoc* (*L. mesenteroides* and *L. fallax*) (Table 4.2).

Fermentation of a mixture of different vegetables (FMV) is aimed to produce a traditional product in several regions, being involved a diversity of microbial fermenting population. However, probiotic *L. plantarum* was the predominant species in all fermented products (Table 4.2). Furthermore, pediococci and other lactobacilli were isolated, such as *L. animalis* (*Ligilactobacillus animalis*), *L. rhamnosus* (*Lacticaseibacillus rhamnosus*), *L. pentosus* (*Lactiplantibacillus pentosus*), *L. fermentum* (*Limosilactobacillus fermentum*), *L. salivarius* (*Ligilactobacillus salivarius*), and *L. brevis* (*Levilactobacillus brevis*) (Table 4.2). FVJ as sources of probiotics using different vegetables such as carrots, cherries, broccoli, ginger, radish, grapes were evaluated and the probiotics isolated from these fermented beverages were diverse, including *Weissella*, lactobacilli, pediococci, lactococci, enterococci, and leuconostocs (Table 4.2). Similarly, probiotics isolated from fermented fruits (FF), such as grapes, pineapple, mango, peaches and plums, revealed a great diversity with the presence of lactobacilli (*L. plantarum, L. brevis, L. casei,* and *L. pentosus*), pediococci (*P. pentosaceus* and P. *parvulus*), *Weissella* sp., *Ln. mesenteroides, Lc. Lactis,* and *O. oeni* (Table 4.2).

4.3.1.2 *Benefits of indigenous probiotics from fermented vegetables and fruits*

Selection of appropriate autochthonous LAB and their application as starter cultures in a controlled fermentation provide advantages to fermentation process and also to human health (Di Cagno et al., 2013). The autochthonous LAB are adapted to their specific plant matrix allowing a rapid decrease of pH (with enough amount of lactic acid) and rapid inhibition of undesirable microbiota instead of traditionally spontaneous fermentation, with the aim to get a final product defined as "functional food" with determined desirable organoleptic, technological and nutritional properties (Di Cagno et al., 2013; La Anh, 2015; Vera-Pingitore et al., 2016). When the selected starters have also a probiotic potential, health benefits are guaranteed, receiving increased attention especially in "health-conscious" developed countries. Among these beneficial effects on health, the modulation of gut microbiota and immune system, which reflect an improvement of chronic low-grade inflammation linked to metabolic diseases (Torres, Fabersani, Marquez, & Gauffin-Cano, 2019), the generation or the increase of bioavailability of nutrients and bioactive compounds (vitamin C and other antioxidant compounds, antitumor glucosinolates, etc.), and also the diminution of anti-nutritive compounds are reported in fermented products with selected probiotic LAB (Cao, Green-Johnson, Buckley, & Lin, 2019; Verón et al., 2019).

4.3.2 Probiotics isolated from fermented fish and fermented meat products

Microorganisms endowed with probiotic features are also being isolated from fermented products of animal origin other than milk and dairy products, such as fermented meat and fermented fish. Although South and East Asia are the main producers of fermented fish, followed by Asia and Europe, the production and consumption of these ethnic foods are increasing and spreading worldwide due to several factors, including consumer's shift towards a cosmopolitan and healthy diet, increasing immigration rates, recommendations of Food Authorities to increase the consumption of fish and spreading of the use of some fermented fish products as sodium chloride substitutes due to their umami and salty taste (Fusco et al., 2015; Tofalo et al., 2020). Defined as products that, with or without sodium chloride, have been subjected to the degradative changes resulting from the activity of endogenous enzymes and microorganisms, fermented fish have a heterogeneous and copious microbiota (Tofalo et al., 2020). Numerous microorganisms with probiotic features have been isolated from fermented fish and their probiotic features have been demonstrated mainly by in vitro studies (Table 4.3).

Indeed, apart from Kuda, Kawahara, Nemoto, Takahashi, and Kimura (2014), who successfully demonstrated the immune-promoting and/or anti-inflammation properties of two LAB strains, and Nakamura et al. (2012), who assessed the inhibitory effects of *Leuconostoc mesenteroides* 1RM3 on A/J mice orally infected with *Listeria monocytogenes*, no in vivo investigation of the probiotic features of microorganisms isolated from fermented seafood have been carried out so far (Table 4.3). While potentially probiotic strains isolated from fermented milk and dairy products have been tested in microscale or in pilot-scale productions of these products and their probiotic features in these matrices have been assessed, to the best of our knowledge no technological exploitation of the potentially probiotic fermented fish isolates has been carried out. However, it should be considered that the majority of the studies dealing with the characterization of the probiotic and safety features of

TABLE 4.3 Potential probiotic microorganisms isolated from fermented fish.

Strain	Species	Source	Tests	References
40 strains	*Lb. plantarum* (11 strains); *P. pentosaceus* (7 strains); *P. acidilactici* (4 strains); *P. lolii* (1 strain); *E. hirae* (2 strains); *E. lactis* (3 strains); *E. faecium* (7 strains); *E. faecalis* (5 strains)	Shidal (Indian fermented fish)	In vitro functional and safety characterization: resistance to low pH, to bile salts, autoaggregation, hydrophobicity, antagonistic activity, antibiotic susceptibility	Gupta, Mohanty, Majumdar (2021)
PMRS35	*Staphylococcus simulans*	Budu (salt-fermented fish)	In vitro functional and safety characterization: resistance to lysozyme and phenol, survival under stomach and intestinal simulated condition, autoaggregation, antagonistic activity, biogenic amine production, detection of pathogenic genes, antibiotic susceptibility, hemolytic activity	Kanjan and Sakpetch (2020)
AAS3	*Lb. plantarum*	Dry fish based fermented food	In vitro functional, technological and safety characterization: resistance to simulated gastric juice, bile salt hydrolase activity, cholesterol assimilation activity, antioxidant activities, hydroxyl radical scavangin activity, beta galactosidase activity, tolerance to sodium chloride, amylase, lipase and protease production, carbohydrate fermentation capabilities, cell autolysis, catalase activity, nitrate reductase activity, haemolytic, DNase and gelatinase activities, antibiotic susceptibility	Aarti and Khusro (2019)
MF067509; KY962905; KY962882	*E. faecium* (MF067509); *E. faecalis* (KY962905); *E. durans* (KY962882)	Traditional dried and salted anchovy fish	In vitro functional and safety characterization: tolerance to simulated gastrointestinal conditions and bile salts, detection of virulence genes, antibiotic susceptibility, hemolytic activity, antimicrobial activities, autoaggregation, hydrophobicity, bile salt hydrolysis, cholesterol removal, conjugated linoleic acid conversion. In vitro investigation of the cytotoxicity, antioxidant and antidiabetic activities in fermented sausage	Alkalbani, Turner, and Ayyash (2019)
FB003	*Lb. plantarum*	Salted-fermented shrimp	In vitro functional and safety characterization: tolerance to gastrointestinal conditions, autoaggregation and coaggregation, adhesion, cholesterol assimilation activity, antibacterial activity, production of biogenic amines, degradation of mucin and hemolytic activity	Le and Yang (2019)
3; 12; 19; 24; 43	*S. cerevisiae*	Suan yu (low salt fermented Chinese fish)	In vitro functional and technological characterization: survival in pH 2.5 medium with 0.3% bile salts, antimicrobial activity, hydrophobic properties, biofilm production, silmulation of transit into gut, catalase activity, urease activity, beta glucosidase activity, growth at different pH values, NaCl amounts, and temperatures, resistance to acetic and lactic acid, proteolytic and lypolytic activities	Zeng, Fan, He, Duan, and Xia (2019)
FB003; FB011; FB081; FB110	*Lb. plantarum* (FB003); *Lb. sakei* (FB011, FB081, FB110)	Salted-fermented shrimp	In vitro functional and safety characterization: tolerance to oro-gastrointestinal conditions, autoaggregation and coaggregation, adhesion, antibacterial activity, protective effect against Vibrio parahaemolyticus	Le and Yang (2018)

Strain	Species	Source	Tests	References
L8; L20; S1	*Lb. platarum* (L8, L20); *Lb. pentosus* (S1)	Pekasam (Malaysian fermented fish)	In vitro functional characterization: adhesion ability and cytotoxic evaluation on Ht-29 and Ccd-18Co intestinal cells	Muryany, Lian, Ahamad Rohi Ghazali, Zamri-Saad, and Rajab (2018)
UK2; UK3; UK10; UK12; UK20; UK25	*S. piscifermentans* (UK2, UK25); *S. condimenti* (UK3); *S. carnosus* (UK10, UK20); *Staphylococcus sp.* (UK12)	Utonga-kupsu (traditional fermented fish)	In vitro functional characterization: gastric acid, bile salt and phenol tolerance, biofilm production, antimicrobial activity, co-aggregation, cytotoxicity against different cancer cells	Singh, De Mandal, Mathipi, Ghatak, and Kumar (2018a)
HNS60	*Bacillus subtilis*	Hentak (traditional fermented fish)	In vitro functional, safety and technological characterization: antimicrobial activity, protease, carboxymethyl, cellulose, amylase and phosphatase activities, pH, lysozyme tolerance, beta galactosidase production, antibiotic resistance, biofilm production	Singh, De Mandal, Lanunmawii, and Kumar (2018b)
LAP2	*Lb. brevis*	Hentak (Indian fermented fish)	In vitro functional characterization: antibacterial substance production and characterization, antibiotic susceptibility, cell viability at low pH,, resistance to simulated gastric juice, tolarence to bile, proteolytic activity, auto-aggregation, cell surface hydrophobicity, antioxidative property (2,2-Diphenyl-1-picrylhydrazyl free radical scavenging activity, tolerance to hydrogen peroxide)	Aarti et al. (2017)
BDU3	*B. coagulans*	Ngari (Indian traditional fermented fish)	In vitro functional characterization: bacteriocin production and characterization, cytotoxicity assay using purified bacteriocin (3-(4,5.dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) assay on mammalian cells)	Abdhul et al. (2015)
BDU7	*E. faecium*	Ngari (Indian traditional fermented fish)	In vitro functional characterization: acid and bile tolerance, auto aggregation, hydrophobicity, production, purification and antioxidant activity of exopolysaccharide	Abdhul et al. (2014)
S-SU1	*Lb. plantarum*	Japanese fermented fish (salted squid)	In vitro functional characterization: acid and bile resistance, antioxidant properties, protective effects of heat killed probiotic cells against hydrogen peroxide toxicity on S: cerevisiae and human enterocyte like HT-29-Luc cells, and inhibitory effect on nitrite production in mouse RAW264.7 macrophage cells induced by Escherichia coli O111 lipoplysaccharide	Kuda et al. (2014)
F66	*P. pentosaceous*	Jeotgal (fermented salted Korean seafood)	In vitro functional characterization: enzyme activities, acid resistance and bile salt tolerance, antibiotic susceptibility, adhesion capacity, cell-surface hydrophobicity, autoaggregation and coaggregation	Lee et al. (2014)
AN6	*Lb. plantarum*	Aji-narezushi (traditional lactic acid-fermented fish)	In vitro bile acid- and cholesterol-lowering activities	Kuda, Yazaki, Ono, Takahashi and Kimura (2013)
1RM3	*Leuc. mesenteroides*	Narezushi (fermented fish with rice)	In vitro and in vivo functionality: inhibitory effects of the potential probiotic strain on Listeria monocytogenes infection to Caco-2 cells and A/J mice	Nakamura et al. (2012)

TABLE 4.4 Potential probiotic microorganisms isolated from fermented meat.

Strain	Species	Source	Tests	References
23K; NS[a]	*Lb. sakei* (23K); *S. xylosus* (NS)	French sausage, Artisanal Tunisian fermented sausages	In vitro functional and technological characterization: antagonistic activity against pathogens, bile salt tolerance. Effect of the potential probiotic strains on physicochemical, microbiological and sensorial characteristics of Tunisian dry fermented sausages	Najjari, Boumaiza, Jaballah, Boudabous, and Ouzari, (2020)
L125	*Lb. plantarum*	Traditional Greek fermented sausage	In vitro functional, safety and technological characterization: resistance in simulated gastro-intestinal conditions, hydrolitic and haemolytic activity, resistance to antibiotics and antimicrobial activity against pathogens. Effect of the potential probiotic strain on physicochemical, microbiological and sensorial characteristics of dry-fermented sausages	Pavli, Argyri, Chorianopolos, Nychas, and Tassou (2020, 2016)
K41; K44; K99	*P. pentosaceus* (K41, K44); *P. acidilactici* (K99)	Pastirma (Turkish cured beef product)	In vitro functional characterization: tolerance to simulated gastrointestinal conditions, auto and coaggregation, cell adhesion, cell hydrophobicity.	Topçu, Kaya, and Kaban (2020)
Sk7-5; Sk7-8; Sk9-15	*E. faecium*	Sokobania sausage (traditional Serbian sausage)	In vitro functional and safety characterization: tolerance to phenol and simulated gastrointestinal digestion, antimicrobial activity, biogenic amine production, antibiotic resistance	Žugić Petrović et al. (2020)
UAM1	*E. faecium*	Vienna sausages	In vitro functional and safety characterization; antibiotic susceptibility, gastrointestinal tolerance, adhesion, auto and co-aggregation	Hernández-Alcántara, Wacher, Llamas, López, and Pérez-Chabela (2018)
CTC204; CTC368	*L. lactis* (CTC204); *L. plantarum* (CTC368)	Meat products (fresh meat, raw, cooked, matured, dried or fermented meat products)	In vitro functional and safety characterization: tolerance to acid and bile salts, survival or growth at different temperatures, biofilm formation, antibiotic resistance, biogenic amine production, thermonuclease, hemolytic and gelatinase activities, antagonistic activity	Moreno et al. (2018), Bromberg, Moreno, Zagnanini, Delboni, and Oliveira (2004)
MZF1; MZF2; MZF3; MZF4; MZF5	*E. faecium*	Dried Ossban (Tunisian fermented meat)	In vitro functional and safety characterization: tolerance to simulated gastrointestinal conditions, autoaggregation,antimicrobial activity, cell adhesion, biogenic amine production, antibiotic resistance, cytotoxicity, detection of virulence and vancomycin resistance genes, detection of amine decarboxylase and bacteriocin structural genes	Zommiti et al. (2018)

Strain	Species	Source	Tests	References
R4	*Lb. brevis*	Harbin dry sausages (traditional Chinese fermented sausage)	In vitro functional characterization: tolerance to simulated gastrointestinal transit, adhesion, auto-aggregation, hydrophobicity, antioxidant activity	Han, Kong, Chen, Sun, and Zhang (2017)
SCH6; BAL6; KL14	*Lb. brevis* (SCH6); *P. pentosaceus* (BAL6, KL14)	Polish raw fermented meat products	In vitro functional and safety characterization: resistance to simulated gastrointestinal conditions, antibiotic resistance, enzyme activities, antimicrobial activities, detection of antibiotic resistance genes	Rzepkowska, Zielinska, Oldak, and Kolozyn-Krajewska (2017)
120	*E. faecium*	Alheira (Portuguese fermented meat product)	In vitro functional, technological and safety characterization: amino acid decarboxylase production, antimicrobial activity, heat resistance, survival to gastrointestinal conditions	Barbosa, Borges, and Teixeira (2014)
62781-3; 46035-1; 46035-4; 14324-8	*P. pentosaceus* (62781-3, 46035-1, 46035-4) *L. mesenteroides* (14324-8)	Ciauscolo salami (traditional Italian fermented sausage)	In vitro functional and safety characterization: acid and bile salts tolerance, adhesion, antibiotic resistance, detection of resistance genes	Federici et al. (2014)
UFLA SAU 14, 20, 34, 52, 91, 172, 285, 187, 238, 258	*Lb. plantarum*	Pork sausages	In vitro functional and safety and technological characterization: tolerance to simulated gastrointestinal digestion, auto and coaggregation, cell hydrophobicity, hemolytic activity, antibiotic resistance, biogenic amine production. Production of pork sausage with the addition of a cocktail of these strains and assessment of the inhibition of the growth of L. monocytogenes.	Dias, Duarte, Santos, Ramos, and Schwan (2013)
Al-128; AL-143	*Lb. paracasei* (Al-128); *Lb. sakei* (AL-143)	Spanish dry-cured sausages	In vitro functional, safety and technological characterization: tolerance to simulated gastro-intestinal conditions,biofilm production, biogenic amine production, antibiotic resistance, detection of virulence genes, nitrate reductase, proteolytic, lypolytic and catalase activities.	Landeta, Curiel, Carrascosa, Munoz, and Rivas (2013)
HN8; NH2	*P. pentosaceus* (HN8); *Lb. namurensis* (NH2)	Fermented meat products	In vitro functional and safety characterization: tolerance to simulated gastrointestinal conditions, auto and coaggregation, cell adhesion, cell hydrophobicity, antibiotic susceptibility	Ratanaburree, Kantachote, Charernjiratrakul, and Sukhoom (2013)
1K; 2K; 6K1; 7K2; 4K1; 2K1	*Lb. plantarum* (1K); *Lb. delbrueckii* (2K); *L. mesenteroides* (6K1); *L. acidophilus* (7K2); *S. xylosus* (4K1); *S. warneri* (2K1)	Slavonski kulen sausage (traditional Croatioan sausage)	In vitro functional, technological and safety characterization: sensitivity to oxgall bile, antimicrobial activity, production of enterotoxins, antibiotic resistance, effect of NaCl concentration and temperature of incubation, proteolytic and lypolytic activities, lactic acid production	Babić et al. (2011)

(Continued)

TABLE 4.4 Cont'd

Strain	Species	Source	Tests	References
Z12P; Z13P	*P. pentosaceus*	Sukuk (Turkish fermented sausage)	In vitro functional, technological and safety characterization: tolerance to simulated gastrointestinal conditions, auto and coaggregation, antimicrobial activity and antibiotic resistance, production of lactic acid, hydrogen peroxide, and exopolysaccharides, proteolytic activity.	Yuksekdag et al. (2010)
3007; 3010	*Lb. fermentum*	Thai traditional fermented pork sausages	In vitro functional and safety characterization: tolerance to simulated gastrointestinal digestion, auto and coaggregation, cell hydrophobicity, hemolytic activity, antibiotic resistance, biogenic amine production	Klayraung, Viernstein, Sirithunyalug, and Okonogu (2008)
KKA; UGA146-3; CICC6078; CK1013; IDCC2102	*P. acidilactici* (KKA, UGA146-3); *E. faecium* (CICC6078, CK1013, IDCC2102)	Iberian dry fermented sausages	In vitro functional and technological characterization: growth under the ripening conditions of Iberian dry fermented sausages, acid, bile salts and pancreatic enzyme tolerance	Ruiz-Moyano, Martín, Benito, Nevado, and De Guia Cordoba (2008)
AL1; Al2; AL3; AL5; BL6; CL1; CL2; CL3; CL5; CL6; CL8; CL9; CL11; DL2; DL3; DL6; EL3; EL6; EL7; FL1; FL2; FL4; GL1; GL2; GL3	*Lb. brevis*, (AL1, AL3, BL6, CL1, CL2, DL2, GL3); *Lb. plantarum-group* (Al2, AL5, CL5, CL6, CL9, CL11, DL3, DL6, FL1, FL2, FL4, GL1, GL2); *Lb. paracasei-group* (CL3, CL8, EL3, EL6, EL7)	Italian fermented sausages	In vitro functional characterization: acid and bile tolerance, adhesion, growth in presence of different prebiotics	Pennacchia, Vaughan, and Villani (2006, 2004)
SO3/1M/1/2	*S. xylosus*	Slovak traditional sausages	In vitro functionaland safety characterization: sensitivity to oxgall bile, lactic acid production and antibiotic susceptibility, antimicrobial and amino acid-decarboxylase activities, adhesion	Simonová et al. (2006)
MF1291; MF1298; MF1300	*Lb. plantarum* (MF1291, MF1298); *Lb. pentosus* (MF1300)	Swedish salami, Norwegian mutton salami	In vitro functional and technological characterization: ability to acidify meat models, acid resistance and bile tolerance, adhesion, antimicrobial activity. Application as starter cultures for the production of fermented sausages, monitoring of the pH and of the probiotic strains, sensorial analysis of the probiotic fermented sausages.	Klingberg, Axelsson, Naterstad, Elsser, and Budde (2005)

microorganisms isolated from fermented fish have been published only in the last four years (2017–2021).

Meat foodstuffs including raw cured and ripened meat products, resulting from a fermentation process piloted by endogenous (of meat) and exogenous (of microorganisms) enzymes are produced worldwide. Also, these matrices are being explored as carriers of probiotic bacteria but technological challenges linked to the high content of curing salt, the low pH and water activity of the final products that may affect the viability of the probiotic microorganisms make hard the choice of the appropriate probiotic to be used. For these reasons, numerous studies in the last decades have focused on the isolation from fermented meat products of indigenous potentially probiotic microorganisms (Table 4.4).

While in fermented milk and dairy products the potentially probiotic strains isolated mainly belong to LAB of the *Lactobacillus* genus (or novel genera, Zheng et al., 2020), in fermented meat, like in fermented fish, strains of other LAB genera, mainly *Enterococcus* and *Pediococcus* and coagulase-negative staphylococci are being isolated and characterized for their probiotic and technological features. This is not surprising given their acidophilic to acidotolerant, halotolerant or halophilic natures, which are consistent with the low to high pH and salt content of the ecological niches they inhabit in the fermented products.

4.4 Conclusion

Fermented foods, especially fermented milk and dairy products but even non-dairy, such as fermented vegetables, fruits, meat and fish, inhabit a copious number of potentially probiotic microorganisms. While a plethora of studies dealing with the isolation, in vitro functional and safety characterization of these indigenous microorganisms, only a few studies have demonstrated in vivo the probiotic features of these microbes. However, the probiotic attributes of indigenous microorganisms may be indirectly assumed based on *in vivo* studies evaluating the effect on the human host of the consumption of fermented foods, which, as above-mentioned, are reservoirs of potentially probiotic microorganisms (Bansal, Mangal, Sharma, & Gupta, 2016; De Filippis et al., 2020; Ghosh et al., 2019; Nuraida, 2015; Sakandar & Zhang, 2021; Şanlier et al., 2019; Tamang et al., 2016a; Valero-Cases, Cerdá-Bernad, Pastor, & Frutos, 2020). A "LAB-food-gut-axis", i.e. the ability of ingested fermented foods-lactic acid bacteria to ingrain in the human gut and take route in the gut microbiome as permanent members, has been highlighted, but further appropriate studies are needed to understand the underlying mechanisms and the effects of this axis on the human health (De Filippis et al., 2020).

Few studies have assessed the technological features of potentially probiotic microorganisms isolated from fermented foods and even less assessed the probiotic and technological potential of these microbes in the fermented products. Based on these few studies, autochthonous potentially probiotic microorganisms of fermented foods seems to possess technological features better than those harbored by probiotic bacteria of human origin, but further adequate studies using these microorganisms as starters in the production of fermented foods and assessing the beneficial effects of the consumption of the probiotic fermented foods on human health should be carried out.

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CHAPTER

5

Foods and supplements as probiotic delivery vehicles

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5.1 Introduction

A frequent occurrence of lifestyle diseases and negative lifestyle habits in today's society has unquestionably triggered food science and nutrition (Sharifi-rad et al., 2020). The change of consumers' preferences is a dynamic process. Consumers have become more health-conscious and concerned about the practical value of food and the food chain's sustainability, thus driving manufacturers to emphasize the promotion of functional foods. Therefore, the key for successful acceptance and marketing of novel foods depends on the food quality and the concept of added value based on the food functionalities (Bryant, 2019).

The growing search for a healthier diet has driven new foods with functional properties, particularly sources of bioactive compounds and containing probiotics (Marrero, Martínez-Rodríguez, Pérez, & Moya, 2019). Probiotics are live microorganisms that have frequently been reported beneficial in preventing various diseases and playing a significant role in treating the existing ailments. A microorganism to be called probiotic must fulfill the criteria below: (1) Its culture can be produced on an industrial scale; (2) it can survive during production and storage; (3) it can tolerate the gut environment of the host, and (4) it exerts health effects when consumed (FAO/WHO, 2002). The positive health impact of probiotic strains

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has been more recognized in the last years. Probiotics are often delivered through foods as part of fermentation, dietary products, nutraceuticals, supplements, and drugs. In this way, probiotic products have been categorized into two major groups: probiotic foods and supplements. Both foods and supplements have conferred the health benefits claimed for them (Homayoni-Rad, Mehrabany, Alipoor, & Mehrabany, 2016).

Regulatory agencies have regulated these products, whether they authorize claims mentioning a disease's diagnosis, prevention, or treatment (Aspri, Papademas, & Tsaltas, 2020; Behera & Panda, 2020; Sharifi-rad et al., 2020). In this sense, the global probiotic market has attracted the attention of the food industry to produce new products containing probiotics, as well as researchers to study specific characteristics of probiotics and their effects on human health (Behera & Panda, 2020; Tangyu, Muller, Bolten, & Wittmann, 2019). The documented benefits of probiotics include alleviation of gastrointestinal disorders, improvements in metabolic syndrome, and modulation of immune system function, among the most critical health issues worldwide (Homayoni-Rad et al., 2016).

The use of probiotics to improve health is based on the following principle: exogenous microorganisms (from food sources or supplements) amplify the beneficial physiological effects of normal (indigenous) intestinal microbiota (Sharifi-rad et al., 2020). A range of lactic acid bacteria (LAB) species are listed as Generally Recognized as Safe (GRAS) and comprises the most used probiotic species in supplements or food matrices (Hill et al., 2014; WGO, 2017). Probiotics used as ingredients, in food products, and as supplements are summarized in Table 5.1.

Together with the *Bifidobacterium* genus (*B. animalis*, *B. longum*, *B. lactis*, *B. bifidum*, *B. infantis*, *B. breve*), species of *Lactobacillus* (*L. acidophilus*), *Lactiplantibacillus* (*L. plantarum*), *Lacticaseibacillus* (*L. casei*, *L. rhamnosus*), *Limosilactobacillus* (*L. fermentum*, *L. reuteri*), and *Ligilactobacillus* (*L. salivarius*) is most commonly used as probiotics. Furthermore, the *Saccharomyces cerevisiae* yeast and some *Streptococcus* and *Bacillus* species are also used as probiotics and explored for incorporation into foods (Alves et al., 2016; WGO, 2017). *Lacticaseibacillus*, *Lactiplantibacillus*, *Ligilactobacillus*, and *Limosilactobacillus* genus previously comprised the *Lactobacillus* genus recently reclassified due to the enormous genomic diversity among the more than 260 species belonging to the genus *Lactobacillus* and the continuous addition of new species. The reclassification was based on phenotypic, genotypic, and ecological features (Salvetti, Harris, Felis, & O'Toole, 2018; Zheng et al., 2020).

Bifidobacteria is phylogenetically distinct from LAB, but it shares some ecological and metabolic traits with them, and, consequently, they have often been associated with this bacterial group. In addition, a few Gram-negatives, such as *Escherichia coli* Nissle 1917, are considered probiotics. Moreover, metagenomic surveys and recent functional studies have hinted at some bacterial members of the human gut microbiome as potential probiotics, such as *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Bacteroides uniformis*, and *Clostridium* cluster IV, XIVa, and XVIII. These microorganisms represent promising candidates for developing so-called next-generation probiotics (El Hage, Hernandez-Sanabria, & Van de Wiele, 2017). They may offer physiological functions that may not be achieved with lactobacilli and bifidobacteria, such as the high propionate and butyrate productions (Cunningham et al., 2021).

These potentially beneficial microorganisms can often represent a diverse community that is not well defined in strain composition and stability. As a result, it is indicated that fermented foods should only be considered probiotics when they meet the recommended

TABLE 5.1 Main probiotic strains used as ingredients and at supplements or probiotic food (dairy and non-dairy) products currently commercialized.

Probiotic product	Company	Type	Strain source	Probiotic microorganisms
Ingredients	Chr. Hansen	Lyophilized strain – - individual package		*Bifidobacterium lactis* Bb-12
				Lactobacillus acidophilus La-05
				Lacticaseibacillus paracasei CRL 431 (former *Lactobacillus paracasei* CRL 431)
				Limosilactobacillus fermentum VRI003 (former *Lactobacillus fermentum* VRI003 (PCC)
				Limosilactobacillus reuteri RC-14 (former *Lactobacillus reuteri* RC-14)
	Lallemand	Lyophilized strain – - individual package		*Lacticaseibacillus rhamnosus* GR-1 (former *Lactobacillus rhamnosus* GR-1)
				Lacticaseibacillus paracasei F19 (former *Lactobacillus paracasei* F19)
				Lacticaseibacillus rhamnosus R0011 (former *Lactobacillus rhamnosus* R0011)
				Lactobacillus acidophilus R0052
	DuPont Nutrition Biosciences ApS			*Lactobacillus acidophilus* NCFM
		Lyophilized strain– - individual package		*Bifidobacterium lactis* Bi-07
				Bifidobacterium lactis HN019 (DR10)
				Lacticaseibacillus rhamnosus HN001 (former *Lactobacillus rhamnosus* HN001 (DR20)
	Morinaga Milk Industry Co., Ltd.	Lyophilized strain		*Bifidobacterium longum* BB536
	Essum AB	Lyophilized strain – - individual package		*Lactiplantibacillus plantarum* LB21 (former *Lactobacillus rhamnosus* LB21)

(*Continued*)

TABLE 5.1 (Cont'd)

Probiotic product		Company	Type	Strain source	Probiotic microorganisms
					Lactococcus lactis L1A
		Probi AB	Lyophilized strain – - individual package		*Lactiplantibacillus plantarum* 299v (former *Lactobacillus plantarum* 299v)
		Next Foods			*Lacticaseibacillus rhamnosus* 271 (former *Lactobacillus rhamnosus* 271)
	Sustenex	Ganeden Biotech Inc.	Lyophilized strain		*Bacillus coagulans* BC30
		Lacteol Laboratory	Lyophilized strain		*Lactobacillus acidophilus* LB
	Cultura	Arla Foods	Lyophilized strain		*Lacticaseibacillus paracasei* F19 (former *Lactobacillus paracasei* F19)
Food Products	DanActive	Danone	Multiple flavors fermented milk		*Lacticaseibacillus casei* DN-114 001 (former *Lactobacillus casei* DN-114 001)
	Activia	Danone	Multiple flavors yogurt		*Bifidobacterium animalis* DN-173 010
	Actimel	Danone	Multiple flavors fermented milk		*Lacticaseibacillus casei defensis* (former *Lactobacillus casei defensis*)
	Yakult	Yakult	Fermented milk		*Lacticaseibacillus casei* Shirota (former *Lactobacillus casei* Shirota)
					Bifidobacterium breve Yakult
	Lifeway kefir	Lifeway	Coconut-chia kefir		*Lactococcus lactis*
					Lactobacillus acidophilus
					Streptococcus diacetylactis
					Bifidobacterium lactis
	Vaalia kids	Vaalia	Multiple flavors yogurt		*Lactobacillus acidophilus*
					Lacticaseibacillus rhamnosus LGG (former *Lactobacillus rhamnosus* LGG)
					Bifidobacterium bifidus

Probiotic product		Company	Type	Strain source	Probiotic microorganisms
	Keiff Kefir	B Corporation	Multiple flavors kefir		*Bifidobacterium infantis*
					Bifidobacterium lactis
					Lactobacillus acidophilus
					Limosilactobacillus fermentum (former *Lactobacillus fermentum*)
					Lacticaseibacillus paracasei (former *Lactobacillus paracasei*)
					Lacticaseibacillus rhamnosus (former *Lactobacillus rhamnosus*)
					Lactococcus lactis
					Lactococcus lactis subsp. *cremoris*
					Lactococcus lactis subsp. *lactis*
					Lactococcus lactis subsp. *biovar diacetylactis*
					Leuconostoc mesenteroides
					Leuconostoc pseudomesenteroides
					Streptococcus thermophilus
					Debaryomyces hansenii
		Nestlé			*Lactobacillus johnsonii* Lj-1 NCC533
					Lactobacillus acidophilus La-1
	LC1 / LC1go	Nestlé	Multiple flavors Yogurt		*Lactobacillus johnsonii*
	Molico	Nestlé	Powdered Milk		*Lactobacillus acidophillus* La-1
					Streptococcus thermophilus
	Chamyto	Nestlé	Fermented Milk		*Lactobacillus acidophillus* La-1
	Probiotic Muffin	Isabella's Healthy Bakery	Muffin	Ganeden Biotech, Mayfield Heights (USA	*Bacillus coagulans*
					Ganeden BC30

(*Continued*)

TABLE 5.1 (Cont'd)

Probiotic product		Company	Type	Strain source	Probiotic microorganisms
	Zena's	Zena's Brownie	Chocolate Chunk Cookies	Ganeden Biotech, Mayfield Heights (USA	*Ganeden* BC30
	Naked Pizza	NKD Pizza	Pizza	Ganeden Biotech, Mayfield Heights (USA	*Ganeden* BC30
		So Delicious	Cultured coconut		*Lacticaseibacillus rhamnosus* (former *Lactobacillus rhamnosus*)
		Dairy Free	Milk		*Lactobacillus acidophilus*
					Bifidobacterium bifidum
					Bifidobacterium animalis
	Happy Bellies	Happy Baby	Oatmeal		*Lactobacillus acidophilus*
					Ligilactobacillus salivarius (former *Lactobacillus salivarius*)
					Lactiplantibacillus plantarum (former *Lactobacillus plantarum*)
					Bifidobacterium lactis
	KIND Breakfast Bars	KIND	Flavorized cereal breakfast bars	Ganeden Biotech, Mayfield Heights (USA)	*Bacillus coagulans* GBI-30 6086
	Mucilon	Nestlé	Oat and rice		*Bifidus* BL
	Bio K Plus	Bio K Plus International Inc.	Rice and soymilk		*Lactobacillus acidophilus* CL1285
					Lactobacillus casei LBC80R
					(reclassified as *Lacticaseibacillus casei* LBC80R)
					Lactobacillus rhamnosus CLR2
					(reclassified as *Lacticaseibacillus rhamnosus*)

Probiotic product	Company	Type	Strain source	Probiotic microorganisms
Vale oats	Velle	Drink-type and yougurt-type		*Lactiplantibacillus plantarum* (former *Lactobacillus plantarum*)
				Leuconostoc mesenteroides
				Pediococcus cerevisiae
				Saccharomyces cerevisiae
				Bacillus subtilis
Proviva	Skane Dairy	Fermented fruit drink with oatmeal		*Lactiplantibacillus plantarum* 299v (former *Lactobacillus plantarum* 299v)
Yosa	Bioferme Ou	Oat bran yogurt		*Lactobacillus acidophilus* La-05
				Bifidobacterium bifidum Bb-12
Avenly vele	Avenly Oy Ltd.	Oat based drink		*Lactobacillus*
				Bifidobacterium
Grainfields wholegrain liquid	AGM Foods Pvt. Ltd.	Grains, beans and seeds		*Lactobacillus acidophilus*
				Lactobacillus delbruekii
Nancy's Soy	Nancy's Yogurt	Soy bean		*Streptococcus thermophilus*
				Lactobacillus bulgaricus
				Lactobacillus acidophilus
				Lacticaseibacillus casei (former *Lactobacillus casei*)
				Lacticaseibacillus rhamnosus (former *Lactobacillus rhamnosus*)
				Bifidobacterium bifidum
Probio Yogurt	Puravida	Coconut milk and red fruits		*Streptocuccus thermophilus*

(*Continued*)

TABLE 5.1 (Cont'd)

Probiotic product		Company	Type	Strain source	Probiotic microorganisms
					Lactobacillus salivarus
					Lactobacillus delbruecki subsp bulgariccus
					Lactobacillus acidophilus
					Lacticaseibacillus rhamnosus (former *Lactobacillus rhamnosus*)
					Lacticaseibacillus casei (former *Lactobacillus casei*)
					Lactococcus lactis
					Bifidobacterium lactis
					Bifidobacterium bifidum
	Good Cacau	Be Good	Chocolat		*Bacillus coagulans*
					Ganeden BC30
	Tipton Mills Probiotic Coffee	Tipton Mills	Instant coffee		*Ganeden* BC30
Supplements / Medicines	Sho Balance	Sho Nutrition	Vegan spheric-gels		*Lacticaseibacillus casei* K-1(former *Lactobacillus casei* K-1)
	Align	P&G	Capsules		*Bifidobacterium* 5624
	Culturelle	DSM	Capsules		*Lactobacillus* GG^{R}
	Florastor	Biocodex	Capsules		*Saccharomyces cerevisidae boulardii*
	Probiatop	Invictus	Capsules – multiple strains		*Lactobacillus acidophilus* SD5221
					Lacticaseibacillus rhamnosus (former *Lactobacillus rhamnosus* SD5675)
					Lacticaseibacillus paracasei F19 (former *Lactobacillus paracasei* SD5275)
					Bifidobacterium lactis SD5674

Probiotic product	Company	Type	Strain source	Probiotic microorganisms
VSL#3	Alfasigma	Capsules – multiple strains		*Streptococcus thermophilus*
				Bifidobacterium breve
				Bifidobacterium lactis (former *Bifidobacterium longum*)
				Bifidobacterium lactis (former *Bifidobacterium infantis*)
				Lactobacillus acidophilus
				Lactiplantibacillus plantarum (former *Lactobacillus plantarum*)
				Lacticaseibacillus paracasei (former *Lactobacillus paracasei*)
				Lactobacillus helveticus (former *Lactobacillus delbrueckii* subsp. *bulgaricus*)
Protectis	Biogaia	Chewable tablets or probiotic drops	Occurs naturally in the human gastrointestinal tract	*Limosilactobacillus reuteri* ATCC 55730 (former *Lactobacillus reuteri* ATCC 55730)
				Lactobacillus acidophillus
Probiotic 10	Now Foods	Capsules – multiple strains		*Lactobacillus*
				Lacticaseibacillus casei (former *Lactobacillus casei*)
				Lacticaseibacillus rhamnosus (former *Lactobacillus rhamnosus*)
				Lacticaseibacillus paracasei (former *Lactobacillus paracasei*)
				Ligilactobacillus salivarius (former *Lactobacillus salivarius*)

(*Continued*)

TABLE 5.1 (Cont'd)

Probiotic product	Company	Type	Strain source	Probiotic microorganisms
				Bifidobacterium longum
				Bifidobacteriium aninalis subespécie lactis
				Bifidobacterium breve
				Streptococcus thermophiles
SimFort	Vitafor	Lyophilized strains – multiple strains package		*Lactobacillus acidophillus*
				Lacticaseibacillus casei (former *Lactobacillus casei*)
				Lactococcus lactis
				Bifidobacterium lactis
				Bifidobacterium bifidum
20 Bi	ProPac Labs	Capsules – multiple strains		*Lactobacillus acidophilus NCFM*
				Lacticaseibacillus paracasei Lpc-37 (former *Lactobacillus paracasei* Lpc-37)
				Bifidobacterium lactis Bi-04
				Bifidobacterium lactis Bi-07
				Bifidobacterium bifidum Bb-02
SimCaps	Vitafor	Capsules – multiple strains		*Lactobacillus acidophilus*
				Bífidobacterium lactis
Lactoflor	EMS	Lyophilized strains – multiple strains package		*Bífidobacterium lactis*

Probiotic product	Company	Type	Strain source	Probiotic microorganisms
				Lactobacillus acidophilus
LactoFos	Invictus	Lyophilized strains – multiple strains		*Lacticaseibacillus paracasei* (former *Lactobacillus paracasei*)
				Lactobacillus acidophilus NCFM
				Lacticaseibacillus rhamnosus (former *Lactobacillus rhamnosus*)
				Bifidobacterium lactis
SimbioFlora	Farmoquímica	Lyophilized strains – multiple strains		*Lactobacillus acidophilus* NCFM
				Lacticaseibacillus rhamnosus HN001 (former *Lactobacillus rhamnosus* HN001)
				Lacticaseibacillus paracasei LPC-37 (former *Lactobacillus para casei* LPC-37)
				Bifidobacterium lactis
Enterogermina / Enterogermina Plus	Sanofi Aventis	Flaconette - aquous suspension		*Bacillus clausii*
	University College		Occurs naturally in the human oral cavity, intestine, and vagina	*Ligilactobacillus salivarius* UCC118 (former *Lactobacillus salivarius* UCC118)

criteria and suggest that foods with no defined microbial composition should be described as "containing live and active cultures," but not as probiotics. However, these products may represent promising sources of new strains with probiotic potential. Nevertheless, the market offers many products with the "probiotic" label without meeting the minimum criteria, such as defined content, adequate viable count at the end of shelf life, and evidence of health benefits (Hill et al., 2014).

Probiotics should demonstrate safety, efficacy, and quality control during processing. Suppose a probiotic product uses a health claim that implies therapy, treatment, relief, prevention, or disease diagnosis. In that case, it is classified as a pharmaceutical or medical product, and it is regulated as such. At the same time, it is a live microorganism and falls in the biologic's regulation. If a probiotic product does not use a health claim, it is classified as a dietary or food supplement. These categories complied with probiotics in capsules or pills and foods and regulated less rigor than pharmaceutical products. There are some cases where the clinical data classify a product as a medical food intended to manage a specific disease (Simone, 2019). The regulations concerning probiotic foods and supplements may vary depending on the country.

In the European Union, supplements and probiotic foods are regulated by the Food Products Directive and Regulation (directive 2000/13/EU and regulation 178/2002/EC). The European Food Safety Authority (EFSA) authorizes the health claims and lists microorganisms with a Qualified Presumption of Safety (QPS). The probiotic cultures included in the list do not require to have safety assessments. Up to date, there are no approved health claims for probiotics in the European Union. In the United States, the regulatory bodies are Food and Drug Administration (FDA) and Dietary Supplement Health and Education Act (DSHEA). Supplements are considered 'food' and are regulated by DSHEA/FDA. The supplements need to follow the Good Manufacturing Practices guidelines, but it is not required to test efficacy or quality. It is not allowed to associate supplements with disease claims. However, they can present functional allegations that are not misleading, truthful, and supported by scientific evidence. If the probiotic is intended for medical therapeutic purposes, it is considered a drug regulated by the FDA. FDA also regulates the utilization of structure claims, health claims, functions, among others. The utilization of health claims must be authorized by FDA (Koirala & Anal, 2021). Safety studies are needed both for probiotic foods and supplements before the efficacy studies, even if the probiotic used is GRAS. When considering marketing purposes, the prohibition or approval is dependent on the claims (Simone, 2019).

In Canada, the needed data is dependent on the claim required for the product. Anyway, health products are a drug subset and need assessment and license before commercialization. In Japan, probiotics are regulated under the "Foods for specified health use" (FOSHU) and may show health claims related to intestinal movement. Furthermore, a category denominated "Foods with functional claim" (FFC) comprises foods excepted FOSHU, and they are generally associated with the promotion of gastrointestinal balance. The approval process for FOSHU typically takes one year, and epidemiological studies and clinical trials must be included. The clinical investigations must be performed with a sufficient number of subjects and for an extended period. On the other hand, FFC does not need pre-approval, and the

scientific evidence can use systematic literature reviews and/or clinical trials with a product (Koirala & Anal, 2021).

China has a list of microorganisms allowed to be used in food products. If the manufacturer only states in the label that the product is processed with a probiotic culture, the probiotic culture is considered a 'nutritional component.' However, if the manufacturer uses a health claim, the product is classified as a healthy food. In this case, a pre-market registration is needed, and the evaluation takes from 18 to 24 months to be concluded. Finally, in Brazil, pre-authorization of claims needs to be registered in Brazilian National Health Surveillance Agency using an online petition system. Some of the information required is proof of the scientific claim, description of the microorganism, hemolytic activity, antimicrobial resistance profile, potential adverse effects, and viability (Koirala & Anal, 2021).

To ensure the beneficial effects on the host, a minimum concentration of live probiotic cells must reach the intestine. The minimum bacterial concentration suggested is around 8 log cfu/g or mL of food at the time of consumption. The decreases observed in survival during gastrointestinal tract (GIT) exposure may not compromise the functionality of probiotics on the host (WGO, 2017). Despite the differences between the daily amounts recommended by the US or European agencies to present health claims, it has been proposed that the daily intake of numbers around 6–9 log CFU probiotic/g or mL is adequate (Grom et al., 2020). However, the required dose may be lower and strain-specific for claiming specific health effects (Hill et al., 2014; WGO, 2017). The use of probiotics in foods needs proof of their safety and effectiveness. The evidence of safety must be carried out using scientific studies that demonstrate a history of safe use and lack of adverse events or virulence and pathogenic factors. Furthermore, the absence of substances or metabolites that cause risks to man's health and lack antibiotic resistance is needed. Finally, susceptibility to at least two antibiotics may be mandatory (Brasil, 2018).

Due to the diversity of yeast and bacteria strains, strict approaches have been designed to assess side effects and post-market surveillance. One of the essential delivery systems of probiotics is food, mainly because of the higher beneficial health effects than pharmaceutical products and the increasing importance of food and nutrition (Sharifi-rad et al., 2020). Generally, designing the probiotic food formulation is more complex than supplements considering the stability of probiotics during the process and storage and achieving appropriate sensory characteristics because probiotics might negatively change the sensory attributes of foods. Also, probiotic foods can have more difficulties from regulatory administration and legislation standpoint than supplements (Meybodi & Mortazavian, 2017).

5.2 Changes in the nomenclature of the genus *Lactobacillus* and consequences for the industry

Lactobacillus genus is widely used in industrial fermentations. Fermentation can improve foods' taste, nutritional value, and texture and add to food safety and shelf life by acidifying and producing antibacterial compounds. Thus, some species of the genus *Lactobacillus* have a long history of safe and legal use in foods. However, other species within the genus do not

benefit from this status, and consequently, proper nomenclature and taxonomy are a prerequisite for safety assessment (Pot, Salvetti, Mattarelli, & Felis, 2019).

In the past, phenotypic criteria such as enzymatic profiles, fermentation patterns, and DNA-DNA hybridization were cornerstone techniques for speciation. But today, the broader high-throughput sequencing technology availability and the relatively small genome size of bacteria resulted in phenotypic testing being replaced by genome sequencing as the primary source of taxonomic information (Pot et al., 2019). In addition, in October 2018, the Lactic Acid Bacteria Industrial Platform – LABIP organized an expert workshop to discuss economic, scientific, and regulatory consequences of the rename of *Lactobacillus* genera (Pot et al., 2019). Therefore, it is clear that the renaming of *Lactobacillus* genera will have considerable economic and legal consequences, which may heavily impact the industry using these organisms to produce fermented foods, food supplements, and feed additives.

LABIP workshop participants recommended keeping the initial "L" for the names of the new genera to minimize confusion. In this way, commercially important species such as *Lactobacillus casei*, *Lactobacillus plantarum*, and *Lactobacillus reuteri* would no longer be lactobacilli. However, they would still be abbreviated as *L. casei*, *L. plantarum*, and *L. reuteri*. The suggestion is corroborated by another change, as "*Clostridium" difficile* has been recently reclassified as *Clostridioides difficile* maintaining the common abbreviation *C. difficile*, preventing confusion (Lawson, Citron, Tyrrell, & Finegold, 2016).

The renaming of most lactobacilli will impact the consumer, the scientific and medical or veterinary communities, regulators, governments, companies, and lawyers. Consumers may be concerned about why an ingredient list no longer contains the name of a friendly microbe and therefore have doubts about the content of the product (feed, food, or food supplement). Scientific literature surveys will be needed for historical and new genus names. Residual use of historical names is likely to occur in books. Lawyers or scientists involved in validating patent applications or trademarks may be confronted with the same issue. Patent officers may not find prior art for the new bacterial genera. In this way, the risk exists that existing patents may be undermined in this way. This risk is real, considering more than 10.000 patents with the term "*Lactobacillus*" in the title or the abstract. Specific taxonomic names are familiar in the medical and veterinary fields, referring to either innocence or danger. New names will undoubtedly require further information to be collected. Regulators and governments will need to compare laboratory reports with biosafety and biosecurity lists (Laulund, Wind, Derkx, & Zuliani, 2017), necessary for considerations on certification or quarantine, to decide on Nagoya protocol requirements, or in the feed and food arena, to verify with QPS-, GRAS-, GMO-, additive-, IDF inventory- or positive national lists, the latter existing in Canada, China, Brazil, Malaysia, and Thailand. In the EU Register of Feed Additives, more than 90% of the listed products with lactobacilli will be impacted by the change in the names.

International Airline Transportation Authorities and truck companies may have problems during border controls. In addition, the import/export of specific products may delay because responsible officers may not be aware of the official name changes of organisms that are usually 'approved for import/export.' For companies, taxonomic names are crucial for their research and manufacturing and master regulatory dossiers, product authorizations, marketing materials, ingredient lists, safety dossiers, product websites, patent monitoring, export certificates, and other documentation (Lodha et al., 2022).

5.3 Factors influencing the probiotic delivery by food or supplements

The scientific literature clearly illustrates the importance of probiotic consumption to increase the quality of life. Therefore, the number of food products or supplements that can be functioned by probiotics is growing. Different parameters including the probiotic viability in the product until the time of consumption as well as during delivery throughout the gastrointestinal tract (efficiency), sensory characteristic of the product, the natural positive imagination of consumers for foods versus supplements, economic point of view, and even the shelf life of products are essential factors that determine the customer's consumption pattern (Meybodi & Mortazavian, 2017).

Nowadays, the probiotic markets have a significant market share, either in the food or supplement industry. However, the impact of ingesting probiotics via food products or supplements is not the same from the consumer's point of view and efficacy (Meybodi & Mortazavian, 2017). The dietary consumption of probiotics in functional food or supplements may improve human health and help to prevent disease. However, the health potential and biological activity of food-delivered probiotics can be severely compromised by the stress conditions encountered by the microorganisms throughout the manufacturing process. It includes preparing probiotic food, subsequent storage, and ingestion (Fiocco et al., 2020). Therefore, the survival of the probiotic culture and its impact on the product's quality characteristics must be evaluated. In this way, the stress factors can have significant adverse effects on probiotic viability and functionality, focusing on food-related environmental adverse conditions (Fiocco et al., 2020).

Foods and supplements seem to have been efficient carriers for beneficial bacteria. However, probiotic foods appear to be preferred to probiotic supplements to promote public health in communities. This may be due to the buffering properties of foods for probiotics during passage through the gut, provision of essential nutrients for maintaining the activity and efficacy of the probiotic bacteria, synergistic effects of food ingredients on probiotic growth, and consumer attitude toward probiotic foods (Fiocco et al., 2020; Homayoni-Rad et al., 2016). In this way, foods have the advantages of being consumed for hedonism than supplements, which is crucially essential for humankind. In addition, food products have an extremely high potential for variation and development compared to supplements and adding probiotics to them leads to many products and market development (Meybodi & Mortazavian, 2017). On the other hand, supplements can transfer high numbers of viable probiotics into GIT without considerable loss during storage.

With the current *in vitro* and *in vivo* data, next-generation probiotics promise to treat diverse medical conditions. They can be more effective than single or multi strains of commercial probiotics. Moreover, several different strains with proven health benefits can also be candidates for next-generation probiotics and other microbiota-based supplements. However, additional research is required for an increased understanding of the interactions among those strains, aiming at producing a successful therapeutic formulation (El Hage et al., 2017). Furthermore, the next-generation probiotics show challenges to be overcome, such as the need for anaerobic conditions and rich growth media, resulting in higher complexity and costs. These factors demand optimization of fermentation and manufacturing processing (Cunningham et al., 2021). Overall, for general usage, probiotic foods with high

TABLE 5.2 Selected clinical trial using probiotic delivery by food.

Clinical trial design	Population/ sample size	Intervention group	Probiotic strain	Probiotic dose	Main results	References
Yogurt/Fermented milk						
Randomized controlled trial	96 healthy adults	4 weeks of two doses (1 or 3 bottles/day)	*L. paracasei* subsp. paracasei CNCM I-1518 and CNCM I-3689 and *L. rhamnosus* CNCM I-3690.	107 to 10^9 CFU/g	The global structure of the gut microbiota as assessed by alpha and beta-diversity, was not altered by consumption of the product for four weeks	Alvarez et al. (2020)
Randomized controlled trial	60 pregnant women.	4 weeks (300 300 g/day probiotic or conventional yogurt	yogurt, enriched with *Bifidobacterium* and *Lactobacillus*	4.8×10^{10} CFU/g	Consumption of 300 g/day probiotic and conventional yogurt can play a role in improving the symptoms of constipation during pregnancy.	Mirghafourvand, Homayouni Rad, Charandabi, Fardiazar, and Shokri (2016)
Randomized controlled trial	78 menopausal women	24 weeks	Yogurt enriched with *L. plantarum* 3547	10^{10} CFU/g	Improves bone health markers in Menopausal women at risk of osteoporosis without pharmacological treatment	Morato-Martínez et al. (2020a)
Randomized controlled trial	70 women with overweight/ obesity	8 weeks (50 g of Kashk/day)	Kashk with *L. acidophilus* La5 and *B. lactis* Bb12	10^6 CFU/g	Significantly decreased Triglycerides and low density lipoprotein cholesterol, reductions in body mass index, fat percent and waist Circumference, systolic blood pressure, and weight.	Razmpoosh et al. (2020)
Randomized controlled trial	44 subjects with metabolic syndrome	8 weeks (300 g of probiotic yogurt/day)	probiotic yoghurt with *B. lactis* Bb12 and *L. acidophilus* La5	10^6 CFU/g	Decrease in weight, body mass index, waist circumference, hip circumference, systolic blood pressure, diastolic blood pressure, triglyceride, and a significant increase in high-density lipoprotein cholesterol	Rezazadeh et al. (2020)

Clinical trial design	Population/ sample size	Intervention group	Probiotic strain	Probiotic dose	Main results	References
Randomized controlled trial	123 children	8 weeks (100 mL of fermented milk/day)	Fermented milk with *L. rhamnosus* SD11	5×10^{10} CFU/mL	A significant reduction of salivary total streptococci *and S. mutans* and increase of salivary lactobacilli	(Pahumunto et al. (2020)
Randomized-crossover trial	19 healthy participants	350 g of yogurt	*Streptococcus thermophilus, Lactobacillus delbrueckii subsp. bulgaricus, Lactobacillus acidophilus LA-5 and Bifidobacterium animalis subsp. lactis BB-12*	-	Riceberry rice yogurt could be a healthy food for improving the postprandial glycemic and antioxidant response in humans	Anuyahong, Chusak, Thilavech, & Adisakwattana, 2020
Randomized controlled trial	76 subjects with irritable bowel syndrome	4-week (200 mL of fermented milk/day)	Fermented milk with *L. acidophilus* La-5 (and *B. animalis* ssp. lactis BB-12	10^7 CFU/g	Improvements in several IBS symptoms and total IBS-QoL score	Šmid et al. (2016)
Kefir						
Randomized controlled trial	48 subjects with metabolic syndrome	11-week	probiotic milk fermented with kefir grains	-	Improved blood pressure, fasting glucose, and lipid levels, reducing oxLDL and the risk of developing cardiovascular events in the next ten years.	Ghizi et al. (2021)
Randomized controlled trial	75 women with overweight or obese premenopausal	8- weeks	milk fermented with kefir grains	-	Significantly lower serum levels/ ratios of lipoproteins	Fathi, Ghodrati, Zibaeenezhad, & Faghih, 2017

TABLE 5.3 Selected clinical trial using probiotic delivery by food.

Clinical trial design	Population/sample size	Intervention group	Probiotic strain	Probiotic dose	Main results	References
Intestinal disease						
Randomized controlled trial	50 subjects with irritable bowel syndrome (IBS)	8 weeks	*B. coagulans*	10^9 CFU/g	Alleviated IBS symptoms.	Abhari et al. (2020)
Randomized double-blind	61 subjects with unconstipated irritable bowel syndrome	4 weeks	*L. paracasei L. salivarius and L. plantarum*	1×10^9 CFU/mL once a day	The mixture of Lactobacilli was effective in the global relief of IBS symptoms	Oh et al. (2019)
Randomized, double-blind	285 subjects with irritable bowel syndrome	12 weeks	*B. longum* or *L. paracasei*	10×10^9 CFU/g	*L. paracasei* and *B. longum* may reduce GI symptom severity and improve the psychological well-being of individuals with certain IBS subtypes	Lewis et al. (2020)
Randomized trial blinded trial	20 subjects with Irritable bowel syndrome and 20 healthy individuals	3 week	*Lactobacillus acidophilus* LLA-10, *L. helveticus* LLH-108, *L. rhamnosus* LLR-L1, *L. fermentum* LLF-01, and *L. bulgaricus* LLB-06	$2{,}5 \times 10^7$ CFU/g	Increased the number of activated NK cells in the peripheral blood as well as decreasing IL-6 and IFN- levels in the fecal samples	Velikova et al. (2020)
Randomized controlled trial	60 subjects with mild to moderate ulcerative colitis	8 weeks	Lactocare (*L. casei, L. acidophilus, L. rhamnosus, L. bulgaricus, Bifidobacterium breve, Bifidobacterium longum, Streptococcus thermophilus*	10^9 CFU/g	Beneficial change on gut microbiota	Amiriani et al. (2020)
Randomized trial	237 subjects with two or more colorectal tumors (adenoma or early cancer) and had all of their tumors removed endoscopically	10 - years	*L. casei* strain Shirota	Biolactis^R 3 g/day	Very long-term treatment with a *Lactobacillus* probiotic preparation suppressed weight loss in the elderly.	Mutoh et al. (2020)
Randomized	46 children with acute gastroenteritis	7 days	*Streptococcus thermophilus, L. rhamnosus, L. acidophilus, Bifidobacterium lactis, Bifidobacterium infantis*, fructo-oligosaccharides	1 capsule/ day during 7 days	Enhance the normalization of stool consistency and reduce the duration of diarrhea	Kluijfhout et al. (2020)

Clinical trial design	Population/sample size	Intervention group	Probiotic strain	Probiotic dose	Main results	References
Randomized, double-blind	101 subjects undergoing Roux-en-Y gastric bypass (RYGB)	90 days	FloraVantage *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* Bi-07)	5×10^{10}/g	Improves the vitamin and lipid profile.	Rizzon et al. (2021)
Brain disease						
Randomized controlled trial	37 children with cerebral palsy and chronic constipation	14- months	*L. reuteri* DSM 17938	10^8 CFU/g	Improved the stool characteristics.	Contreras, Garibay, Ramírez, Morris, and Rizo (2020)
Randomized trial	78 subjects with low-to-moderate depression	8 weeks	*L. helveticus* R0052 (CNCM strain I-1722) and *B. longum* R0175 (CNCM strain I-3470)	10×10^9 CFU/g	Improved depression scores	Heidarzadeh-Rad et al. (2020)
Randomized double-blind	82 subjects with depressed individuals	28 days	*B. bifidum* W23, *B. lactis* W51, *B. lactis* W52, *L. acidophilus* W22, *L. casei* W56, *L. paracasei* W20, *L. plantarum* W62, *L. salivarius* W24 and *L. lactis* W19	7.5×10^{10}/g	Improve the microbiota composition	Reininghaus et al. (2020)
Double blind randomized	10 women with Major Depressive Disorder	8 week	*L. helveticus* R0052 and *Bifidobacterium longum* R0175	3×10^9 CFU/ per sachet	Significant improvements in affective clinical symptoms	Wallace and Milev (2021)
Pregnant women						
Randomized controlled trial	88- pregnant women in gestational week 20	week 20, 32 and postpartum (4 days after delivery)	*L. reuteri* DSM 17938	10^9 CFU/g	Number of circulating NK cells was decreased in peripheral blood during pregnancy.	Forsberg et al. (2020)
Elderly subject						
Randomized double-blind	78 menopausal women aged between 50 and 60 years and; osteopenia not being treated pharmacologically	24 weeks	*Lactiplantibacillus plantarum* 3547	1×10^{10}/g	Improved the bone health	Morato-Martínez et al. (2020b)

(*Continued*)

TABLE 5.3 (Cont'd)

Clinical trial design	Population/sample size	Intervention group	Probiotic strain	Probiotic dose	Main results	References
Double blind randomized controlled trial	32 geriatric patients treated with home enteral nutrition (HEN)	60 days	Proxian *Lactiplantibacillus plantarum* LP01 (LMG P-21021), *Lentilactobacillus buchneri* Lb26 (DSM 16341), *Bifidobacterium animalis subsp. lactis* BS01 (LMG P-21384)	1×10^{10}/g	It appears not to change the incidence of infections or modulate intestinal inflammation in elderly treated with HEN.	Orlandoni et al. (2021)
Atopic dermatitis						
Randomized controlled trial	109 subjects with atopic dermatitis (AD)	3- months	*Bifidobacterium bifidum* CCFM16 and *L. plantarum* CCFM8610	10^9 CFU/g	*L. plantarum* CCFM8610 exerts the strain-specific amelioration effects on patients with AD.	Fang et al. (2020)
Obesity						
Randomized trial	39 subjects with raised total cholesterol (TC) ≥ 200 mg/dL	4 years	*Lactiplantibacillus plantarum* strains CECT7527 (KABP011), CECT7528 (KABP012), and CECT7529 (KABP013)	10^9 CFU/g	reduction in LDL-C and TC	Guerrero-bonmatty et al. (2021)
Randomized trial	20 subjects overweight/obese and had a mean BMI of 33.5 kg/m^2	3 months	*Lactobacillus acidophilus* DDS-1, *Bifidobacterium lactis* UABla-12, *Bifidobacterium longum* UABl-14, and *Bifidobacterium bifidum* UABb-10.	15×10^9 CFU/mg	Increased an abundance of beneficial microbial species.	Sergeev, Aljutaily, Walton, and Huarte (2020)
Randomized	270 overweight and obese pregnant women	until 6 months postpartum	*Bifidobacterium animalis* spp lactis 420 (DSM 22089; Dupont, Niebuell, Germany) and *L. rhamnosus* HN001 (ATCC SD5675; Dupont, Niebuell, Germany)	10^{10} CFU/g	The specific gut microbiota species do not contribute to GDM in overweight/obese women.	Mokkala et al. (2021)
Infant						
Randomized trial	150 - newborns, 38–40 weeks gestational age by cesaria	12 h/ day 5 or 6 after delivery	*Bifidobacterium breve* PB04 and *L. rhamnosus* KL53A	2×10^6 CFU/g	Increases numbers of lactobacilli and bifidobacteria in their gut and thus it mimics the natural colonization of newborns born naturally	Hurkala et al. (2020)

Clinical trial design	Population/sample size	Intervention group	Probiotic strain	Probiotic dose	Main results	References
Randomized trial	45 exclusively breastfed infants	28 days	*L. rhamnosus* (ATCC 53103)	5×10^9 CFU/g	Reductions in crying time and fecal calprotectin levels, with increased total bacteria and *Lactobacillus* spp.	Savino et al. (2020)
Oral disease						
Randomized	51 subjects with periodontitis stage I and II	30 days	*L. salivarius* SGL03	-	Reduction in the periodontal depths pockets	Nedzi-Góra et al. (2020)
Double-blind method	77 subjects induced oral mucositis (OM) in nasopharyngeal cancer (NPC)	7 weeks	*L. plantarum* MH-301, *B. animalis* subsp. *Lactis* LPL-RH, *L. rhamnosus* LGG-18, and *L. acidophilus*	10^9 CFU	Reduces the severity of OM by enhancing the immune response of patients with NPC and modifying the structure of gut microbiota	Xia et al. (2021)
Urogenital disease						
Randomized trial	30 women with symptomatic vaginal vaginosis	30 days	*L. acidophilus, L. rhamnosus GG, B. bifidum* and *B. longum*	2×10^9 CFU/g	44.8% of women cleared BV one-month post-treatment	Happel et al. (2020)
Athlete						
Double-blind test	21 healthy people middle- and long-distance runners	3 weeks	*Bifidobacterium longum* subsp. *longum* Olympic No. 1 (OLP-01)	5×10^9 CFU/g	Improved exercise performance	Lin et al. (2020)
Randomized, double-blind	77 subjects performed moderate or intense physical activity	3 months	*Bifidobacterium lactis* W52, *L. brevis* W63, *L. casei* W56, *Lactococcus lactis* W19, *Lactococcus lactis* W58, *Lactobacillus acidophilus* W37, *Bifidobacterium bifidum* W23 and *L. salivarius* W24	2.5×10^9 CFU/g (1 capsule)	A positive effect on the parameters of body composition	Smarkusz-Zarzecka et al. (2020)
Randomized double-blind	365 athletes	12 weeks (one capsule/day/20 mg of iron)	*Lactobacillus plantarum* 299v LP299V	10^{10} CFU/g	Reduce GI side effects during intense exercise and/or following consumption of iron supplements	Axling et al. (2020)

viability standards are superior to supplements, but for specific therapeutic applications, the latter is more efficient and, therefore, preferred (Meybodi & Mortazavian, 2017).

5.4 Evidence from human intervention trials the probiotic delivery by food or supplements

Both supplements and foods have been used as delivery vehicles in clinical trials to investigate the health benefits of probiotics. Tables 5.2 and 5.3 show evidence from human interventional trials about probiotic delivery by food or supplements.

Regarding clinical trials using food as probiotic delivery, we found more studies with yogurt, fermented milk, or kefir. As mentioned before (Table 5.1), the probiotics primarily used in this type of food are *L. paracasei* subsp. paracasei, *L. rhamnosus*, *L. plantarum*, *L. acidophilus* La-5 and *B. animalis* ssp. lactis BB-12. It is essential to highlight that the clinical results depend on the evaluated strain and disease. *L. acidophilus* La-5, and *B. animalis* ssp. lactis BB-12 decreased triglycerides and low-density lipoprotein cholesterol, and reduced body mass index, fat percent and waist circumference, systolic blood pressure, and weight in women with overweight/obesity (Razmpoosh, Zare, Fallahzadeh, Safi, & Nadjarzadeh, 2020), or metabolic syndrome (Rezazadeh, Alipour, Jafarabadi, & Gargari, 2020). On the other hand, yogurt enriched with *L. plantarum* showed improved bone health markers in menopausal women at risk of osteoporosis without pharmacological treatment (Morato-Martínez, López-Plaza, Santurino, Palma-Milla, & Gómez-Candela, 2020). In addition, a recent meta-analysis showed that probiotics consumption, especially fermented soy milk with *L. plantarum*, may reduce inflammation and oxidative stress biomarkers by significantly decreasing high sensitivity-C reactive protein (hs-CRP) and malondialdehyde (MDA) and increasing glutathione total antioxidant capacity (GSH TAC) in diabetic nephropathy patients (Bohlouli et al., 2021).

Several studies showed that probiotic fermented milk has an anti-obesity effect by reducing body mass index (Mohammadi et al., 2021). In this way, Mousavi, Saboori, and Asbaghi (2020) showed in systematic review and meta-analysis of randomized controlled clinical trials that daily probiotic yogurt consumption for more than eight weeks has a beneficial effect on serum CRP levels in overweight people. Interestingly, recent meta-analysis studies show specific clinical evidence of probiotic microorganisms alone or in combination, but these studies focus on the disease rather than probiotic strain. For example, Hadi et al. (2021) showed a positive impact of probiotics on anthropometric and metabolic characteristics in adults with metabolic syndrome. In this way, probiotic fermented milk, which is an excellent source of protein and other nutrients, is inexpensive and available compared to probiotic capsule supplements (Mohammadi et al., 2021). Thus, probiotics have been used for nearly 10,000 years, mainly as fermented milk in earlier centuries in Europe. In the Balkan regions, longevity and healthy living were attributed to fermented yogurt. Thus, probiotic foods have the advantage of the food matrix that can provide functional nutrients beneficial to health (Reid et al., 2018; Saxelin, 2008).

However, in recent years, the use of probiotics in the form of supplements is growing. Sales of probiotic supplements are estimated to reach $7.1 billion by 2027 (Businesswire, 2021). Thus, Table 5.3 shows the clinical evidence for the delivery of probiotics through

supplements. We can observe that probiotics have a positive effect on intestinal and extra-intestinal diseases (Table 5.3). Looking at intestinal disorders, clinical trials in patients with irritable bowel syndrome (IBS) showed that *B. coagulans* (Abhari et al., 2020), *L. paracasei* associated with *L. salivarius* and *L. plantarum* (Oh, Jang, Kang, Chang, & Min, 2019), or *B. longum* (Lewis et al., 2020) alleviated IBS symptoms. On the other hand, mixtures of probiotic strains seem to positively affect colitis (Amiriani et al., 2020) and reduce the diarrhea duration in children with acute gastroenteritis (Kluijfhout, Trieu, & Vandenplas, 2020; Mutoh et al., 2020). In addition, a remarkably interesting long-term study showed that *L. casei* strain Shirota suppressed weight loss in the elderly with two or more colorectal tumors (Mutoh et al., 2020).

Several studies have demonstrated a clear link between gut microbiota and brain function. Therefore, the gut microbiota appears to be a key regulator of mood and behavior. Furthermore, due to its effects on the gut microbiota, probiotic supplementation seems to positively impact the management of brain disorders such as anxiety (Heidarzadeh-Rad, Gökmen-Özel, Kazemi, Almasi, & Djafarian, 2020) and depression (Reininghaus et al., 2020). Interestingly, the use of probiotics, especially *L. plantarum* isolated or combined with *L. buchneri* Lb26 and *B. animalis* subsp. lactis BS01 improved bone health in menopausal women with osteopenia (Morato-Martínez et al., 2020) and reduced intestinal inflammation in patients treated with home enteral nutrition (Orlandoni et al., 2021).

In the field of extra-intestinal diseases, the use of probiotics in oral diseases has recently been investigated. In this way, the study of Nedzi-Góra, Wróblewska, and Górska (2020) showed that *L. salivarius* SGL03 could reduce periodontal disease. In addition, in recent years, growing evidence has identified the gut microbiota as a potential factor in the pathophysiology of both obesity and related metabolic disorders (Muscogiuri et al., 2019). As a result, dietary strategies have been investigated for weight loss and reducing obesity-related metabolic diseases. In this sense, Table 5.3 shows that *L. plantarum* strains can improve the lipid profile (Guerrero-bonmatty, Gil-Fernández, Rodríguez-Velasco, & Espadaler-mazo, 2021).

It is important to emphasize that probiotic therapy has been indicated for adults and newborns, infants, and children. It is already known that gut microbiota colonization is a progressive process. In recent years, controversy has arisen over whether the colonization starts intrauterine, with the microbiota present in the placenta and the amniotic fluid, or during the delivery process. However, available studies agree that the delivery mode modifies the microbiome, with lower proportions of *Bifidobacterium* and *Lactobacillus* and a delayed establishment of gut microbiota in newborns delivered by C-section. In this way, probiotic therapy has been indicated to infants (Navarro-Tapia et al., 2020). The benefits of premature ingestion of probiotics are many, such as the colonization of the microbiota with beneficial microorganisms (Hurkala, Lauterbach, Radziszewska, Strus, & Heczko, 2020) even reducing crying in newborn babies (Savino, Montanari, Galliano, Daprà, & Bergallo, 2020). It is essential to highlight that the use of probiotics is not limited to specific pathologies but has also been used in healthy individuals, such as athletes, for performance improvement (Axling et al., 2020; Smarkusz-Zarzecka et al., 2020).

Tables 5.2 and 5.3 show more clinical trials with probiotic delivery by supplement than food. One of the reasons may be the operational complexity and expense of running a clinical trial has increased dramatically over the past decades (Eichler & Sweeney, 2018). This increase

is driven by an ever-growing "superstructure" that includes more intensive and complex monitoring, reporting rules, and governance. Thus, supplements can facilitate the delivery of probiotics since it is not necessary to process any food. In addition, when only the microorganism is being evaluated in supplement, the matrix will not be a bias in the effect of the probiotic.

Finally, with the growing interest in self-care and integrative medicine, recognizing the link between diet and health has never been stronger. As a result, the market for probiotics provided by foods, or supplements that promote health in addition to providing essential nutrition, is flourishing. In addition, clinical evidence shows that probiotics offer numerous health benefits regardless of the mode of delivery.

5.5 Conclusion

This chapter discussed the effect of probiotic foods and supplements on human health. Probiotic foods have advantages, such as lower cost, positive consumer attitude, possible synergistic effects between the ingredients and probiotics, and the high potential for variation. The consumption of probiotic foods, mainly yogurt, fermented milk, and kefir, may improve the symptoms of constipation, bone health markers, lipid profile, body mass, blood pressure, and postprandial glycemia. Probiotic supplements have as advantages the transfer of high numbers of viable probiotics into the gastrointestinal tract without considerable loss during processing and storage and the lower difficulties from regulatory and legislation standpoints. In addition, probiotic supplements may relieve intestinal disease symptoms, improve depression scores and oral health, and alleviate atopic dermatitis and urogenital disease. In a general view, probiotic foods may be recommended for general use and supplements for specific therapeutic applications.

We observed more studies with probiotic supplements than probiotic foods, which may be associated with the high operational complexity and expense of running a clinical trial and regulatory issues. Supplements can facilitate the delivery of probiotics since it is not necessary to process any food. In addition, when only the microorganism is being evaluated in supplement, the matrix will not be a bias in the effect of the probiotic. However, probiotic foods may be suitable probiotic carriers with a positive impact on consumers due to hedonism. In this way, further studies are needed, mainly with non-dairy products or dairy products other than fermented milks and yogurts.

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CHAPTER

6

Technology aspects of probiotic production and live biotherapeutics

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6.1 Probiotics and health

Probiotics are defined as "living microorganisms, which when administered in adequate amounts confer a health benefit to the host health" (Hill et al., 2014; WHO, 2002). These microorganisms can be consumed both incorporated into functional foods and as dietary supplements (Makinen, Berger, Bel-Rhlid, & Ananta, 2012), as pure or mixed cultures. Different microorganisms have been employed as probiotics either in food or in non-food matrices (e.g., silage and active packaging) (Moradi, Guimarães, & Sahin, 2021). They include lactic acid producing bacteria (genera *Lactobacillus, Bifidobacterium*, *Streptococcus* and *Lactococcus*), non-lactic acid producing bacteria (genera *Propionibacterium*, and *Acetobacter*), some genera of yeasts (*Saccharomyces*, *Picchia, Issatchenkia* and *Kluyveromyces*) (Diosma, Romanin, Rey-Burusco, Londero, & Garrote, 2014; França et al., 2015; Romanin et al., 2016), as well as certain sporeforming bacteria (genera *Bacillus*, *Clostridium*, *Sporolactobacillus* and *Brevibacillus*) (Homayoni Rad, Mehrabany, Alipoor, Mehrabany, & Javadi, 2012; Jafari et al., 2017).

The health promotion by probiotics is strain dependent and occurs at different levels by preventing various medical conditions, including inflammatory bowel diseases, autoimmune disorders, infectious diarrhea, *Helicobacter pylori* infection, antibiotic-associated diarrhea, necrotizing enterocolitis, and chronic kidney disease (Sharma, Bajwa, Manhas, & Sarwan, 2020). The main mechanisms of action of probiotics include antagonism through the production of antimicrobial compounds, immunomodulation of the host, interaction with toll-like receptors and cell cascade signaling, inhibition of toxin production, competition with pathogens for adhesion to the epithelium or for nutrients and psychobiotics (Hill et al., 2014). Besides the well documented properties of probiotic bacteria, there is increasing evidence

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about health benefits not necessarily associated with viable bacteria. In this regard, dead or injured probiotics, as well as their metabolites have also demonstrated to produce health benefits. These observations conducted to a change of paradigm, providing great dynamism to the traditional definition of probiotics. This led scientists to define two new concepts, paraprobiotic and postbiotics.

The term paraprobiotics (or ghost probiotics) was introduced by Tavertiti and Guglielmetti (2011) and is associated to inactivated microbial cells of probiotics (intact, ruptured [killed or tyndallized] or crude cell extracts with complex chemical composition) (Piqué, Berlanga, & Miñana-Galbis, 2019), which when administered regularly and in appropriate amounts are able to confer benefits on human or animal health (de Almada, de Almada, & Sant'Ana, 2018). The inactivation of cells can be achieved by application of heat, high pressure, ultraviolet, irradiation, sonication, drying, acid, pH changes and formalin. Less employed methods include pulsed electric field, ohmic heating, and supercritical CO_2 (de Almada, de Almada, Martinez, & Sant'Ana, 2016; Siciliano et al., 2021).

The most important advantage of paraprobiotics over probiotics is that the formers retain the health benefits of probiotics, being stable during storage over a wide temperature range and easily transported and handled (de Almada et al., 2018; Siciliano et al., 2021). Further advantages include their longer shelf-life, no need of cold chain and no loss of activity when used together with antibiotics or antifungal agents (de Almada et al., 2016). Likewise, the application of paraprobiotics in foods could offer different advantages. Among them, the lesser or no interaction with other food components contributes to extend the shelf-life. In addition, as they can be added before thermal processes without alteration of their activity, paraprobiotics enable greater food processing possibilities (including scale-up). All these advantages offer a greater convenience for the administration of paraprobiotics as supplements to immune-compromised people (de Almada et al., 2016; Siciliano et al., 2021). All these characteristics support the use of paraprobiotics in processes that are normally considered deleterious to probiotics.

The mechanisms of action of paraprobiotics involve an increase in their adhesion and concomitant inhibition of pathogens, secretion of metabolic products, and modulation of the immune system. Other benefits include immunomodulatory effects, protection against enteropathogens, mucosal protection, modulation of intestinal microbiota, recovery of intestinal injuries, reduction of bacterial translocation and preservation of intestinal barrier, reduction of lactose intolerance, reduction of cholesterol, management of dermatological or respiratory allergic diseases, improvement in alcohol-induced liver diseases, inhibition of growth of cancer, and treatment of atopic dermatitis (Nataraj, Ali, Behare, & Yadav, 2020).

Another relatively recently emerged concept is that of postbiotics. Although it is still under development, it refers to bioactive soluble factors (products or metabolic by-products) produced by live probiotic microorganisms or released after cell rupture, that confer any physiological benefit to the host (Barros et al., 2020; Mantziari, Salminen, Szajewska, & Malagón-Rojas, 2020). Such soluble factors include short chain fatty acids, organic acids (e.g., propionic, butyric, formic, lactic and acetic acids), enzymes, peptides, teichoic acids, peptidoglycan-derived mucopeptides, S-layer proteins and other cell surface proteins, bacteriocins, endo- and exopolysaccharides, and plasmalogens (Teame et al., 2020). Although the role of postbiotics on the host well-being is still under study, there is an increasing number of reports aiming to elucidate the mechanisms involved. It is accepted that different postbiotics stimulate and

modulate the immune and acquired host response by interacting with different pattern recognition receptors (e.g., nucleotide-binding domain and oligomerization domain like receptors and toll-like receptors) (Mantziari et al., 2020). The benefits of organic acids, bacteriocins, proteases, peroxides and exopolysaccharides are mainly related to their antibacterial and antifungal properties. Because of such inhibitory properties, many of them have realistic technological potential in biopreservation, food packaging, biodegradation of contaminants and biofilm control (Moradi et al., 2020).

The role of short chain fatty acids produced by different species of lactobacilli and bifidobacteria has been thoroughly investigated (Bengoa et al., 2019; Bengoa, Dardis, Gagliarini, Garrote, & Abraham, 2020; Bengoa, Errea, Rumbo, Abraham, & Garrote, 2020; Garrote, Abraham, & Rumbo, 2015; Iraporda et al., 2017; Iraporda et al., 2016; Iraporda et al., 2015; Iraporda, Romanin, Rumbo, Garrote, & Abraham, 2014). Short chain fatty acids and organic acids interfere with the cytoplasmic membrane structure and nutrient transport. This mechanism is strongly determined by the pH. When pH is below the pKa, they are non-dissociated and can penetrate bacterial membranes. Once inside the cell, they dissociate into anions and protons. As the cytoplasm has a neutral pH, bacterial cells try to export the extra protons out from the cell, and this results in the depletion of cellular energy (Mantziari et al., 2020). In turn, bacteriocins belong to the group of bioactive peptides and their inhibitory activity against pathogens (either a bacteriostatic or as bactericidal agents) is well-known. S-layer proteins have been reported to be responsible for preventing *Salmonella* invasion (Golowczyc, Mobili, Garrote, Abraham, & De Antoni, 2007).

Exopolysaccharides produced by probiotic bacteria include homopolysaccharides from glucose (dextrans, mutans, alternans and reuterans), fructose (levans and inulin-type) or galactose (poly-galactans). Heteropolysaccharides include combinations of those monomers with arabinose, mannose, rhamnose, fucose, glucosamine and xylose. Their production is strain dependent and some of them have been reported to inhibit a-glucosidase and a-amylase enzymes, thus having antidiabetic activity, to possess dermal wound healing properties in rats, to have antiviral properties, antibiofilm activity, anti-inflammatory, antioxidant and to stimulate innate immune response, among the main beneficial properties (Angelin & Kavitha, 2020).

Finally, it is important to point out the role of phenolic-derived metabolites on the gut microbiota. Phenolic compounds are part of several food products, and the digestive process promotes chemical reactions leading to the hydrolysis of ester or carboxylate groups (Cortés-Martín, Selma, Tomás-Barberán, González-Sarrías, & Espín, 2020). The role of the resulting products, also filling the definition of postbiotics, on the gut microbiota is gaining special attention in the last years. Elucidating such mechanisms is particularly relevant because it would explain the link between polyphenols consumption and the host-gut microbiota interactions. In this regard, it is accepted that phenolic-derived postbiotics have a higher bioavailability than dietary polyphenols, thus, they would, at least partially, mediate the health benefits of dietary polyphenols, including the gut microbiota modulation. The modulation of gut microbiota includes not only the bacteria-polyphenols derived interaction but also their degradation by the host microbiota. Indeed, gut microbiota can deconjugate glycosides, glucuronides, and organic acids, leading to the corresponding aglycones. Such aglycones further react through ring and lactone fission reactions, dehydroxylation, reduction, decarboxylation, demethylation, isomerization, among others. These transformations are determined both by

the phenolic structure (flavonoids or not flavonoid, polymerization degree, spatial configuration) and by genetic factors. These latter are particularly relevant as the different human metabotypes are associated with a differential gut microbial metabolism of polyphenols. These variations have opened new research scenarios to explain the inter-individual variability upon polyphenols consumption (Cortés-Martín et al., 2020).

6.2 Challenges of probiotics production

Probiotics are generally incorporated into different food matrices, mainly but not only, in dairy products (e.g., fermented milks, yogurts, cheeses and dairy-based desserts) (Casarotti & Penna, 2015; Cassani, Gomez-Zavaglia, & Simal-Gandara, 2020). In the last years, there is a growing interest in the development of non-dairy probiotic containing products (e.g., fruits, vegetables, cereals, soybeans, cereals, breads, spreads, bars and even coffee/tea) (Gupta & Abu-Ghannam, 2012; Sloan, 2018), particularly relevant for vegetarian or vegan consumers, as well as for those who are lactose intolerant, allergic to milk proteins or hypercholesterolemic (Gawbowski & Chikindas, 2013; Shori, 2016). Probiotics incorporated into functional foods can also promote improvements in their flavor, aroma and texture (Cassani et al., 2020), as well as act as biopreservatives to inhibit non desirable microorganisms.

According to the international regulation organizations, for a product to be considered as probiotic, it must contain at least 10^6–10^7 CFU per gram or milliliter at the moment of being consumed (Aquilina et al., 2013). Considering that the target of probiotics is the gut and before arriving they must overcome harmful environments (e.g., technological, storage and digestive conditions), the need to maintain the viability and metabolic activity of probiotic microorganisms is clear (Hill et al., 2014; Romano et al., 2014). Different factors can reduce probiotic viability during shelf-life when incorporated into food products. Among them, acidic foods, such as fruit juices (Saarela et al., 2011), freezing or temperature variations during storage often result in membrane damages, reducing the viability of these microorganisms (Ranadheera, Evans, Adams, & Baines, 2013). Furthermore, the permeability of packaging to oxygen also contributes to reduce the viability of probiotic bacteria in food products (Cruz et al., 2013), which is particularly relevant considering that most probiotic strains are anaerobic or microaerophilic (Ranadheera et al., 2013). The heat and dehydration sensitivities of probiotic bacteria are additional factors to be considered when defining processing and storage conditions. For these reasons, obtaining probiotic viable cells in the gut is a challenge, requiring the engineering of specific processes to optimize the products.

In the last years, certain spore forming bacteria have been increasingly used as probiotics, appearing as a valuable solution to overcome processing and storage problems associated with the stability of certain species of lactobacilli and bifidobacteria (Rodriguez Ayala et al., 2017). The high resistance of microbial spores to high temperatures, low pH and high pressures, together with their stability during storage, rapid germination and refractance toward stomach conditions, make them suitable ingredients for the formulation of several probiotics containing foods (Jafari et al., 2017). The health benefits associated to spore forming probiotics require a first activation of spores in the stomach. Then, the gut is the anatomical site rich in nutrients and enzymes that promotes their germination and multiplication (Hong, Le, & Cutting, 2005).

The application of spores of *Bacillus* species as probiotic dietary supplements or in pharmaceutical forms (capsules, tablets and liquid suspensions) is expanding extensively and rather rapidly due to some health benefices, such as immune stimulation, antimicrobial activities and competitive exclusion. However, only a few strains of *Bacillus coagulans* or *Bacillus subtilis* var. Natto have been approached as probiotics for human consumption. Some examples include the stability of *B. coagulans* MTCC 5856 toward baking, brewing, refrigerating and freezing of functional food products with various nutritional profiles (e.g., banana muffins, waffles, chocolate fudge frostings, peanut butters, fruit preserves and vegetable oils) for up to 24 minutes (Jafari et al., 2017; Majeed et al., 2016). Although it is a relatively recent approach and there is still too much to be investigated, the specific characteristics of spore forming probiotics in terms of heat and stress tolerance, as well as their refractance to digestive conditions, convert them in a promising alternative for the production of probiotic foods.

6.3 Technological strategies for probiotics production

Technological production of probiotics requires that processing procedures avoid bacterial damage or death. Therefore, the main challenges to be overcome for the industrial production of probiotics occur during:

- Production at large scale (e.g., freeze-drying, freezing, and spray-drying);
- Storage of dehydrated microorganisms;
- Rehydration;
- Industrial food processing and composition of food matrices;
- Food shelf-life; and
- Passage through the gastrointestinal tract (e.g., exposure to low gastric pH, bile salts, and digestive juices).

For this reason, suitable preservation strategies to avoid bacterial damage or death are strongly required during the production of lactic acid bacteria as industrial starters.

6.3.1 Production at large scale of dehydrated probiotics

Innovative production strategies are required to retain the viability and metabolic activity of probiotics during shelf-life. To achieve this goal, probiotic cell suspensions are usually exposed to drying processes driving to obtain powder formats. These formats facilitate the ease incorporation into functional foods, handling, storage, and transport, and overall, increase their shelf-life. High and low-temperature drying technologies are frequently applied for this purpose. The former refers to spray-drying and fluid bed-drying as the most extensively used, whereas the latter include freeze-drying, spray-chilling, among others (Liu et al., 2019). Spray-drying is one of the most widely employed methods to produce probiotic starters at large scale due to its cost-effectiveness (approximately 10 times cheaper than freeze-drying), easy scale-up and high production rate in a relatively short time (Frakolaki, Giannou, Kekos, & Tzia, 2020; Liu et al., 2019). This technique involves the emulsion or dispersion of probiotics in an aqueous carrier solution, the atomization into droplets through a nozzle in a drying chamber and its evaporation by a controlled flow of hot air, conducting to the

production of small microcapsules of probiotics (Frakolaki et al., 2020). However, during the drying process, probiotics suffer several stresses associated with dehydration, shear, osmotic and oxidative stresses, leading to the loss of viability (Cassani et al., 2020). Freeze-drying is the low-temperature drying method most widely applied for thermolabile components, including probiotic cells. It consists of freezing the probiotic suspensions and then drying by sublimation under high vacuum (Liu et al., 2019). The main advantage of this technique over spray-drying is the higher survival rate of probiotic cells obtained at mild thermal conditions. However, the bacterial cells integrity is affected due to water removal from the extracellular ice crystals formed during freezing, which directly impacts the structure of sensitive proteins, cell wall and physical state of lipid membranes (Tymczyszyn, Gómez-Zavaglia, & Disalvo, 2007), causing decrease in probiotic viability. Moreover, freeze-drying is expensive and time-consuming. To overcome the main drawbacks of these drying techniques some strategies are applied to protect probiotic cells and enhance their viability during processing, storage and consumption, where the most widely applied are the adjustment of the processing parameters, application of sublethal stress before drying and addition of protective agents to encapsulate cells (Cassani et al., 2020).

6.3.2 Storage of dehydrated microorganisms

To retain a high survival rate of probiotic during long-term storage, some important factors (e.g., temperature, moisture content, water activity -a_w-, exposure to light and oxygen) should be carefully controlled. For instance, although low storage temperatures (4–7°C) and avoiding temperature fluctuations ensure a higher viability of encapsulated probiotics during long-term storage, it involves high energy and transportation costs (Dianawati, Mishra, & Shah, 2016). Therefore, storage of dry probiotics at room temperature is ideally expected to overcome such problems. When storage temperature is selected, the glass transition temperature (Tg, temperature at which a given material passes from a glassy/amorphous state to a rubbery one) of protective agents should be considered. Carbohydrates are typically used as protective agents during dehydration of probiotics because of their ability to form glassy states (characterized by high viscosity), which limits chemical reactions and decreases the rotational and vibrational molecular movements. Hence, dehydrated microorganisms get embedded into a glassy matrix that ensures their stability during drying processes and storage. For this reason, dehydrated products containing probiotic strains should acquire an amorphous state during storage. This can be accomplished by adding dehydration matrices with high Tg (e.g., maltodextrins and inulins) and storing samples at temperatures below Tg.

Water activity of the dehydrated probiotics is another key factor that can affect bacteria viability during storage. Several authors reported that probiotics should be stored at the optimal a_w range (0.07–0.20) and this a_w value should be kept as low as possible and constant. This contributes to reduce oxidation processes, enhancing the probiotic stability at room temperature for extended storage periods (Marcial-Coba, Pjaca, Andersen, Knøchel, & Nielsen, 2019). For instance, Garcia-Rodenas et al. (2011) reported that a_w values lower than 0.15 are desired for a powdered nutritional formula containing *Bifidobacterium breve*, as bacterial viability, physiology and functionality are enhanced in low a_w matrices (Vesterlund, Salminen, & Salminen, 2012). In this line, Dianawati and Shah (2011) observed a better enzymatic functionality of *Bifidobacterium animalis* ssp. *lactis* Bb-12 at a_w values within 0.07 and

0.10 than at a_w 0.20. Despite the advantages of storing probiotics at very low a_w values, in many cases, it is difficult to achieve or maintain these values during long-term storage. Therefore, using moisture barrier packaging or applying microencapsulation techniques could allow protecting probiotic strains from such stresses.

Oxygen is detrimental to probiotic survival since most of the species are strictly anaerobic and thus triggers an intracellular production of reactive oxygen species (ROS) that directly affect the cell membrane of oxygen-sensitive probiotic strains. In this context, the strategies to overcome these drawbacks include finishing fermentation under vacuum (for fermented products), the addition of ascorbic acid as oxygen scavengers, controlling a_w, using vacuum packaging with low oxygen permeability, which also limits the light exposure of probiotic powders, and controlling the production process to reduce the incorporation of oxygen into the product (Cassani et al., 2020; Mohammad Mortazavian, Mohammadi, & Sohrabvandi, 2012) Gaudreau, Champagne, Remondetto, Bazinet, and Subirade (2013) observed that adding green tea extracts (62.5–500 μg/mL) in culture media greatly stimulated the growth of *Lactobacillus helveticus* R0052 under aerobic conditions by decreasing the redox potential of the medium. These authors also attributed these results to the possible interaction between catechins (hydrophilic flavonoids) and polar head groups of lipid membranes at the water interface through hydrogen bonds. This would provide certain protection against oxidants, contributing to preserve the structure and function of biological membranes (Gaudreau et al., 2013).

6.3.3 Rehydration

In order to avoid a decrease of probiotic viability, some factors should be controlled during the rehydration processes, namely the composition of the encapsulation material employed, rehydration media, pH, volume, temperature and kinetics of rehydration (Bodzen et al., 2020). Rehydration represents a critical step in the recovery of dehydrated probiotic cells, allowing their reactivation and growth. As a result, dehydrated bacteria should be able to repair the damages produced by sub-lethal stresses during the drying processes. In this regard, Bodzen et al. (2020) observed that rehydration of freeze-dried *Lacticaseibacillus casei* ATCC 334 under a mixture of gases (N_2:H_2:CO_2 at 85:5:10), not containing oxygen, improved the probiotic survival by preserving cells from oxidative stress. In addition, Alves, Messaoud, Desobry, Costa, and Rodrigues (2016) proposed that the selection of protective agents during dehydration also affects the rehydration time of an orange juice containing *L. casei* NRRL B-442 spray and spouted bed-dried. These authors observed that powders containing Arabic gum as drying agent require significantly higher rehydration time than those obtained using maltodextrin due to its low water solubility (Alves et al., 2016).

6.3.4 Industrial food processing and composition of food matrices

Traditionally, food products containing probiotics were directly associated with fermented dairy products. However, in the last years, an increasing trend toward the incorporation of probiotics in food matrices from different origins has been observed. This responds the demands of people with dietary restrictions to dairy foods, including lactose intolerant, milk protein allergic and vegans. In this line, novel fruit-based formulations, chocolates, meat and derivatives, cereals and soy products containing probiotic strains have been tested Table 6.1.

TABLE 6.1 Recent developments of foods containing probiotics.

Food carrier	Probiotic strains	Incorporation method of probiotic into food matrices	Effect on probiotic viability	References
Apple	*Lacticaseibacillus rhamnosus* and *Bifidobacterium animalis* subsp. *lactis*	Probiotic strains were incorporated into alginate-based prebiotic coatings. Apple cubes were immersed into alginate coating-forming solutions and drained. Packed samples were stored in darkness at 5 ± 1°C for 8 days.	Both probiotics successfully remained above 9.0 log CFU/g in the product after 8 days of storage.	Alvarez, Bambace, Quintana, Gomez-Zavaglia, Moreira (2021)
Blueberries	*Lacticaseibacillus casei* CECT 9104 and *Bifidobacterium animalis* subsp. *lactis* CECT 8145	Probiotic strains were incorporated into alginate-based prebiotic coatings. Fresh blueberries were immersed in theses coating-forming solutions, dried at room temperature, packed and stored in darkness at 5 ± 1°C for 14 days.	The viability of both probiotic strains was maintained above the minimum recommended levels (10^6 CFU/g) up to the end of storage.	Bambace et al. (2021)
Bread	*Bacillus coagulans*	The bread dough was inoculated (before baking) with probiotic strains, then the dough was placed in a food warmer at 40 °C/1 h for fermentation and then baked in the oven at 180 °C for 20 min. The loaves were packaged and stored at room temperature for seven days.	Probiotics incorporated into bread were able to survive when exposed to gastro-intestinal conditions even at the end of the shelf-life	Soares et al. (2019)
Cheesecake	*Lacticaseibacillus rhamnosus* HN001	Probiotic counts ($10^{9\text{-}10}$ CFU/mL) were directly added to the mixed cheesecake cream. This was then added on the cheesecake sub-base, packaged with a sealed container and stored at -20°C for 120 days and 4°C for 5 days.	Probiotic added to cheesecake was found higher than 6 log CFU/g at the end of storage, regardless the storage conditions applied.	Hasgucmen and Sengun (2020)
Coffee	*Bacillus coagulans* MTCC 5856	*B. coagulans* spores (15×10^9 CFU/g) powder was added to the ground unroasted green coffee or roasted coffee and then blended at room temperature. A serving of coffee containing probiotics was brewed using an electric brewer. The temperature of coffee was recorded at 80 ± 2 °C. Brewed coffee was stored at 4 ± 2 °C for 72 h.	A significant resistance of probiotic spores was observed at coffee brewing conditions. The probiotic spores added to coffee were also able to germinate in hostile gastro-intestinal environment.	Majeed et al. (2019)
Dehydrated Strawberry	*Bacillus coagulans* BC4	Strawberries were added with probiotics by two alternative methods (impregnation and alginate coating) and then submitted to two drying methods (freeze-drying and oven-drying). After processing, all treatments were packed and stored at 25°C and 50% RH.	The combination of impregnation and freeze-drying was the one that resulted in the highest probiotic viability through storage (8 log CFU/mL along 6 months)	Oliveira et al. (2021)

Food carrier	Probiotic strains	Incorporation method of probiotic into food matrices	Effect on probiotic viability	References
Dried date paste	*Bacillus coagulans* BC4	Freeze-dried probiotic spores were incorporated in dried date pastes to obtain a final concentration of approx. 10^8 CFU/g. Samples were stored at 25 °C in sealed aluminum laminated bags containing anaerobic or aerobic environmental atmosphere.	No significant change regarding viability was detected in pastes after 45 days of storage regardless of the storage conditions.	Marcial-Coba et al. (2019)
Low-fat fermented sausage	*Lacticaseibacillus casei*	The probiotic inoculum was added after mixing all ingredients. Then, the mixture was stuffed in cellulose cases. Temperature and relative humidity parameters were controlled during storage.	Probiotic strains showed high viability in the low-fat Italian type salami, reaching 8 log CFU/g after 18 days.	Bis-Souza, Penna, and da Silva Barretto, (2020)
Orange juice	*Pediococcus acidilactici* CE51	Direct inoculation of 10^6 CFU/mL into juices. Then, samples were stored at 4°C and 30°C during 35 days.	Probiotic counts, under both storage conditions, were between 7.2 and 8.5 log CFU/mL at the end of storage.	de Oliveira Vieira et al. (2020)
Whey and pineapple juice	*Lactobacillus acidophilus* NIAI L-54	Probiotics (9.8×10^9 CFU/mL) were directly inoculated into the mixture of pasteurized pineapple juice and whey (75:25) in sterilized glass bottles. Samples were incubated at 37 °C for 5 h.	Probiotic viability was >80% during in vitro gastrointestinal digestion up to 56 days of storage.	Islam et al. (2021)

Despite the wide variety of food products that can act as potential carriers of probiotic bacteria, their ability to effectively deliver viable cells to the human gut can vary considerably depending mainly on the stability of probiotic strains for industrial production, the physicochemical properties of the employed food matrices, food processing and storage conditions (Fig. 6.1) (Terpou et al., 2019).

As mentioned before, at least 10^6 to 10^7 CFU/g of viable probiotic microorganisms should be ingested to confer their beneficial effects on human health (Aquilina et al., 2013). Hence, when developing functional probiotic foods, not only the microorganism viability and metabolic activity should be guaranteed, but also similar physicochemical and sensory characteristics as those of the conventional products should be maintained during the product shelf-life.

In general, probiotics are incorporated into food products as microencapsulated forms. This improves their resistance to harmful environments and conditions, such as food matrices, long-term storage, and passage through the gastrointestinal tract. The several techniques usually employed to encapsulate probiotics can be classified into three groups, namely, dehydration, extrusion, and emulsion (Cassani et al., 2020). The design of an effective probiotic delivery system is challenging as it must ensure both the physical protection of microorganisms against

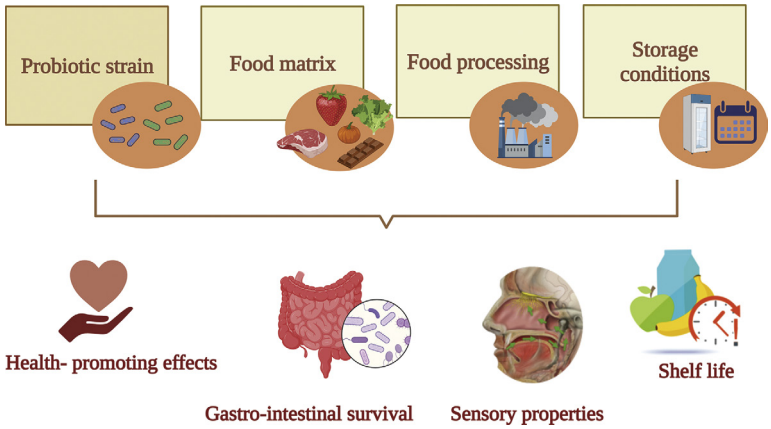


FIG. 6.1 Stability of probiotic strains for industrial production, the physicochemical properties of the employed food matrices, food processing, and storage conditions. No Permission Required.

the harsh conditions of the stomach, bile acids, and digestive enzymes in the small intestine, and also be unstable in the gut to facilitate bacterial release. In addition, the encapsulation material should be food grade and generally recognized as safe (GRAS).

Regarding the food matrices, fruit and vegetable-based foods are natural sources of healthy nutrients, free of lactic allergens and consumed by all age groups, which make them adequate carriers for probiotic cultures. However, the low pH and high organic acid content, as well as the presence of oxygen and preservatives employed at industrial scale can negatively affect the survival of probiotic strains (Pimentel, Klososki, Rosset, Barão, & Marcolino, 2019). Nualkaekul, Salmeron, and Charalampopoulos (2011) studied the impact of some influencing factors on survival of *Bifidobacterium longum* in model acidic solutions and fruit juices during 6 weeks of storage. These authors reported that high levels of pH, citric acid, protein and dietary fiber enhanced the cell survival during cold storage, while high content of phenolic compounds (e.g., in pomegranate and strawberry juices) negatively impacted the probiotic survival. Citric acid could be used as a nutrient source for probiotic growth and dietary fiber could interact with bacterial cells to physically protect them (Nualkaekul et al., 2011). In this line, Borges et al. (2016) found that dried banana is a better carrier for *Lactiplantibacillus plantarum* 299v, than strawberry and apple, as it enables a higher probiotic survival. These results were attributed to the high levels of dietary fiber present in banana, which may contribute to bacterial protection during processing. Cereal-based foods are a good source of water-soluble fiber with a prebiotic-like effect and many phytochemicals able to provide interesting technological properties, such as high viscosity and water-holding capacity. Meat products have a high nutritional profile due to their high-fat content, which confers a good buffer capacity and solid or gel structure capable of protecting probiotic strains into their structure (Gomand et al., 2019). Nevertheless, the low pH, low water activity, low contents of naturally occurring sugars, competing microorganisms and curing salts of most of these products can affect the probiotic survival during processing (Cassani et al., 2020).

Considering that some factors (e.g., pH, matrix buffering capacity, water activity, physical food structure, oxygen and nutrient contents) affect the viability of probiotic cells, an ideal food matrix for direct and effective probiotic delivery should meet some requirements, namely: i) to be neutral or slightly acidic pH (5–6); ii) to exert a high buffering capacity (e.g., high fat content) reducing the impact of different stresses (mechanical, physicochemical and temperature); iii) to show a solid or gel structure enhancing the entrapment of probiotic cells, thus protecting them from adverse factors during storage; iv) to have a high content of fiber facilitating the bacterial adhesion to the matrix or fermentable sugars that promote bacterial growth during processing (Gomand et al., 2019). However, it is difficult that one product can meet all these requirements, thus indirect probiotic delivery appears as an interesting strategy to fill this gap.

Indirect bacterial delivery refers to the incorporation of probiotic cultures into edible biopolymeric matrices to develop active edible films and coatings (Espitia, Batista, Azeredo, & Otoni, 2016). Several polymeric matrices, such as alginate, gellan, gelatin, agar, methylcellulose, pullulan and starches, have been successfully used for this purpose. Prebiotic compounds have also been incorporated into edible films, enhancing the stability of probiotics entrapped in the film. In this regard, Bambace, Alvarez, and Moreira (2019) proposed an alginate-based coating enriched with inulin and oligofructose to deliver *Lacticaseibacillus rhamnosus* CECT 836 (Bambace et al., 2019). This coating was then used to coat fresh blueberries contributed to retain probiotic viability after 21 days of cold storage without altering the sensory and quality attributes of blueberries. These results encouraged the authors to apply the prebiotic-alginate coatings to other probiotic strains (e.g., *L. casei* CECT 9104 and *Bifidobacterium animalis* subsp. *lactis* CECT 8145) with success (Bambace, Alvarez, & Moreira, 2021).

Regarding the food processing, temperature and the strain resistance are two critical factors that should be considered. Probiotic strains from genera *Lactobacillus* and *Bifidobacterium* are the most widely used in food products, but in general most of the strains are sensitive to the processing conditions (Oliveira et al., 2021). Fermentation is an ancient food preservation method that ensures the microbial safety and offers technological, nutritional, sensorial, and health benefits. In fermented food products, the incubation temperature should be controlled in the 37 to 43°C temperature range depending on the probiotic strain to ensure the optimal bacterial growth (Cassani et al., 2020). In general, these fermented products are not pasteurized and probiotic strains have to compete against native microflora. In non-fermented products, some foods are subjected to pasteurization/sterilization techniques to extend their shelf-life by significantly reducing their native microflora and inactivating undesirable enzymes. However, these processes may lead to the loss of important nutrients naturally present in foods and may alter their sensory properties (Cassani, Tomadoni, Viacava, Ponce, & Moreira, 2016). For this reason, non-thermal techniques (e.g., ultrasound, high-pressure processing, pulsed electric field, among others) have been investigated in the last years to obtain food products with extended shelf-life and better retention of sensory and nutritional properties when compared to thermal treatments. The use of these emerging techniques has been extended to probiotics food manufacturing with promising results in terms of quality attributes maintenance (Asaithambi, Singh, & Singha, 2021). However, the application of these emerging technologies to food production containing probiotics at a large scale is still rare due to their high costs.

When probiotics are added into non-fermented foods, the microorganism viability must be retained until consumption (Oliveira et al., 2021). Thus, probiotic cultures should be added after thermal processing (e.g., heating, cooking, and pasteurization). For instance, da Costa et al. (2017) incorporated *Lacticaseibacillus paracasei* into orange juice fortified with ascorbic acid and oligofructose, previously pasteurized at 80°C for 20 min. These authors observed that the probiotic juice showed similar quality attributes (physicochemical characteristics, acceptance and stability storage but lower turbidity) than the controls, without viability decay after processing. In contrast, Sheehan, Ross, and Fitzgerald (2007) evaluated the addition of different *Lactobacillus* and *Bifidobacterium* strains in orange juice before thermal pasteurization (76°C for 30 seconds or 90°C for 1 minute), observing that none of the probiotic strains (neither the most thermotolerant ones) were able to survive after thermal processing. As thermal treatments were proved to be unsuitable to ensure probiotic survival, these authors applied alternative high-pressure processing (400 MPa for 5 minutes) to the orange juices after the probiotic cultures addition, finding unexpected results since significant inactivation of the studied strains were reported with counts lower than the recommended levels (<6.0 log CFU/mL). This underlines the importance of correct engineering and processing conditions of food products containing probiotics.

In emulsified cooked meat products, thermotolerant lactic bacterial strains are usually employed as starters, being inoculated during the preparation of sausage batter, that is, before thermal treatment. Therefore, the thermotolerant strains are able to survive after sausages are cooked and also, to exhibit an antagonistic effect against enterobacteria. Pérez-Chabela, Díaz-Vela, Reyes-Menéndez, and Totosaus (2013) reported that the inoculation of *Pediococcus acidilacti* UAM15c, *L. plantarum* UAM10a, *Pediococcus pentosaceus* UAM17, and *Lactobacillus curvatus* UAM18 to fat and salt reduced cooked sausages resulted in an improvement of moisture retention, texture properties and stability without negatively affecting the physicochemical parameters (Pérez-Chabela et al., 2013). This was ascribed to the biostabilizing effect of the exopolysaccharides produced by probiotic strains.

6.3.5 Food shelf-life

Consumers expect that the food products they buy contain the recommended level of probiotic bacteria at the time of being consumed. Hence, the food industry is concerned about the evolution of bacterial viability during food shelf-life. In this regard, some factors should be considered, including the presence of oxygen, storage temperature, relative humidity and water activity. In general, foods containing probiotic strains are recommended to be stored at low temperatures (<4°C). However, these conditions result in higher costs of transportation and storage, together with a risk of losing probiotic viability if the temperature fluctuates during storage. In this sense, room temperature is preferred, but it must be taken in mind that high storage temperature might accelerate the probiotic metabolic activity, leading to promoting oxidation processes and decreasing bacterial viability (Min, Bunt, Mason, Bennett, & Hussain, 2017). Min et al. (2017) assessed the viability of certain probiotic strains (*Lactobacillus acidophilus* ATCC4356T, *L. plantarum* RC30 and *Bifidobacterium longum* ATCC15707T) incorporated into non-dairy foods during 4 weeks of storage at 20°C and 20% relative humidity (RH) or 30°C and 50% RH. They observed that increasing the storage time, temperature, and relative humidity led to decrease probiotic survival. *B. longum* ATCC 15707T showed the

greatest survival under both storage conditions, followed by *L. plantarum* RC30 and *L. acidophilus* ATCC4356T (Min et al., 2017). In addition, food products with low a_w were those retaining the enzymatic activity of probiotics during storage, which may lead to improvements in their survival (Min et al., 2017).

Oxygen can be incorporated in several processing steps of probiotic containing foods, namely mixing, homogenization and cooling after pasteurization. The presence of oxygen can be significantly reduced by fermenting under vacuum, addition of strains acting as oxygen scavengers (e.g., *S. salivarius* subsp. *thermophilus*), addition of antioxidants (e.g., ascorbic acid and catechins) and correct packaging (Dinkçi, Akdeniz, & Akalin, 2019). In this regard, some aspects should be considered to select appropriate packaging, to cite: i) thickness of the materials, gas (e.g., O_2, CO_2 and water vapor); ii) light permeability through the material; and iii) packaging technique (e.g., vacuum, modified, active or intelligent packaging systems) (Neffe-Skocińska, Rzepkowska, Szydłowska, & Kołożyn-Krajewska, 2018).

6.3.6 Passage through the gastrointestinal tract

The biological site of action of probiotics is the gut where these beneficial microorganisms provide health-promoting effects. In their way to the gut, probiotics are exposed to harmful factors that can negatively affect their survival. Among them, lysozyme in saliva, the low pH in stomach, high bile salts concentration and digestive enzymes in the small intestine and colonic environment (presence of other competing microorganisms, including pathogens) (Mohammad Mortazavian et al., 2012) are the most important stresses that probiotics must overcome to reach the intestine viable and in high numbers (Neffe-Skocińska et al., 2018).

The physical food structure influences its disintegration rate in the stomach and release of nutrients, which is lower for tough foods. Changes in food structure produced by processing operations, such as boiling, roasting, pasteurizing and frying, may affect the digestion rate of food containing probiotics and impact the bacterial survival during their passage through the gastrointestinal tract (Gomand et al., 2019). In this line, Vinderola et al. (2012) concluded the food matrix used for probiotic delivery may affect some parameters of cell functionality of *B. animalis* subsp. *lactis*, such as the ability to tolerate the stressful gastrointestinal conditions, adherence to intestinal epithelium and its biological activity. These authors found that the probiotic survival during the gastrointestinal transit was enhanced when fruit purees (apple-banana puree and peach-apple puree) were used as protective carriers. In contrast, when orange juice was used for probiotic delivery, a significant reduction in cell counts of 3 log cycles was observed, despite having similar pH and nutrients than fruit purees. On the other hand, Saxelin, Korpela, and Mäyrä-Mäkinen (2003) observed a better recovery of *L. rhamnosus* in human feces when delivered in a juice carrier than when delivered as a powder. These authors also found that cheese was the best carrier for *L. rhamnosus*, followed by unfermented milk and fermented milk. In this context, it seems that the denser, more viscous or complex food matrices the better could be the probiotic protection during their way to the gastrointestinal tract.

The organic nature and physicochemical characteristics of the encapsulation materials used as delivery matrices of probiotics may affect the bacterial tolerance to digestive stresses, but it has been scarcely studied (Melchior, Marino, Innocente, Calligaris, & Nicoli, 2020). Different microencapsulating materials from a wide variety of food-grade compounds have been proposed,

including polysaccharides, proteins and lipids, either isolated or associated to form complex delivery systems (Cassani et al., 2020). However, few works have evaluated the protective effect of such supporting materials on bacterial strains when exposed to gastrointestinal conditions. In this regard, Larsen, Cahú, Saad, Blennow, and Jespersen (2018) reported that high-methoxylated pectins have a protective effect on *Lactobacillus fermentum* PCC, *L. reuteri* RC-14, *L. rhamnosus* LGG, and *L. paracasei* F-19 exposed to simulated gastrointestinal digestion. Besides that, other characteristics associated with pectins were observed to improve probiotic survival during their gastrointestinal transit, such as less negative zeta-potential, higher molecular weight, lower values of hydrodynamic sizes, viscosity and degree of branching (Larsen et al., 2018).

Melchior et al. (2020) investigated the protective effect of methylcellulose, sodium alginate, and whey protein-based structures on the viability of *L. rhamnosus* and *Streptococcus thermophilus* during storage and in vitro digestion, reporting that methylcellulose leads to a significant reduction in viability of both strains after exposure to gastrointestinal conditions. These results suggest that the physicochemical characteristics of the network formed by methylcellulose in water (e.g., high viscosity) preclude probiotic protection during digestion. This finding is in agreements with results published by other authors, using chitosan, locust bean gum and guar gum (Melchior et al., 2020). Using sodium alginate as protective agent also leads to a significant reduction in the viability of *L. rhamnosus* and *S. thermophilus* exposed to gastric and intestinal conditions. Such reduction was ascribed to the high porosity of sodium alginate capsules, which allows digestive fluids and moisture to penetrate through the bead walls (Melchior et al., 2020). The microencapsulation of both probiotics in whey protein-based system resulted in the best option to retain bacteria viability during cold storage and the in vitro digestion process. These authors concluded that the pre-treatments and composition of the material system are crucial for protecting probiotics during their transit through the gastrointestinal tract. In this sense, the heat treatment that whey-protein was subjected may have caused some structural modifications in the hydrophobic sites, allowing probiotic protection and avoiding their release in stressful digestion conditions. Therefore, it is necessary to monitor probiotic survival both along long-term storage and in in vitro gastrointestinal digestion conditions to ensure the recommended levels of probiotics at the time of consumption.

6.4 Probiotics in biotherapeutics

The well-recognized properties of probiotics, paraprobiotics and postbiotics support their use in biotherapeutics. This knowledge, together with the recent advances in Process Engineering and Molecular Biology has enabled the rational design of probiotic-containing products for food and biomedical applications (Zuo, Chen, & Marcotte, 2020). Therefore, probiotics are being increasingly used in clinics and prophylaxis against different diseases, namely relief of gastrointestinal symptoms, prevention and control of obesity, diabetes, some types of cancer, and more recently against psychiatric disorders (Barros et al., 2020).

6.4.1 Immunomodulation by probiotics

Probiotics play important roles for the improvement and maintenance of the immunologic system as most of the immune cells also exist in the intestine. Different studies have provided

unequivocal evidence that specific strains of probiotics stimulate and regulate several aspects of natural and acquired immune responses. In fact, several probiotic strains widely used in food industry (e.g., *L. rhamnosus*, *L. casei*, *B. animalis*, *Lactobacillus johnsonii*, *B. lactis* and *Saccharomyces cerevisiae*) are able to modulate the host immune response (Ashraf & Shah, 2014; O'Connell Motherway et al., 2019). In addition, some strains up-regulate the expression of genes leading to anti-inflammatory responses and down-regulation of pro-inflammatory genes linked to inflammatory signaling pathways (Plaza-Diaz, Gomez-Llorente, Fontana, & Gil, 2014). It was reported that the stimulation of the activity of Toll-like receptor 2 (TLR2) in *L. plantarum* OLL2712 depends both on the culture conditions and on the capacity to induce IL-10 activity (Toshimitsu, Ozaki, Mochizuki, Furuichi, & Asami, 2017).

Supplementing the pregnant nutrition with probiotic organisms can affect fetal immune parameters, such as cord blood interferon (IFN)-γ levels, altering the levels of growth factor (TGF)-β1 and immunoglobulin A (IgA) in breast milk. Infants consuming formula with *B. lactis* Bb12 have higher levels of secretory IgA in feces, which helps them to fight against chronic inflammation (Ashraf & Shah 2014). Probiotics can also enhance nonspecific immune response by activation of macrophages, natural killer cells, antigen-specific cytotoxic T-lymphocytes, and the release of different cytokines in a strain and dose-dependent manner (Stojanov,Berlec & Štrukelj, 2020). In the last year, different authors have reported that probiotics consumption improves the host response to respiratory infections, including SARS-COVID-19. The effects of probiotics on different aspects of immune responses and their antiviral properties in both pre-clinical and clinical contexts have been also reported (Mahooti, Miri, Abdolalipour, & Ghaemi, 2020).

Although the direct interaction of probiotics directly with intestinal epithelial cells and other cell types of the immune system has been deeply investigated, there is a growing number of studies suggesting that probiotics also secrete or release different bioactive molecules with immunomodulatory activity (Hemarajata & Versalovic, 2013; Ruíz García, Hevia, Bernardo, Margolles Barros, & Sánchez García, 2014). These bioactive molecules include cell structural components (lipoteichoic acid [LTA] and exopolysaccharides [EPS]), peptides (secreted or shed, including lactocepins), metabolic products (polyphosphates, lactate, butyrate, tryptophan metabolites, histamine and quorum sensing molecules). In fact, probiotics or their metabolites (e.g., short-chain fatty acids also called volatile fatty acids) interact with diverse immune cells (e.g., antigen presenting cells and T cells) and confer their immunoregulatory functions, thereby maintaining the immune homeostasis by balancing pro-inflammatory and anti-inflammatory immune responses (Kang & Im, 2015). In addition, butyrate produced by different probiotic bacteria leads to the improvement of allergic disease symptoms by increasing the ratio of cytokines (e.g., IL-4, IL-10/IFN-γ, Treg/TGF-β), reducing serum eosinophil levels and the expression of metalloproteinase-9 (Eslami et al., 2020).

6.4.2 Probiotics and obesity

Obesity is a metabolic disorder resulting from unhealthy eating behaviors with consequences in the control of energy intake, consumption and storage that is widely spread all over the world both in developed and developing countries. There is sounded evidence about the composition of the gut microbiota in obesity-related disorders, as it contributes to differences in body weight and body mass indexes (Cerdó, García-Santos, Bermúdez, & Campoy, 2019).

The first studies suggesting the relationship between gut microbiota and obesity reported that germ free adult mice (devoid of gut microbiota) increased *ca.* 60% of the body fat content after having been recolonized with healthy cecal microbiota (Kobyliak, Virchenko, & Falalyeyeva, 2015). It has been proposed that the capacity of the microbiota to obtain energy from food components and regulate the energy balance of the host would be responsible for that. In fact, the Firmicutes and Bacteroidetes ratio (the two prevalent bacterial phyla in the gut microbiota) has been suggested as a marker for obesity (obese individuals usually have a higher proportion of Firmicutes and decreased proportion of Bacteroidetes) (Turnbaugh et al., 2006). Degradation of dietary polysaccharides and fiber by Firmicutes and Bacteroidetes results in the production of short-chain fatty acids, namely acetate, propionate and butyrate. Acetate is a substrate for the synthesis of cholesterol at peripheral tissue (Mazloom, Siddiqi, & Covasa, 2019). In turn, butyrate and propionate are important sources of energy for epithelial cells in the colon and for the *de novo* synthesis of lipids and glucose in the liver, respectively (Shahid & Irfan, 2018). This supports the role of Firmicutes and Bacteroidetes metabolism in the final increase of cholesterol, lipids or glucose, with different consequences in obesity disorders. For this reason, modulating the composition of the intestinal microbiota might be a strategy for the treatment of obesity. In this regard, probiotics stimulate the development of gut microbiota, counterbalancing the changes associated with high-calorie diets by improving beneficial and reducing pro-inflammatory bacteria, and thereby restoring the previous composition of the microbiota (Ejtahed et al., 2019; Kong, Gao, Yan, Huang, & Qin, 2019; Mazloom et al., 2019).

The consumption of yogurts containing probiotic strains of *L. acidophilus, L. casei, B. lactis,* and *S. thermophilus* together with a low-calorie diet enhances the expression of toll-like receptor-2 and FOXP3 genes in obese people, thus initiating inflammatory responses and antigen-specific adaptive immunity (Yazdani et al., 2018). *L. reuteri* strains are also capable of improving pro-inflammatory factors and obesity indicators by modifying expression of insulin and lipid metabolism genes in white adipose tissues (Chen et al., 2018). *Enterococcus faecium* and *B. longum* incorporated into a fermented soy beverage also reduced adipocytes and enhanced the levels of IL-10 and IL-6 in a mice model (de Carvalho Marchesin et al., 2018). In turn, *L. casei* incorporated into a fermented milk decreased the body weight of adult mice and increased the number of IgA positive cells and macrophages in the small intestine (Núñez, Galdeano, de LeBlanc, & Perdigón, 2014). Similarly, diet-induced obese rats showed a reduction of fat, size of adipocytes, expression of lipoprotein lipase, modulated leptin level and increased the expression of receptor-activated adiponectin and peroxisome proliferator-activated receptors-g when their diet was supplemented with probiotic *B. longum* strains (Karimi et al., 2017).

All these findings soundly support the key role of probiotic strains on the Firmicutes/Bacteroidetes ratio, and thus as healthy products for the treatment of obesity.

6.4.3 Probiotics as antibiotic replacers

The indiscriminate use of antibiotics has stimulated the development of different mechanisms of bacterial resistance. This situation is of great concern worldwide because of its high impact on animal and public health. In order to control this problem, the European Food Safety Authority (EFSA) and also the health authorities of different European countries have

strongly restricted the use of antibiotics in poultry feed (Haque et al., 2020). Despite such regulations, at least more than 60% of all antibiotics currently produced are used in livestock production, including poultry (van Boeckel et al., 2015). These problems have strongly encouraged the development of antibiotic alternatives not only from a clinical viewpoint but also from a fundamental research one. In this regard, almost all funding agencies worldwide have considered antibiotic resistance as a priority topic and probiotics appear as an appropriate strategy because of their inhibitory capacity against pathogenic bacteria, as well as livestock growth promotors (Adhikari & Kim, 2017; Dunislawska et al., 2017).

The prophylactic and therapeutic properties of probiotics can be exerted in different ways, namely by modulating the innate and acquired immunity, by directly acting on pathogens or by producing compounds with antibiotic properties (bacteriocins). These mechanisms are determined by the bacterial metabolism, cell surface molecules, the capacity to release antibacterial compounds and the bacterial genetic machinery (Oelschlaeger, 2010). Lactobacilli, enterococci, bifidobacteria, pediococci, streptococci and leuconostocci species are normal inhabitants of the gastrointestinal tract and the genera generally incorporated into functional fermented food products to facilitate their delivery to the gut (Plessas et al., 2017; Priyodip, Prakash, & Balaji, 2017).

In animals, such genera of bacteria can also be incorporated into capsules as freeze-dried powders or as aqueous solutions. PoultryStar is a commercially synbiotic product very useful to diminish the colonization of *Salmonella* Enteritidis and *Campylobacter jejuni* (Ghareeb et al., 2012). CLOSTAT is a probiotic containing *Bacillus subtilis* able to improve the feed conversion, intestinal morphology, ameliorate the immune response and inhibit gastrointestinal tract colonization by *Escherichia coli*, *Salmonella Minnesota* and *C. jejuni* (Abudabos, Alyemni, Marshad, & B., 2013; Melegy, Khaled, El-Bana, & Abdellatif, 2011). Floramax B11 is a combination of *Ligilactobacillus salivarius* and *Pediococcus parvulus* that improves the gain of weight, bone properties, intestinal morphology and immune response, as well also decrease the colonization by *S. Enteritidis* (Fuentes et al., 2013).

Besides bacteria, yeasts (particularly *Saccharomyces boulardii*) can also be used to prevent and treat infectious bacterial diseases, including typhoid, paratyphoid and non-typhoidal *Salmonella* (Tomičić et al., 2016). One of the main advantages of using yeasts is that they are not affected by drugs administered against intestinal pathogenic bacteria, as probiotic bacteria do. For this reason, they have been recommended as a complementary therapy (Martins, Veloso, Arantes, & Nicoli, 2009).

In summary, probiotics have a positive effect on feed digestion, regulation of the gastrointestinal tract microbial communities, physiology and the immune system. However, deep research is still needed to understand the molecular mechanisms involved on the probiotic inhibitory properties, as well as the interaction between probiotics, pathogens and epithelial cells.

6.4.4 Probiotics in diabetic disease

Diabetes disease has been considered as a growing epidemy that requires the development of strategies at the molecular level. Type 2 diabetes mellitus is the most common form of diabetes and is characterized by an increased concentration of glucose in blood that leads to systemic damage, especially at vessels and nerves levels (WHO, 2006). The disease is

associated to a severe dysbiosis (Tilg & Moschen, 2014) and it has been suggested that the overgrowth of certain Gram-negative bacteria can determine the risk of diabetes on inflammatory pathways (Gulnaz et al., 2021). Indeed, an excessive availability of lipopolysaccharides from Gram negative microorganisms may promote the leakage of the intestinal barrier and lead to chronic systemic inflammation (Ghareeb et al., 2012). In addition, the gut microbiota can modulate the production of enteroendocrine peptides, such as glucagon-like peptide-1 (GLP1) in the gut, by L-cells, thus having an important role on glucose metabolism. The secretion of GLP-1 is associated with a reduction in gastric emptying time and food intake, and an increase in insulin secretion (Hasain et al., 2020).

All this background supports the intervention of probiotics to restore normality of the gut microbiota. For this reason, using probiotics (mainly lactobacilli and bifidobacteria) in clinics enabled the improvement of glycemic parameters (Hsieh et al., 2018; Sun et al., 2020). As the mechanisms of action are species and strain dependent, cocktails including multiple species are generally used until deeper investigations shed light on specific strains. The efficiency of such treatments is improved when long-duration treatments (*ca.* 8 weeks) of appropriate concentrations of probiotics (more than 6 log CFU/g) are employed (Shah & Swami, 2017).

6.4.5 Probiotics and cancer

Probiotics may be dietary constituents that reduce the risk and incidence of cancer risk (Perillo et al., 2020; Śliżewska, Markowiak-Kopeć, & Śliżewska, 2021). There exists reported evidence about the capacity of probiotics to modulate the proliferation and apoptosis of gastric and colonic cancer cells as well as that of myeloid leukemia cells (Han, Lee, Park, & Paik, 2015; Saxami et al., 2016; Tiptiri-Kourpeti et al., 2016). Several researchers reported a significant antiproliferative and/or induction of apoptosis on colonic cancer cells in vitro (e.g., HGC-27, Caco-2, DLD-1, HT-29) (Altonsy, Andrews, & Tuohy, 2010; Sadeghi-Aliabadi, Mohammadi, Fazeli, & Mirlohi, 2014), together with a decrease of IL-8 levels (Lopez, Li, Kataria, Russell, & Neu, 2008).

In vivo studies have been mainly carried out on animal models. The treatment of *Bacillus polyfermenticus*, *Bifidobacterium infantum*, *B. bifidum*, *L. acidophilus*, *L. casei*, *L. lactis*, *L. plantarum*, *L. rhamnosus*, and *L. salivarius* significantly inhibited the development of colonic cancer in rats or mice injected with the carcinogenic 1,2-dimethylhydrazine (Chang, Shim, Cha, Reaney, & Chee, 2012; Zhang et al., 2015). The in vitro (eukaryotic cell lines) and in vivo (animal model studies) beneficial effects of probiotics support the use of probiotics-based products on cancer prevention and/or as adjuvants in anticancer chemotherapy treatments (El-Nezami et al., 2006; Rafter et al., 2007; Rasouli, Ghadimi-Darsajini, Nekouian, & Iragian, 2017).

Although the anticarcinogenic effect of probiotics is strain dependent, there is no doubt that several probiotic strains play key roles in preventing, treating and reducing the progression of several types of cancer (e.g., liver, breast, cervical, colon, colorectal and bladder). The mechanisms behind this anticarcinogenic effect include the modification of the intestinal microbiota composition and its metabolic activity, the production of compounds with anticarcinogenic properties (e.g., conjugated linoleic acid and short-chain fatty acid), the induction of cancer cells' apoptosis and inhibition of their proliferation, the influence on mutagenic and carcinogenic factors, the degradation of carcinogenic compounds present in the intestinal lumen, immunomodulation, and improvement of the intestinal barrier (Śliżewska et al., 2021).

6.4.6 Probiotics in psychiatric disorders

The concept of psychobiotics is relatively novel (Dinan, Stanton, & Cryan, 2013) and denotes a new class of probiotics acting as carriers of neuroactive compounds (Barros et al., 2020). A psychobiotic is a live organism that, when ingested in adequate amounts, produces health benefits in patients suffering from psychiatric diseases. Psychobiotics produce and deliver compounds with neuroactive properties (e.g., gamma-amino butyric acid and serotonin) that regulate the activity of the hypothalamic-pituitary-adrenal axis and the vagus nerve, leading to the improvement of psychiatric disorders (Dinan et al., 2013). Wallace and Milev (2017) found that a 4-week treatment with probiotic strains acting on the gut-brain axis (*Lactobacillus helveticus* R0052 and *Bifidobacterium longum* R0175) alleviates psychotic symptoms of mood, anhedonia and sleep disturbance, and these results endure up to 8 weeks. Other probiotic species (e.g., *L. rhamnosus* JB-1 and *L. plantarum* PS128) demonstrated anxiolytic and antidepressive effects in mice, and these psychotropic effects are related with their capacity to modulate the levels of serotonin, dopamine and their metabolites (Liu et al., 2019).

As this psychobiotic effect of probiotics is relatively novel, there are still a lot of unknown mechanisms, thus opening the possibility of thorough studies aiming to unravel biological mechanisms and also their biotherapeutic potential (Tremblay, Lingrand, Maillard, Feuz, & Tompkins, 2021).

6.4.7 Para and postbiotics in biotherapeutics

Paraprobiotics and postbiotics also provide biotherapeutic health benefits, including effectiveness in treatment and prevention of alcohol-induced liver disease, respiratory and infectious diseases, diarrhea, colitis, atopic dermatitis, dental caries, allergies, inhibition of pathogens, improvement of anxiety and stress, modulation of the gut microbiota, immune system, and gut microbiota (Barros et al., 2020). In particular, their immunomodulatory activity may represent a safer alternative when the use of live probiotic bacteria is not indicated, as the case of immunodeficient individuals. For this reason, using paraprobiotics and postbiotics in these cases reduces the risks of:

a. Development of opportunistic infections (fungemia, bacteremia, endocarditis and sepsis);
b. Increased inflammatory responses to vaccine or allergens (fever and arthritis);
c. Deleterious metabolic effects due to the degradation of mucine and production of deconjugated bile salts and D-lactate, which can cause gastrointestinal disorders, cholestasis and/or colorectal cancer and D-lactic acidosis, respectively;
d. Horizontal transference of antibiotic resistance genes to other commensal or pathogenic bacteria in the gut; and,
e. Microbial translocation.

6.5 Industrial perspectives of probiotic production

The worldwide probiotics market size was *ca.* 51.72 billion USD in 2019, being expected to expand over 6.9% in the 2020–2027 period, reaching *ca.* 88.20 billion USD in 2027 (Global probiotics market). The well-demonstrated benefits of probiotic consumption and the increas-

ing incidence of different digestive disorders are the main factors leading to the increasing demand of probiotic containing products. In addition, the awareness of consumers for ingesting functional foods, stimulated by the increasing access to information, strongly contributes to the sustainable rise of the global probiotics market. Because of their high nutritional value, probiotics are employed not only as functional ingredients but also as dietary supplements, and this stimulates the growth of both the food and nutraceutical industries. All these reasons encourage researchers and technologists to get insights into their applications supporting the development of innovative products.

Dairy products (e.g., yogurts, fermented milks, cheeses, and ice-creams) have been traditionally the main food matrices for the incorporation of probiotics at a global level. Innovation in Food Science and Technology, together with the evolving regulatory framework, the increasing number of people allergic to milk proteins and growth of vegan population, created business opportunities for the incorporation of probiotics in other food matrices, namely drinks, juices, fruit and vegetables, cereals, and soybean products (Cassani et al., 2020).

In developed countries and emerging markets, probiotics supplements represent another group of products with an increasing demand. The presence of a sophisticated retail infrastructure and the high income of consumers are the main factors conducting to the expansion of the probiotics market in Europe and USA. China and Japan are the markets having the highest growth in Asia Pacific, including both the food and nutraceuticals. The expanding middle class in certain countries of Africa (e.g., South Africa) and America (e.g., Brazil) is another factor pushing the market growth in those countries because of the willingness to spend toward health and wellness.

A segment of the probiotic market is occupied by animal feed, whose size was 3.56 billion USD in 2018 and is projected to reach 6.24 billion USD by the end of 2026 (CAGR 7.30%). This segment is, in turn, subsegmented into poultry, swine, ruminants, aquaculture, pets, and others. The poultry segment is projected to dominate the probiotics in the animal feed market in 2025 and the Asia Pacific region is expected to be that with the highest demand because it of its large population and the increase in a number of urbanized populations opting for protein-rich food options including poultry products (e.g., eggs and meat). Besides that, probiotics are being increasingly used as sustainable growth promoters in replacement to antibiotics (Probiotics in Animal Feed market, Global Forecast to 2025). The consciousness of producers and the regulatory requirements for sustainable production have also brought clean-label needs. Therefore, animal owners are going for natural offerings that improve their animal productivity. Hence, the demand for probiotics in the animal feed industry is expected to increase.

Regarding para and postbiotics, the market is still under development and no official information about their growth is available, mainly because of two reasons. From the one side, the concepts of para and postbiotics are relatively new (2011) (Barros et al., 2020; Mantziari et al., 2020). From the other side, in the case of postbiotics, they include a heterogeneous group of compounds (e.g., organic acids, bacteriocins, enzymes and polysaccharides) that are already being commercialized. Among them, bacteriocins are the postbiotics most widely expanded as commercial products. In particular, nisin, and bacteriocin produced by *Lactococcus lactis* subsp. *lactis* is currently commercialized by different American and European enterprises as natural preservatives with inhibitory action against foodborne enteropathogens, such as *Clostridium difficile* (Nataraj et al., 2020). Although the commercial

potential of para and postbiotics is doubtless, their current production is possible at laboratory scale or under small-scale production. At industrial levels constrains could occur due to a change in processing conditions or scaling that can lead to structural modifications and variations on their physiological function. In addition, standardized methods for their production are necessary to warrant a uniform production, and thus the effectiveness in potential prophylactic and therapeutic treatments. For these reasons, innovation in the production of post and paraprobiotics is still needed to identify processes that ensure their stability and bioactivities (Cuevas-González, Liceaga, & Aguilar-Toalá, 2020).

6.6 Conclusions

Although the concept of probiotics is not new, the dynamism of research on fundamental and applied studies provides a continuous support for novel technological and therapeutic perspectives. Furthermore, the awareness of consumers for healthy food products, especially plant-based ones, enhances the demand for innovative products with great impact on market development. This chapter aimed to present the most novel features of technological and biotherapeutic aspects of probiotics, paraprobiotics and postbiotics as triggers for future innovations in a field where the market is increasingly demanding worldwide.

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CHAPTER

7

Probiotics and bioactive metabolite production

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7.1 Introduction

Until recently, it has become prominent that food is more than a source of energy and nutrients. More clearly, food is now quoted as "medicine." The food is realized to perform the functions of maintaining health and well-being, owing to their antioxidant, anti-inflammatory, antimicrobial, and immune-modulating activities. A long list of bioactive compounds sourced from plants to microorganisms, are generally involved in the underlying mechanisms. The Russian Nobel laureate É lie gave the canvas to this theory by showing the health benefits and longitivity of ingestion of a number of bacteria. Whilst scientists are still examining the human health effects and exact implications of peculiar type of gut microflora (Mai & Draganov, 2009). FAO/WHO, 2001 defined the probiotics as "*live microorganisms which when administered in adequate amounts confer a health benefit on the host*" ("FAO/WHO Joint Expert Consultation Report," 2001). Bacteria such as *Lactobacillus* and *Bifidobacterium* and yeast like *Lactococcus, Streptococcus, Enterococcus, Propionibacterium,* and *Saccharomyces* are predominant and widely existing probiotic microorganisms (Azad, Sarker, Li, & Yin, 2018). A wide range of probiotic functional foods are prepared in the form of dairy products such

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as milk, acidified milks, yoghurt, cheese, cream, and ice cream, also other products like meat and meat products, bread and cereal fiber snacks, juice and various fruit products (Terpou et al., 2019). Probiotics produce a wide range of bioactive compounds; confer enormous health benefits involving varying mechanisms. Probiotics carry out competitive inhibition of intestinal pathogenic bacteria by changing the pH and reducing the availability of oxygen (Schepper et al., 2017), production of bacteriocins for non-competitive inhibition (Lopetuso et al., 2019), synthesis of essential micronutrients like vitamins, amino acids and enzymes, the enhancement of the bioavailability of various dietary nutrients (Pandey, Naik, & Vakil, 2015), host immune system stimulation (Kober & Bowe, 2015), and increase the metabolism of undigested carbohydrates (Kerry et al., 2018). In this sequence, this chapter deliberates the probiotic microflora, production of various bioactive metabolites including their health-promoting effects and their supplementation in diet.

7.2 Defining the gut microbiome

The gut microbiome or microbiota can be stated as the group of mutualistic, symbiotic and pathogenic microorganisms and the presence of their cumulative genetic material in gastrointestinal (GI) tract (Alexandre & Centre, 2018). The human gut microbiota inhabited approximately 10^{13} to 10^{14} microorganisms and can further be divided into four main phyla, that is, *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* (Sender, Fuchs, & Milo, 2016). Strict anaerobes in colons comprise majority of 97% from *Firmicutes* (64%), *Bacteroidetes* (23%), *Proteobacteria* (8%), *and Actinobacteria* (3%). Fungi and archaea make less than 1% of total gut microbiota (Cardinelli, Sala, Alves, Torrinhas, & Waitzberg, 2015).

It was assumed that microbiota begins from birth itself, but this belief is challenged by few studies where microbes were detected in the placenta. After development over exposure with illness, antibiotic treatment, change in diets and lifestyle post birth, GI tract become colonized swiftly and changes were observed in microbiota (Rodríguez et al., 2015). Also, composition of microbiota will be affected by the procedure of delivery, as microbiota of newborn observed to have high load of *Lactobacilli* which directly indicates similarity toward vaginal flora (Avershina et al., 2014), whilst in newborns delivered by C-section, colonization of *Bacteroides* is hampered and delayed while facultative anaerobes like *Clostridium* are present (Jakobsson et al., 2014). The composition of microflora is further impacted by the nutritional, immunological and chemical factors in the gut. For instance, high amount of acid, oxygen and antimicrobials supports the growth and survival of facultative anaerobes that grow at fast rate and adhere to intestinal epithelia unlike other bacteria (Donaldson, Lee, & Mazmanian, 2016).

A wide range of benefits are provided to host by gut microbiota due to its substantial genomic content and metabolic complement as shown in Fig. 7.1. The microorganisms present in the gut aid to maintain the mucosal barrier integrity and provide nutrients to safeguard from pathogens as well (Thursby & Juge, 2017). Some bacteria after colonization, secrete carbohydrate-active enzymes which help to ferment complex carbohydrates resulting in generation of metabolites like short chain fatty acids, that is, acetate, propionate and butyrate (Musso, Gambino, & Cassader, 2010). The changes in composition of gut microbiota may lead to inflammatory bowel disease, obesity, type 2 diabetes, cardiovascular disease and cancer (Alexandre & Centre, 2018). The diet is considered to play a key role in composition

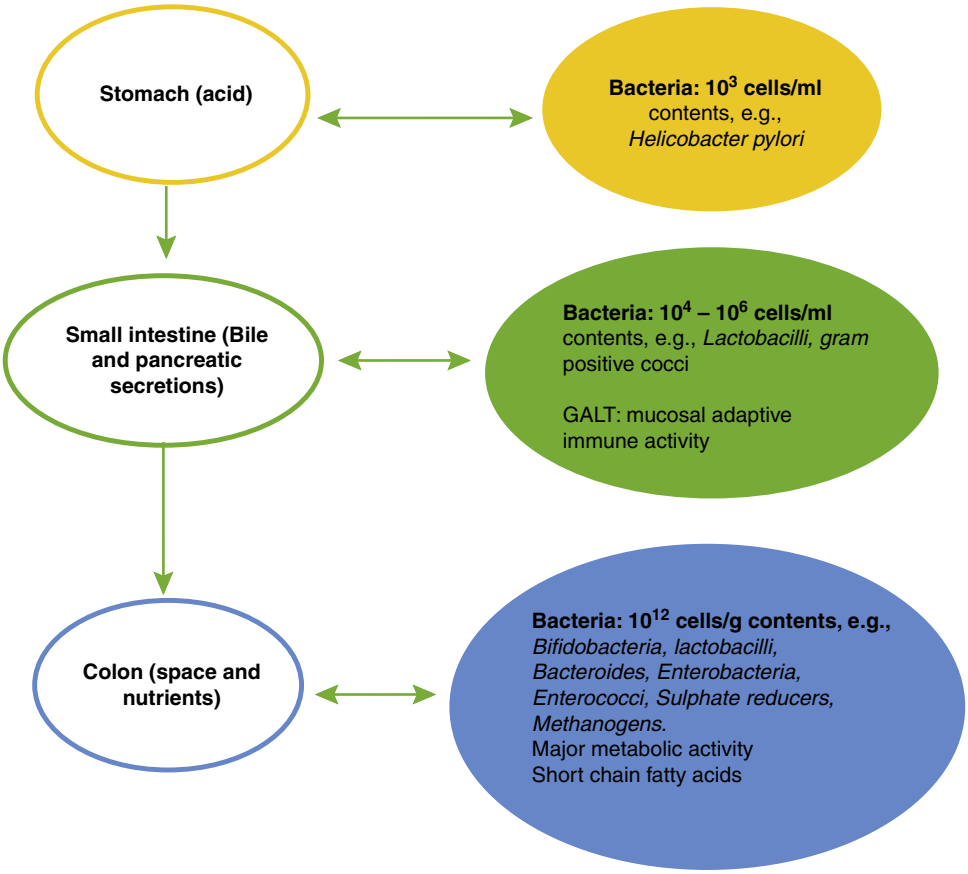


FIG. 7.1 Reciprocal relationship between human gut bacteria and the host.

and diversity of gut microbiota. Not only the microorganisms present in the diet but also the lifestyle, dietary behavior and geographical conditions impact the changes in microbiome. Conclusively, the change in food habits, dietary components, including probiotics and prebiotics can help in regulating gut microbiota. In humans, studies have shown the impact of diet on microbiota on the basis of three dietary components, that is, fats, carbohydrates and proteins. Majorly intestinal flora uses these dietary components as an energy source and further influence the gut microbiota (Graf et al., 2015). In terms of dietary products, Wu et al. (2011) reported that *Bacteroidetes* and *Actinobacteria* show positive relation with fat but negative with fibers and opposite in case of *Firmicutes* and *Proteobacteria*.

Various strains of *Lactobacillus* and *Bifidobacteria* are widely used as probiotic microorganisms (Pandey et al., 2015). Most of the groups of lactic acid bacteria, present in fermented foods like milk or yogurt are evident to increase total microbiota when included periodically in diets (Singh et al., 2017). To experience long term effects, continuous consumption of probiotics in appropriate amounts is advised as most of the probiotic microorganisms do not colonize in the gut of the host (Alexandre & Centre, 2018).

7.3 Probiotic bioactive metabolites and their mechanism of action

Probiotics, by their ability to synthesize bioactive metabolites in the host intestine assists in creating an environment that knocks out potential pathogenic microorganisms and encourages the host by flourishing its resistance to new harmful bacteria colonization. Probiotic mediated immune modulatory complexes promotes the secretion of different cells through various pathways such as Nuclear factor kappa B and mitogen activated protein kinase. Furthermore, they bring about the differentiation of cells and release the immune cells into the host body. Probiotic strains such as *Bifidobacterium bifidum, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus rhamnosus* and *Saccharomyces boulardii* produce an array of bioactive metabolites ranging from proteins, carbohydrates, vitamins, enzymes, oligosaccharides, exopolysaccharides, short-chain fatty acids, bacteriocins, and immunomodulatory compounds (Kodali & Sen, 2008). For these reasons, the human gut microbiome is speculated to be an emerging source of unique therapeutic compounds to combat the issues related to obesity, microbial infections, metabolic disorders and host immunity (Kodali & Sen, 2008).

The various bioactive agents produced by probiotic strains as displayed in Fig. 7.2 are described in detail in following sections.

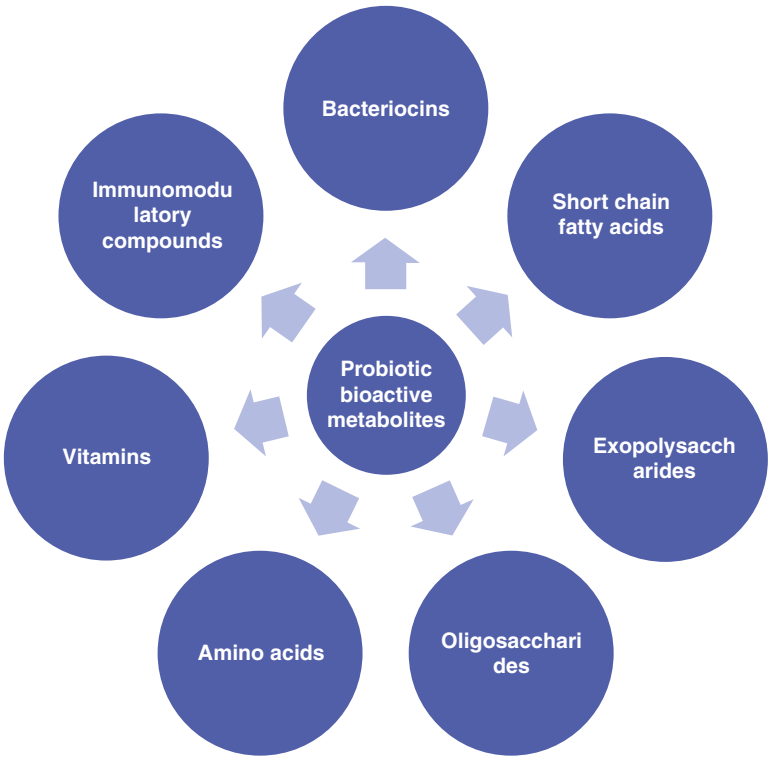


FIG. 7.2 Various bioactive metabolites released by probiotic strains.

7.3.1 Bacteriocins

Bacteriocins are heterogeneous low molecular weight polypeptides (comprising 20–60 amino acid residues) synthesized by beneficial bacteria present in the gut environment. Both gram-positive and gram-negative bacteria have the ability to produce these antimicrobial compounds that will prevent the invasion of pathogenic microorganisms in the host intestine and thus help in establishing a specific niche for the probiotic bacteria (Zacharof & Lovitt, 2012). Each bacteriocin is unique in nature with respect to its size, structure, antimicrobial potency, immunity mechanisms and target cell receptors (Iwatani, Zendo, Yoneyama, Nakayama, & Sonomoto, 2007). They are non-toxic and exhibit a narrow spectrum of activity (Mokoena, 2017; Yang, Lin, Sung, & Fang, 2014). Bacteriocins may often behave differentially by either functioning as colonizing peptides, antimicrobial (killing) peptides or signaling peptides (Dobson, Cotter, Ross, & Hill, 2012).

In recent times, the bacteriocins derived from lactic acid bacteria have captivated food technologist's attention to prevent food spoilage by food-borne Gram-positive pathogenic bacteria owing to their non-toxic behavior and safety aspects (Morisset, Berjeaud, Marion, Lacombe, & Frère, 2004). These bacteriocins could either be bactericidal or bacteriostatic in their action based on the level of dose used, extent of purification, physiological state of target cells and experimental conditions (Parada, Caron, Medeiros, & Soccol, 2007). These effects are brought up possibly due to their association with the receptor of target cell, and thereby inducing pore formation, degeneration of cellular DNA and suppression of peptidoglycan synthesis (Deegan, Cotter, Hill, & Ross, 2006). Furthermore, these have found numerous applications in human therapy either by working as antibiotic replacements or supplements (Fricourt, Barefoot, Testin, & Hayasaka, 1994; Ogunbanwo, Sanni, & Onilude, 2003).

7.3.1.1 Types of bacteriocins

Apart from several probiotic strains, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus* are known for the synthesis of majority of different bacteriocins. These antibacterial peptides are colorless, odorless, and tasteless. These compounds can be classified into different groups on the basis of their molecular weight, sensitivity, thermostability, post-translational modification and mechanism of action (Deegan et al., 2006).

Class I bacteriocins (Lantibiotics): The peptides belonging to this group are low molecular weight (generally <5 kDa) and stable to heat. They possess amino acids with thio-ester groups such as lanthionine and methyllanthionine along with other altered amino acids namely dehydrated serine and threonine.

Lantibiotics, depending on their structural similarities can be segregated into two sub-classes.

Subclass Ia: This class encompasses the positively charged peptides which associates with the negatively charged phospholipids present in the cytoplasmic membrane of the target cells and creates pores, for example, Nisin

Subclass Ib: The peptides belonging to this group either carry no net charge or negatively charged. Because of their structural complexity, they exhibit their action by interrupting with enzymatic reactions of target cell.

Class II bacteriocins (Non-lantibiotics): These thermo-stable compounds have diverse range of molecular weight, usually <10 kDa. Bacteriocins belonging to this class are further divided into four sub-classes:

Subclass IIa: The antimicrobial activity of this group against *Listeria* genera is particularly due to the presence of the YGNGV sequence at N-terminal. Examples are Pediocin PA-1, Sakacin P, Enterocin NKR-5-3C, Enterocin A, Munditicin, and Leucocin A.

Subclass IIb: This type of bacteriocin has two peptides that act in a synergic manner to provide either little or no activity. Examples are Lactacin F and Lactococcin G.

Subclass IIc: Such peptides are short and require a leader peptide for their transportation. Their mechanism of action is controlled by the presence or absence of intramolecular disulfide bonds. Examples are Diverginin A and Acidocin B.

Subclass IId: These bacteriocins are sec-dependent and do not require any leader peptide for their function. Examples are Lacticin Q, Z, Weissellicin Y and M, and Leucocin Q and N.

Class III bacteriocins: These are large sized; heat labile bacteriocins generally having molecular weight of approximately 430 kDa. Examples are Helveticins J and V, Acidofilicin A, and Lactacins A and B.

Class IV bacteriocins: These peptides are associated with either lipid or carbohydrate moiety (Tenea & Yépez, 2016).

7.3.1.2 Mechanism of action

In case of gram-positive bacteria, bacteriocin acts as colonizing peptides which accelerates the interaction of probiotic with the microbiota of host cell. Immediately after the interaction, they may try to reduce the pathogenic load or directly kill the peptides which correspond to the pathogens. Nisin which is widely recognized as the first biopreservative (Gürakan, 2007), was produced by *Lactococcuslactis* ssp. *Lactis*. Despite of numerous technological interventions, Nisin has proven to be the sole commercial bacteriocin which is permitted by World Health Organization and US Food and Drug Administration for its exploitation as a food additive (Perez, Zendo, & Sonomoto, 2014). This peptide with 34 amino acids and molecular weight of 5 kDa can exist in two forms Nisin A and Z. Their lethality is a function of cationic nature, hydrophobicity and heat stability. Small size bacteriocins remain functional from pH 3 to 9. The heat stability of Nisin is linked to the significant number of intramolecular linkages (monosulfide and disulfide bonds) present that helps in stabilizing its secondary structure. Conclusively, Nisin exhibits superior antimicrobial activity at wide range of pH and at higher processing temperatures than its counterparts, thereby making it ideal for use in food preservation (Oscáriz & Pisabarro, 2001). Nisin acts principally by inhibiting the cell wall biosynthesis of spore forming bacilli in a manner that it first binds to the peptidoglycan (Lipid II) which transports it to the site of cell wall synthesis via cytoplasm and leads to the death of cells. Alternatively, Nisin can act by molecular docking process that begins with the process of membrane insertion to create pores and ultimately kills the cell (Gillor, Etzion, & Riley, 2008). Moreover, other low molecular weight metabolites produced by *B. Longum* BL 1928 species exhibit no direct influence over the inhibition activity but it reduces the binding efficiency of *E. coli* present in GI tract. They are active against gram negative bacteria. Colicin is another high molecular weight peptide obtained from *E. coli* strain. It was found that Colicin E1 and K inhibit the synthesis of macromolecules without compromising the respiration rate whereas colicin E2 leads to DNA denaturation (Gillor et al., 2008). It has been observed that some of the bacteriocins act as protein pheromone, which helps in regulating the gene expression under the influence of signal transduction in various quorum systems. Bifidocin B, yet another bacteriocin produced by *Bifidobacterium bifidum* NCFB 1454 possess

dynamic activity toward gram-positive bacteria (Bermudez-Brito, Plaza-Díaz, Muñoz-Quezada, Gómez-Llorente, & Gil, 2012).

7.3.2 Short chain fatty acids (SCFAs)

Human body lacks certain complex-carbohydrate digesting enzymes because of which a fraction of carbohydrates remain undigested. This problem can be overcome with the help of ingestion of probiotic bacteria which are bestowed with the enzymes that possess the capability to ferment these undigested carbohydrates. During the fermentation process, the complex sugars are converted into a range of SCFAs, gases (like methane, carbon dioxide and hydrogen) and energy. As a result, the host cells acquire antimicrobial activity and simultaneously utilize the energy released to accomplish various metabolic activities (LeBlanc et al., 2017; Thursby & Juge, 2017; Yoo & Kim, 2016). In a study, den Besten et al. (2013) reported that this fermentation reaction contributes to the 10% of caloric requirement on day-to-day basis. Butyrates, being the predominant SCFA, are responsible for approximately 60 to 70% of this energy and thus known as a source of fuel for brain, colon and muscle cells. Additionally, these SCFAs exhibits beneficial effects in managing diseases such as antibiotic-linked diarrhea, colon cancer, inflammatory bowel disease (IBL), and heart diseases (Gill, M., Muir, & Gibson, 2018; Moss, Williams, & Ramji, 2018; Sharma & Shukla, 2016; Tominaga et al., 2018).

Often, these compounds are known as volatile fatty acids, and consist of anions of carbon atom ranging from 1 to 6 in numbers with acetates, propionates and butyrates being produced in larger proportions (Table 7.1). Although, SCFAs can be obtained from various sources in direct form but its predominant source remains the fermentation of complex carbohydrates in the gut. Depending on the nature of the diet, population density of microorganisms in the intestine and transit time in the colon, the production of SCFAs in the intestine is roughly around 500 to 600 mmol per day (Gill et al., 2018). The production of SCFAs in the form of propionate, and butyrate is significantly higher when administered with strains such as *Lactobacillus salivarius ssp. Salicinius* (Meimandipour et al., 2010). Moreover, the combination of probiotics along with different strains such as *Lactobacillus rhamnosus* leads to the production of large amount of SCFAs (LeBlanc et al., 2017).

The rate of production of SCFAs in the intestine depends upon the underlying factors:

Nature of fiber: A study conducted by Govers, Gannon, Dunshea, Gibson, and Muir (1999) and McIntyre, Young, Taranto, Gibson, and Ward (1991) reported an increase in

TABLE 7.1 List of some of the short chain fatty acids with their chemical formula (Markowiak-Kopeć & Śliżewska, 2020).

SCFAs	Chemical formula	Molar mass (g/mol)
Acetic acid	CH_3COOH	60.05
Butyric acid	$CH_3 (CH_2)_2 COOH$	88.11
Caproic acid	$CH_3(CH_2)_4COOH$	116.16
Formic acid	HCOOH	46.03
Propionic acid	CH_3CH_2COOH	74.08
Valeric acid	$CH_3 (CH_2)_3COOH$	102.13

SCFA concentration in rats and pigs after consuming wheat bran along with resistant starch that is quickly fermented in comparison to the animals that were just fed on resistant starch only.

Gut transit time: As the transition time across the colon changes, so is the rate of fermentation, thereby affecting the change in SCFA absorption levels in the proximal region of colon. El Oufir et al. (1996) observed an increase in concentrations of butyrate in fecal matter of humans if the transition rate across the colon is fast and there is no change in intake of dietary fiber. Further, an effective increase/decrease in the levels of SCFA scan be observed on taking up the drugs that hastens (e.g., cisapride) or slow down (e.g., loperamide) the colonic transit, respectively.

7.3.2.1 *Mechanism of action*

Fermentation of dietary fiber leads to the production of SCFAs which are further absorbed by colonic cells of host cell by means of passive diffusion or exchange with carbonates which are then partially oxidized into carbon dioxide. Those SCFAs which are not absorbed by colonic cells, they are being transported to liver through basolateral membrane for release of energy in hepatocyctes via oxidation reaction (Dalile, Van Oudenhove, Vervliet, & Verbeke, 2019). During fermentation, a mixed population (*E. coli*, *Salmonella*, *Shigella*, etc.) of microbiota is responsible for the breakdown of carbohydrate into several intermediate metabolites which subsequently helps in the metabolism of other components. It also causes the loss of substances like ethanol, lactate, pyruvate for the maximum production of SCFAs (Markowiak-Kopeć & Śliżewska, 2020).

The positive effects of SCFAs on gut health are attributed to several effects such as upholding the integrity of the intestine membrane, impeding inflammation and diminishing the danger of colorectal cancer (Macfarlane & Macfarlane, 2003). Butyrate plays a key role as energy source for human colonocytes which can further initiate apoptosis of colon cancer cells and induce intestinal gluconeogenesis (De Vadder et al., 2014). They are necessary for epithelial cells to maintain oxygen balance in the gut and hence avert gut microbiome dysbiosis, which can otherwise lead to diet induced obesity and other metabolic problems (Byndloss et al., 2017). On the other hand, propionate regulates gluconeogenesis in liver (De Vadder et al., 2014) and acetate aids cholesterol metabolism and lipogenesis after reaching to peripheral tissues and acts as an important metabolite for bacterial growth (Frost et al., 2014).

7.3.3 Exopolysaccharides (EPS)

Exopolysaccharides are synthesized by various probiotic strains in large amounts, where those exclusively produced by lactic acid bacteria have attained GRAS (generally recognized as safe) status in recent years (Kodali, Das, & Sen, 2009; Mikkili et al., 2016). EPS are produced from the precursors of sugar nucleotides by the action of enzymes. These high molecular weight components of carbohydrates can be substituted with proteins, phospholipids, and some non-carbohydrates components as well.

Synthesis of EPS from probiotics can be done in two different ways such as extracellular biosynthetic pathway and intracellular biosynthetic pathway. Homopolysaccharides are synthesized by extracellular biosynthetic pathway whereas as heteropolysaccharides are

synthesized via both intra and extracellular pathways. In an extracellular biosynthetic pathway, the monomers of sugar molecules are released outside the bacterial cell and then polymerized extracellularly in the presence of glycosyl transferases for the synthesis of EPS polymer. For example, sucrose sugar which reduces into glucose and fructose inside the bacterial cell and then releases out of the cell for further mechanism. These residues then undergo polymerization and turns into glucan and fructan in the presence of glucan sucrase and fructan sucrase respectively (Zannini, Waters, Coffey, & Arendt, 2016). In case of intracellular biosynthetic pathway, necessary modification of the polymer occurs within the cell. The sugar residues are transported into cell, changed into various monomeric units which gets polymerize partially and then gets attached to lipid carrier membrane, that is, isoprenoid. After the modification, monomers are released outside the cell and assembled as polysaccharides. The synthesized heteropolysaccharides are relatively complex whose activity depends on various factors like enzymes, carriers, and transporter proteins encoded by genes of chromosomal or plasmid origin (Patten & Laws, 2015).

7.3.3.1 *Mechanism of action*

Lately, the exopolysaccharides of microbial origin have gained popularity owing to their anti-tumor effects, ability to reduce blood cholesterol levels, immune-stimulatory and modulatory behavior and antioxidant activity (Ghan, Elhafez, & Hamouda, 2014; Tsai et al., 2014). Strains like *Lactobacillus acidophilus, Lactobacillus plantarum* and *Lactobacillus rhamnosus* are reported to produce EPS which act against cancerous cells and oxidation reactions. EPS produced by *Lactobacillus* strain promotes the activity of innate and acquired immunity whereas *Bifidobacterium* stimulate the immunomodulatory activity (Angelin & Kavitha, 2020). EPS produced by lactic acid bacteria promotes the immunomodulatory activity of antigen presenting cell via TLR2 and TLR4 signals. High molecular weight EPS helps in the initiation and differentiation of dendritic cells, which regulates the formation of cytokines. Consequently, it promotes the differentiation of T-cells into Tregs which possibly control the excessive response from T-cells. Moreover, EPS may perhaps efficiently stimulate production of cytokines by macrophages which have potency to cause homeostasis via reduction of pro-inflammator (Saadat, Khosroushahi, & Gargari, 2019). Immunomodulatory components released by lactic acid bacteria promote the stimulation of mucosal immune response via immunoglobulin A, which support the mucosal barrier layers against the antigens due to which the binding efficiency of the cells to the receptor site reduces. Therefore, it acts efficiently to protect the cells from invasion (Matsuzaki et al., 2015). Notararigo, de Las Casas-Engel, de Palencia, Corbí, and López (2014) reported that strains of *Pediococcus parvulus 2,6* and *Lactococcus lactis* recombinant type produced 2-substituted β-(1→3)D-glucan that exhibited immunomodulation activity possibly by activating human macrophages and encouraging the production of cytokines. EPS promotes the antibody mediated immunity produced by lymphocytes which helps in eliminating the antigens. Furthermore, they reduce the chances of infection, inflammatory diseases, and other GI tract diseases. Furthermore, *Chinese Rhizobium* produces β-D-(1→4) with 1:2 β-D-(1→6) branched glucan with anti-tumor and immune-boosting activities as revealed in mice samples (Zhao, Chen, Ren, Han, & Cheng, 2010). Besides their health benefits, these compounds being good emulsifiers have found numerous applications in food formulations as well (Peele, Ch, & Kodali, 2016).

7.3.4 Oligosaccharides

Oligosaccharides are considered as the food source for probiotic microorganisms, thus support their growth and regulate the production of various bioactive metabolites. Present in plants as non-digestible cross-linked polymers, these oligosaccharides differ in terms of the number of monomeric units attached and the type of glycosyl moieties (Meyer, Miguel, Fernandez, & Ortiz, 2015). Some of the examples of oligosaccharides available are inulin, glucooligosaccharides, fructooligosaccharides, and xylooligosaccharides (Yoo & Kim, 2016). Hou, Huo, and Dignam (2013) reported the protective role of glucooligosaccharide on the development of tumors. Moreover, galactooligosaccharides exerts laxative effect and diminishes the severe effects observed during constipation and irritable bowel syndrome especially in the aged people (Rao, Yu, & Fedewa, 2015).

7.3.5 Enzymes

Human food contains many complex compounds (such as anti-nutritional factors in legumes, non-starch polysaccharides etc.) that remain unbroken by the enzymes present in the digestive system, thereby impairing the digestion process and causing under-utilization of nutrients. In such cases, microbial enzyme preparations can dispose of these anti-nutritional components and complex carbohydrates either partially or completely and thus boost up feed utilization (Ndwigah, Bogai, Wanyoike, & Mwirichia, 2015). Enzymes such as amylases and peptidases produced by *Lactic acid bacteria* and *Bifidobacterial* strains assist in the biochemical reactions of the host cells (Savaiano, 2014). α-glucosidase, ß-glucosidase, and fructofuranosidases are involved in the breakdown of dietary fibers. It was reported that the activity of enzymes depends upon the diversity of microflora present in probiotic food. The metabolites produced from carbohydrate and dietary fiber metabolism possess the capability to inhibit the activity of hazardous pathogens present in GI tract (Sugahara et al., 2015). Subsequent release of organic acid plays an important role to control the growth and division of putrefactive bacteria. Moreover, microbial enzymes in gut aids in generating secondary and unconjugated bile acids to impact host pathways by acting as signaling molecules and metabolic regulators (Long, Gahan, & Joyce, 2017). It has been observed that probiotic microbes *(Lactobacillus casei* and *Lactobacillus plantarum)* are capable of reducing cholesterol levels in the human body by the virtue of microbial enzyme bile salt hydrolase that associates with the cholate solution to bring this effect (Piemolini-Barreto, Antônio, & Echeverrigaray, 2015). The absence of lactase enzyme in some individuals leads to the development of lactose intolerance. Probiotic bacteria, in this condition, are found extremely useful where the bacterial lipases could be used to convert the lactose (milk sugar) into glucose and galactose. Similarly, the bacterial culture used to produce yoghurt reduces its sugar content owing to their hydrolytic activity (Vonk, Reckman, Harmsen, & Priebe, 2012). Besides the lactase enzyme, some of the species belonging to lactic acid bacteria family diminish the effects of lactose intolerance by the activity of β-galactosidase enzyme (Montalto et al., 2006).

7.3.6 Amino acids

Fermentation of proteins by probiotic bacteria in the gut of host cells helps in production of small peptides, amino acids, lactones, and indoles and thus improve host's health in various

ways. Firstly, these amino acids act by inducing the synthesis of SCFAs and other metabolites such as indoles and phenols through the fermentation process (Feng, Ao, & Peng, 2018). As a result, these metabolites aids in maintaining energy balance and provide resistance to host cells against invasion of various pathogens. Secondly, the amino acids and their derivatives are capable of controlling the lipids and carbohydrates metabolism (den Besten et al., 2013). Thirdly, many of the aromatic and flavorful compounds (such as acids, alcohols, aldehydes and esters) can be derived from these amino acids by various biochemical pathways. Consequently, many of the synthetic flavors used in food and dairy products are derived from methionine, phenylalanine, threonine, and other branched-chain amino acids (Smit, Smit, & Engels, 2005). Lastly, these amino acids help in managing various disorders such as D-tryptophan, being an immunomodulatory compound, diminishes the hyperactivity induced in allergic responses (Kepert et al., 2017).

7.3.7 Vitamins

Vitamins are group of substances that are essentially required by the human beings for their growth and development. As humans are incapable to synthesize vitamins, they are needed to be provided in the diet failing which may lead to some type of deficiencies (Fitzpatrick et al., 2012). Probiotics can be considered as the natural means of vitamin enrichment in human beings (Miller et al., 2013). Most of the micro-organisms isolated from dairy products, animal and plant products, gastrointestinal tract etc lead to the production of different vitamins such as Vitamin B1, B9, B12, B5, and B6. The basic response of vitamins released after the metabolism of probiotic is depicted in Fig. 7.3.

Extensive research has been undertaken in the production of riboflavin in bacterial strains such as *Escherichia coli* and *Bacillus subtilis*. Riboflavin is needed for the better absorption of iron and pyridoxine in the digestive system (Lin et al., 2014). It also plays a key role as a precursor of the coenzymes flavin mononucleotide and flavin adenine dinucleotide in several biological oxidation and reduction reactions. The concentration of riboflavin changes due to the processing technologies imply to dairy products. It was found to be increased or decreased due to the incorporation of different strains for the production of fermented dairy products (Burgess, Smid, Rutten, & van Sinderen, 2006). Furthermore, *Bifidobacteria* is recognized as a source of B-complex vitamins, particularly pyridoxine and thiamine, which are required in supporting the gut of a host (Markowiak & Śliżewska, 2017). Folic acid, belonging to B-group of vitamins is produced exclusively by *Lactobacillus plantarum* in large amounts (Patel, Shah,

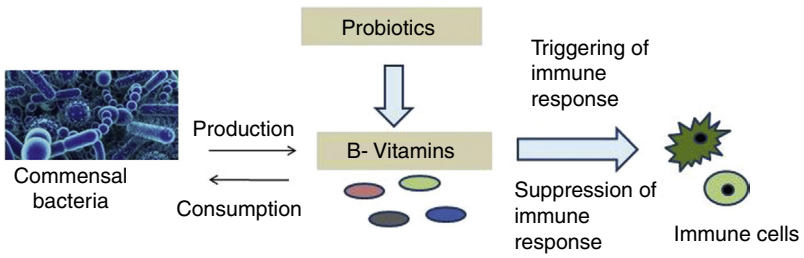


FIG. 7.3 Mechanism of action of vitamin metabolites released after the metabolism of probiotics.

& Prajapati, 2013). It is one of the essential vitamins which are required by bacteria for their metabolism. Folate helps in numerous metabolic functions such as synthesis of nucleotides, DNA replication and repair (LeBlanc et al., 2013). The gene involved in bacterial metabolism and biosynthesis of folic acid was identified as folate gene cluster in *Lactococcus lactis* MG1363 (Stanton, Ross, Fitzgerald, & Van Sinderen, 2005). There are no detailed pathways for the direct synthesis of these metabolites. However, this leads to the release of different metabolites such as shikimate and chorismate which act as precursor for the synthesis of folate. Bacterial fermentation is the sole source of Cobalamine (vitamin B12) which is essentially required for the proper functioning of nervous system and formation of blood in body. Probiotic strains such as *L. reuteri* (Mohammed, Lee, Kang, & Du, 2014) and *Propionibacterium shermani* (Piwowarek, Lipińska, Hać-Szymańczuk, Kieliszek, & Ścibisz, 2018) are able to produce the cobalamin. Even *Clostridium* species along with *Lactobacillus reuteri* ATCC55730 are involved in the release of cobalamin during the production of sourdough (LeBlanc et al., 2013). Thiamin was acquired as one of the metabolites when the cereals were fermented by *Lactobacillus sanfranciscensis*. Some bacterial species such as *Escherichia coli, Salmonella typhimurium, Bacillus subtilis, L. plantarum,* and *L. reuteri* have played significant role in the synthesis of thiamine (LeBlanc et al., 2017). Vitamin K, known for its blood clotting power can be obtained in the form of menaquinone (K2) from gut microbiota (Patel et al., 2013). Through technological interventions such as genetic engineering, genes responsible for β-carotene production are incorporated into *E. coli* strain Nissle 1917 (EcN-BETA). Malnourished children are provided with these probiotics that will cause the increase in vitamin A content in the intestine.

7.3.8 Immunomodulatory compounds

The immune system of human beings is boost up by the functionality of the probiotic bacteria in the manner that these strains are involved in the production of immunomodulatory compounds like antibodies, cytokines, interleukins and lymphocytes (Nagpal et al., 2012). As the pathogens invade the host cells, the probiotic microorganisms interact with the epithelial cells of the intestine and trigger the controlled production of chemokines and cytokines in the gut (Hardy, Harris, Lyon, Beal, & Foey, 2013). Kawashima et al. (2018) reported the production of IgA antibody in mucosal lining of the host cell as a defence operation of host against the pathogens. Furthermore, Ozdemir (2010) studied the depreciation in the levels of IgE antibodies in allergic diseases, thereby suggesting that probiotics are of paramount importance in modulating the immune system and associated diseases. It has been concluded that the oral uptake of probiotics is associated with restoring normal inflammatory response (O'Mahony et al., 2005), alleviate antioxidant activity (Kullisaar et al., 2003), controlling brain sensitivity in terms of sensation and emotion (Tillisch et al., 2013) and reduce the levels of anxiety (A. V. Rao et al., 2009). These effects are the result of communication between probiotic bacteria residing in the human gut and the central nervous system (Bercik, Collins, & Verdu, 2012; Gill et al., 2006) which influences the release of pro- and anti-inflammatory cytokines (Cryan & Dinan, 2012). Additionally, *Lactobacillus* and *Bifidobacterium* strains possess the potential to synthesize neurotransmitters (gamma-aminobutyric acid (GABA) and acetylcholine) that attacks the central nervous system (Barrett, Ross, O'Toole, Fitzgerald, & Stanton, 2012). In this regard, probiotics are considered as potential novel therapeutic agents

for managing various neuro-developmental disorders (Gilbert, Krajmalnik-Brown, Porazinska, Weiss, & Knight, 2013).

7.4 Food supplementation with probiotic microflora

With advent in technology and understanding of the inter-relationship between the gut microbiota and food beyond nutrition, probiotic foods are gaining wide acceptance among the masses. Probiotic foods are the rapidly flourishing area of novel functional foods development. Varied food matrices of animal and plant origin are being utilized as potential delivery vehicle for probiotics. A brief classification of probiotic foods is depicted in Fig. 7.4. Dairy products have been long thought to be the most effective carrier of probiotics. However, in recent years, a shift in paradigm of consumers toward plant and meat based probiotic foods has been observed. This shift of interests has led to the development of a number of other foods in addition to dairy products.

Types of foods employed for supplementation with probiotic microflora

7.4.1 Animal origin foods

Foods of animal origin encompass foods derived from animal source such as meat, egg, milk, yogurt etc.

7.4.1.1 Meat-based products

Probiotic meat products are a relatively recent and underappreciated segment of the meat industry. No doubt, meat matrices in particular fermented products like sausages, salami are quite suitable for the growth as well as viability of microflora as they are very rarely heated. Technical

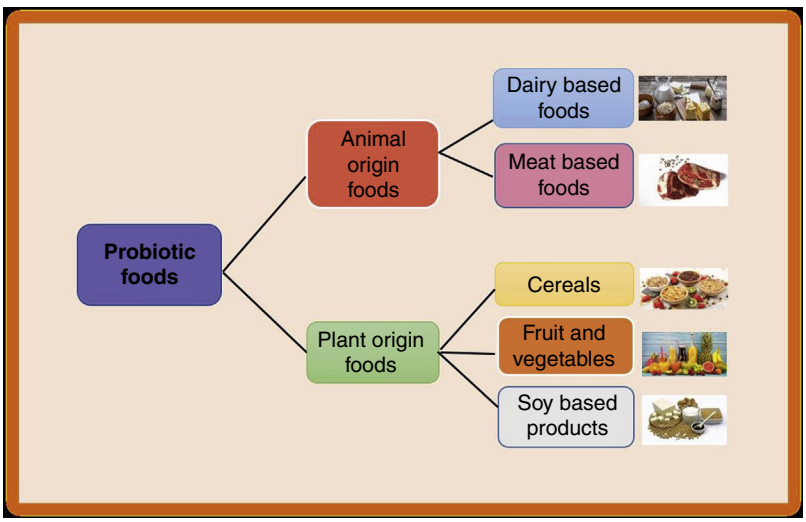


FIG. 7.4 **Classification of probiotic foods.**

TABLE 7.2 Application of probiotic microflora in meat-based products.

Product(s)	Probiotic microflora	References
Dry, fermented pork loins and sausages	*Lactic casei bacillus rhamnosus* LOCK900	(Neffe-Skocińska et al., 2020)
Dry-fermented sausages	*Lactobacillus casei Lactobacillus casei* ATCC 393 (free or immobilized)	(Sidira et al., 2014; Sidira et al., 2019)
Beef sausages	*Lactobacillus plantarum* TN8 & *Pediococcus acidilactici*	(Slima et al., 2018)
Fermented sausages	*Lactobacillus paracasei* & *Lactobacillus rhmanosus*	(Bis-Souza et al., 2019)
Italian salami sausage	*Lactobacillus acidophilus* & *Bifidobacterium lactis*	(Ruiz et al., 2014)
Bovine salami	*Lactobacillus plantarum* 299v	(Blaiotta et al., 2018)

challenge with these products are that the microflora may get deactivated or killed due to higher concentration of curing salts, spices, other microbiota, acidic environment and decreased water activity. Furthermore, the micro-organisms which are added should be resistant to the process of fermentation and should be able to survive the acidic ambiance in stomach. Fermented sausages are contemplated as favorable transporter of probiotics because of the protective action rendered to these by the fat molecules against low pH and bile juices. A number of studies indicating the inculcation of probiotics in meat products are summarized in Table 7.2.

7.4.1.2 Dairy-based products

Probiotic yogurt, ice cream, cheese, milk are some of the terms often used to describe the functionality of dairy based probiotic foods worldwide. Besides providing basic nutrition, dairy based probiotic foods also exhibit several physiological benefits. Milk possesses a unique position in functional foods as it is loaded with phytosterols, conjugated linoleic acid (CLA), vitamins and minerals. Also, the presence of high lactose content helps in the growth of probiotic microbiota. Inherent properties of milk and being stored at low temperature make milk and milk products outstanding carrier of probiotic microorganisms (Sperry et al., 2018) (See Table 7.3).

TABLE 7.3 Examples of dairy-based probiotic foods.

Product(s)	Probiotic microflora	References
Yogurt	*Lactobacillus casei* and prebiotics	(Marinaki, Kandylis, Dimitrellou, Zakynthinos, & Varzakas, 2016)
Probiotic yoghurt flavored with organic beet, carrot, cassava, sweet potato or corn juice.	*L. casei* and starter cultures *Streptococcus thermophilus* and *Lactobacillus delbrueckii ssp. bulgaricus*	(Gilmara Barboza Januário et al., 2017)
Rice incorporated synbiotic yoghurt	*Bifidobacterium animalis ssp. lactis BB-12*	(Kumari, Ranadheera, Prasanna, Senevirathne, & Vidanarachchi, 2015)
Goat milk-based fermented beverage	*Lactobacillus acidophilus, Bifidobacterium animalis ssp. lactis*	(Ranadheera, Evans, Adams, & Baines, 2016)

Product(s)	Probiotic microflora	References
Fermented skimmed milk	*Streptococcus thermophilus, Bifidobacterium animalis ssp. Lactis & Lactobacillus acidophilus- DVS ABT-4 Lactobacillus rhamnosus GG Lactobacillus johnsonii* La1	(Maganha et al., 2014)
Fermented milk	*S. thermophilus, L. delbrueckii ssp. bulgaricus, L. acidophilus and B. animalis ssp. lactis*	(Sgouras et al., 2005)
Probiotic fermented milk beverages with fruits		(Barat & Ozcan, 2018)
Beverage	*Lactobacillus paracasei* LC-37 (LC-37)	(Sun et al., 2021)
Probiotic ice-cream	*Bifidobacteriumlactis* (Bb-12) and prebiotics (inulin)	(Villalva et al., 2017)
	Lactobacillus rhamnosus B 442, Lactobacillus rhamnosus 1937, Lactococcus lactis JBB 500	(Góral, Kozłowicz, Pankiewicz, & Góral, 2018)
	Lactobacillus acidophilus	(Ferraz et al., 2012)
Minas frescal cheese	*L. casei* 01	(Sperry et al., 2018)
Goat coalho cheese	*L. rhamnosus* EM1107	(Araújo, 2018)
synbiotic soy cheese	*L. acidophilus* KLDS 1.0738 (LA-1.0738) with different prebiotics	(Zhang et al., 2020)
γ-aminobutyric acid enriched cheese	*Streptococcus thermophilus 84C* & *Lactobacillus brevis* DSM 32386	(Carafa et al., 2019)

7.4.2 Plant origin foods

7.4.2.1 Soy-based foods

Soybean is a type of legume which is native to eastern Asia. It is considered as the main component of our diet and has been consumed from thousands of years. Soybean products have gained enormous popularity among the consumers as a judicious alternative to milk-based products. For instance, soymilk is favored by consumers for its excellent protein quality, absence of cholesterol and lactose, and functional properties. Very recently (Cui, Chang, & Nannapaneni, 2021) exhibited that soymilk is a better substitute to cow milk for the proliferation of probiotic bacteria. On the other hand, the presence of low molecular weight polysaccharides (i.e., non-digestible oligosaccharides) in soybean may cause distended stomach, loosening of bowels and abdominal discomfort thereby limiting their consumption in moderation (Battistini et al., 2018). Recent research studies have highlighted that probiotic microflora like *Bifidobacterium* and *Lactobacillus acidophilus* are potent to digest non-digestible oligosaccharides (Donkor, Henriksson, Vasiljevic, & Shah, 2007; Hou, Yu, & Chou, 2000). A number of studies have been carried out in past years to exhibit that soybean-based products can be used as a carrier of probiotic microflora (Table 7.4).

TABLE 7.4 List of soy-based probiotic foods.

Product(s)	Probiotic microflora	References
Probiotic soy-based product similar to petit suisse cheese	*Lactobacillus acidophilus La-5, Bifidobacterium animalis Bb12, Streptococcus thermophilus,*	(Matias, Bedani, Castro, & Saad, 2014)
	Lactococcus lactis spp. *lactis Lactococcus lactis* spp. *Lactis diacetylactis, Lactobacillus brevis, Leuconostoc* spp. *& Saccharomyces cerevisiae*	(Baú, Garcia, & Ida, 2014)
Functional soy product with soy fiber and fermented with probiotic kefir culture	*Lactobacillus rhamnosus K4E (KX950834) & Lactobacillus helveticus K14 (KU644578)*	(Mishra, Hati, Das, & Prajapati, 2019)
Soy yoghurt	*Streptococcus thermophilus & Lactobacillus delbrueckii* ssp. *bulgaricus Lactobacillus delbrueckii* ssp. *bulgaricus (LB), Streptococcus thermophilus, Bifidobacterium animalis* ssp. *lactis BB-12 (BB), L. acidophilus La-5 (LA) & L. rhamnosus (LGG)*	(Jayarathna et al., 2020)

7.4.2.2 *Cereal-based foods*

Recent research has implied that use of cereal products as probiotic carrier is accruing impressive results (Budhwar, Sethi, & Chakraborty, 2020). Cereals are the main source of human nutrition all over the world. Cereals allow the inclusion of dietary fiber and non-digestible oligosaccharides in addition to probiotic thereby helping in the enumeration of lactic acid bacteria already present in colon (Lamsal & Faubion, 2009). *Lactobacillus* strains have been identified as a class of probiotics that need minerals, vitamin B complex, nucleic acids, amino acids and carbohydrates to multiply, and thus cereal fermentation may be considered as a low cost alternative to act as a substrate for the development of probiotics (Enujiugha & Badejo, 2017). Moreover, cereal-based products belong to a highly nutritive culinary recipe category. Various cereal based probiotic products developed in recent times are portrayed in Table 7.5. Since most of the probiotic strains are heat labile, these are assimilated into the

TABLE 7.5 Cereal-based probiotic foods.

Product(s)	Probiotic microflora	References
Cereal-based functional food using cereal-mix made up of sorghum, pearl millet, and wheat	*Pichia kudriavzevii OG32*	(Ogunremi, Agrawal, & Sanni, 2015)
Probiotic cereal beverage from barley and malt	*L. acidophilus*, and *L. plantarum*	(Rathore, Salmerón, & Pandiella, 2012)
Cereal food Bsissa	*Lactobacillus rhamnosus GG (LGG)*	(Yahyaoui, Bouzaiene, Aouidi, Aydi, & Hamdi, 2017)
Oat milk drink	Microencapsulated *Lactobacillus plantarum*	(Ravindran & RadhaiSri, 2020)
Soymilk in combination with apple juice	*Lactobacillus acidophilus*	(İçier, Gündüz, Yılmaz, & Memeli, 2015)

baked foods only after baking is done. However, probiotic bacteria like *Bacillus subtilis* produce imperishable spores that survive baking process and temperature and they again becomes viable in the gut. An outstanding strain of this bacteria *Bacillus subtilis* R0179 is commonly used during baking of bread and cookies for rendering the baked products a healthy attribute (Côté, Dion, Burguière, Casavant, & Eijk, 2013) (See Table 7.5).

7.4.2.3 *Fruit- and vegetables-based probiotic foods*

Foods obtained from fruits and vegetables are the novel sources of a number of beneficial nutrients such as minerals, phytochemicals, vitamins, antioxidants, and dietary fiber. These have higher concentration of sugar as well as nutrients, which is vital for development of probiotics. In addition, they also help probiotics to move through the jarring acidic environment quickly, thereby, serving as an ideal food matrix for carrying probiotics. Fruits and vegetable products in comparison to milk-based products are deprived of lactose, certain allergens as well as lack cholesterol which has an adverse effect on particular groups of masses. Fruit and vegetable juices, fermented vegetables, dried fruits and a variety of desserts are all examples of fruit and vegetable based probiotic foods that have been researched and developed. A few representations of fruit and vegetable based probiotic foods are compiled in Table 7.6.

7.4.2.4 *Chocolate*

Chocolate is a high-end food relished by people of all ages. Its heavenly aroma, good digestibility, and instant source of energy make it highly preferable among the consumers. Recent trend in the chocolate industry is solicitation of functional chocolate. Chocolate is prepared from cocoa, which is rich in flavanols, antioxidants, magnesium etc, thereby possessing nutritional and therapeutic properties (Scapagnini et al., 2014). A number of studies have

TABLE 7.6 Representations of fruit- and vegetable-based probiotic foods.

Product(s)	Probiotic microflora	References
Apple juice	*Lactobacillus plantarum* ATCC14917	(Li et al., 2018)
Cashew apple fermented juice	*Lactobacillus* spp., *Leuconostoc mesenteroides* & *Bifidobacterium longum*	(Kaprasob, Kerdchoechuen, Laohakunjit, & Somboonpanyakul, 2018)
Shalgam, a Turkish fermented beverage	*Lactobacillus plantarum*	(Tangüler & Erten, 2012)
Vegetable fruit fermented beverage	*Lactobacillus plantarum*	(X. Yang et al., 2018)
Cornelian cherry juice	*Lactobacillus plantarum* ATCC	(Mantzourani et al., 2018)
Fermented pineapple juice	*Bifidobacterium lactis* Bb12, *Lactobacillus plantarum* 299V, and *Lactobacillus acidophilus* La5	(Nguyen et al., 2019)
Fermented apricot juice	*Bifidobacterium lactis* Bb-12, *Bifidobacterium longum* Bb-46, *Lactobacillus casei* 01 & *Lactobacillus acidophilus* La-5	(Bujna, Farkas, Tran, Dam, & Nguyen, 2018)

indicated that chocolate can act as a potent base for probiotics. In a study by Ramakrishna (2013), it was deduced that milk chocolate can be prepared by substituting skimmed milk powder with yoghurt powder to impart it a probiotic character. Another interesting study by Succi et al. (2017) concluded that viable count of probiotics can be maintained in dark chocolates by encapsulation.

7.5 Challenges in the industrial production of probiotics products

In the recent years publicity and perception toward probiotics related experiments has become more rational and gain wider acceptance in the market. As the insight into human microbiome is getting deeper with the modern scientific tools novel probiotic taxa got identified, that is, next generation probiotics leads to examine range of human microbiome and characterize their potential health benefits. From identification of novel microbiome to convert them into a commercially and economically viable probiotic is a challenging task, which needs thorough study the requirement of their growth media and environmental conditions, standardize fermentation and manufacturing process (O'Toole, Marchesi, & Hill, 2017) Firstly, while developing novel probiotic product, proper care should be taken related to safety of new species and which required their complete characterization (Rouanet et al., 2020). With the technological advancement less bias and more reproducible protocols should be developed and good statistical design & analysis is key for any microbiome study (Casals-Pascual et al., 2020). In the recent time "omics" technologies becomes meta technologies and revolutionized complete chain from identification to industrial production of probiotics (Lloyd-Price, Arze, & Huttenhower, 2019). As mentioned, industrial production of probiotics is challenging task, starts from selection of appropriate strain, optimization of pH, entrapment and microencapsulation of culture for better survival, assessment of effect of probiotic cells on the sensory properties of food each and every step needs standardization which varies from strain to strain and their commercial needs (Champagne, Gardner, & Roy, 2005). Strain selection is an important criterion, so these should be examined for their in-vitro biological functions, that is, sensitivity, stability, resistance to the enzymes of digestive system (Godward et al., 2000). Selection of food matrix and effect on ingredients or additives also have significant influence on the growth of probiotics. Heating before packaging above 45°C will destroy part of probiotic population, which can be overcome by addition of the cultures after the pasteurization leads to aseptic packaging. In the recent time, fruit juices reported as potential medium for probiotics but maintaining the viability and activity of probiotics at low pH is major drawback due to the presence of organic acids (Perricone, Bevilacqua, Altieri, Sinigaglia, & Corbo, 2015). However, Ranadheera, Prasanna, and Vidanarachchi (2014) suggested that addition of lactic acid bacteria can solve this up to certain extent. Storage temperature negatively affects viability of probiotics microbes which can be overcome by microencapsulation method (Sohail, Turner, Prabawati, Coombes, & Bhandari, 2012). Ding and Shah (2008) suggest that microencapsulation can save the probiotic bacteria from the fluctuation in the external environment by creating favorable anaerobic conditions. Level of oxygen in the product should always be minimum since, it can damage product by producing reactive oxygen species & ultimately leads to toxicity and death of probiotic culture (Corbo, Bevilacqua, Petruzzi, Casanova, & Sinigaglia, 2014), this can be overcome either by increasing CO_2 concentration in head space

(Tripathi & Giri, 2014) or by addition of antioxidant compounds (Nag & Das, 2013). An important consideration should be taken care of while adding probiotics to the food or fruit juices is sensory quality, since sometimes it leads to change the taste, odor, etc., which can be overcome by adding pleasant aroma and volatile compounds. Media required to grow probiotics needs to be supplemented with amino acids and vitamins, which increases the production cost enormously (Figueroa-González, Quijano, Ramírez, & Cruz-Guerrero, 2011). In order to reduce it at make it feasible to produce at commercial scale, agro-industrial effluents, which has high amount of proteins, lipids, carbohydrates & vitamins can be used (Gutiérrez-Uribe, Rojas-García, & García-Lara SandSerna-Saldivar, 2010). It is often difficult to in-vitro culture the obligate anaerobic isolates present in human intestine, cultivate and subsequently store them (Ross, Desmond, Fitzgerald, & Stanton, 2005). Therefore, novel approaches for safer delivery of probiotics strains is key to success for production of probiotic products.

7.6 Conclusions

The gut microbiota delivers gigantic health benefits by enhancing physiological function of the gut through metabolites' production, such as bacteriocins, metabolic enzymes, amino acids and peptides, short chain fatty acids, vitamins, antioxidants, anti-inflammatory and immune-modulating agents, as well as exopolysaccharides. The interventions between bioactive metabolites produced by gut microbiome, their mechanism of action and its significance in relation to host health, are the imperative chunk to be studied further. The substantial exploration regarding formulation/composition of already existing and newly identified variants of probiotic bacteria for modulating the host immune system to treat and protect various conditions is the need of the time. The clinical studies are essential to ascertain the clear-cut connections between probiotic strain, dosage and type of illness, including their safety and limitation as well. Adoption of probiotics in bio-therapies can be seen as an influential tool for treating different infections. Eventually, with the growing consumer awareness and rising popularity of probiotic food products, the probiotic food segment is turning out as one of the largest functional food markets. This growing food industry is also fulfilling the consumer demands by incorporating new processed products with improved sensory appeal, but the challenge remains in food supplementation with probiotics to maintain the viability of the microbial cells while processing, packaging, and storage.

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CHAPTER

8

Probiotics and human gut microbiota modulation

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8.1 Intestinal microbiota and its functions

Since in the year 2010, De Filippo et al. (2010) published an article reporting the differences in the microbiota composition between Italian children and children living in Burkina Faso, establishing a link between these changes and the diet, the interest on the role of the intestinal microbiota in the human health has notably increased (more than a 1000%) to date, not only in the number of published articles but also in the number of citations. The autochthonous microbiota refers to the set of microbial communities that colonize the epidermal surface and the ducts and body cavities that communicate with the outside, whereas the terms microbiome refers to the whole habit, including the microorganism, their genes, and the external environmental conditions (Alvarez-Calatayud & Suarez, 2014). Bacteria, also called by some authors collectively the bacteriome, is the part of the microbiome best characterized (Vemuri, Shankar, Chieppa, Eri, & Kavanagh, 2020). However, the other communities referred to as "dark matter" of microbiomes such as viruses (virome), fungi (mycobiome), archaea (archaeome), and helminths have not been completely elucidated (Vemuri et al., 2020). However, these microorganisms also play an important role in human health, for example, viruses possess a high capacity to modify the intestinal composition as well as the bacterial activity. Intestinal bacteriophages have been discovered as the main component of the gut virome, accounting for about 90% of its composition (Reyes, Semenkovich, Whiteson, Rohwer, & Gordon, 2012).

Regarding the composition along the human gastrointestinal tract (GIT), the microbiota differs in number and concentration through the whole GIT (Fig. 8.1). Thus, whereas in the stomach there is a small number of microorganisms due to the acid pH, the colon is the region with the highest density of microorganisms since the conditions are more favorable to their

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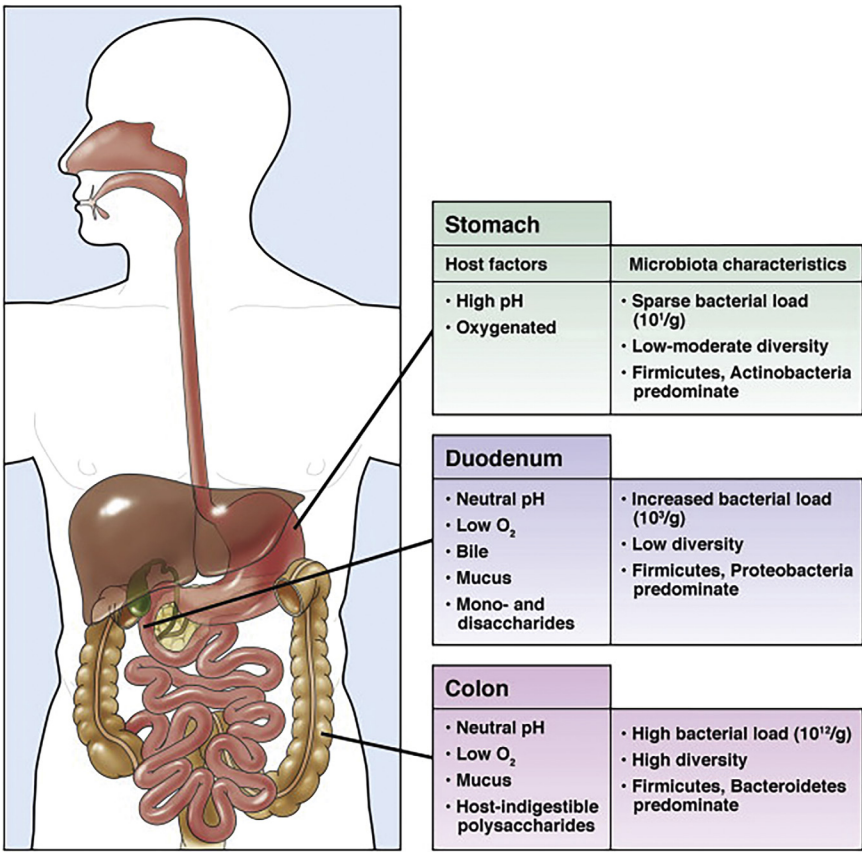


FIG. 8.1 Regional differences in the gastrointestinal tract affect microbial niches. Reproduced from Ohland and Jobin (2015).

survival, and where the changes in its compositions possess a higher implication on the human health.

Regarding its composition, the human gut microbiota is mostly composed of these two bacterial phyla, representing more than 90% of the total community (Magne et al., 2020).

The beneficial functions of the human gut microbiota on the host health include participation in nutrient metabolism as well as in xenobiotic and drug metabolism, maintenance of structural integrity of the gut mucosal barrier, immunomodulation, and protection against pathogens (Jandhyala et al., 2015). It is essential to know all these functions for understanding the role of functional ingredients such as probiotics.

Metabolic function. The metabolic activity of the gut microbiota complements that of the host. Thus, it plays an important role in the modulation of glucose and lipid homeostasis and energy production, besides being able to produce vitamins (ex. vitamin k) and using non-digestible carbohydrates as substrates through the action of enzymes not encoded by the human genome (Rowland et al., 2018).

The impact of the intestinal microbiota on host health has been shown in in vitro studies using human fecal fermentations or more complex continuous culture gut models, as well as in comparative studies with germ-free and colonized animals and clinical studies (Marchesi, Adams, Fava., Hermes, & Hirschfield., 2016; Rowland et al., 2018). The survival of bacteria inhabiting the colon, mainly depends on the presence of non-digested dietary substrates in the upper digestive tract. Saccharolytic bacterial fermentation produces generally beneficial metabolites such as short-chain fatty acids (SCFAs), whereas if non-digestible carbohydrates are restricted in the diet, increasing for example protein or lipids, other metabolites more detrimental to human health can be produced (Boyd, Liu, Wang, Martin, & Dunn-Walters, 2013).

Bacterial cross-feeding. The enormous variety in food preferences by bacteria provides complex interactions among them, especially in ecosystems as complex as the large intestine. The study of these interactions allows a better understanding of the evolution of microbial populations, their resilience to changes to this environment, changes in nutrient supply, and how new interactions might emerge.

There are many forms of nutrient exchange among bacteria and the language used to classify these interactions is varied, but "metabolic cross-feeding" can be defined as an interaction between bacterial strains in which molecules resulting from the metabolism of one strain are further metabolized by another strain (Nick, Shorten, Altermann, Roy, & McNabb, 2019). In this sense, certain metabolites produced by some species of bacteria are used by others, being detected in small quantities in healthy humans (Rowland et al., 2018). This is the case of succinate produced by species as *Bacteroides vulgatus*, *Fecalibacterium prautsnitzii* or *Akkermansia municiphila* and consumed by other species as *Bacteroides tetaiothaomicron* or *Veillonella parvula* (Fernandez-Veledo & Vendrell, 2019).

Short-chain fatty acids. The major short-chain fatty acids (SCFAs) produced by the intestinal microbiota from the fermentation of complex carbohydrates are acetate, propionate, and butyrate (Pascale et al., 2018). SCFAs have distinctive physiological roles such as shaping the gut environment, influencing the physiology of the colon, and being an energy source for host colonocytes (Ríos-Covián et al., 2016).

Acetate is the most abundant organic acid since it is produced by many bacteria, but propionate and butyrate tend to be produced by specific bacteria (Louis, Young, Holtrop, & Flint, 2010; Reichardt et al., 2014), which in many cases needs acetate to survive. This is the case of some butyrate producers, as *Faecalibacterium prausnitzii*, which is not able to grow in pure culture in the absence of acetate (Duncan et al., 2004). Acetate contributes in a significant way to butyrate producers.

The main butyrate-producing bacteria in the human gut microbiota belong to the phylum Firmicutes, such as *Faecalibacterium prausnitzii, Clostridium leptum*, and *Roseburia intestinalis* (Louis & Flint, 2009). Propionate is produced by some *Clostridium* species, as well as by Bacteroides such as *Bacteroides eggerthii* and *Bacteroides fragilis* (Shimizu et al., 2018).

The intestinal microbiota and the immune system maintain a symbiotic relationship where the first is fundamental for the correct development of the second and this when operating correctly exert protective functions against pathogens and foreign substances, but maintains tolerance to innocuous antigens as those related to the (Belkaid & Hand, 2014). Along with the intestinal epithelium, the microbiota forms a protective barrier against infectious threats and plays an active role in promoting and maintaining immune homeostasis during intestinal

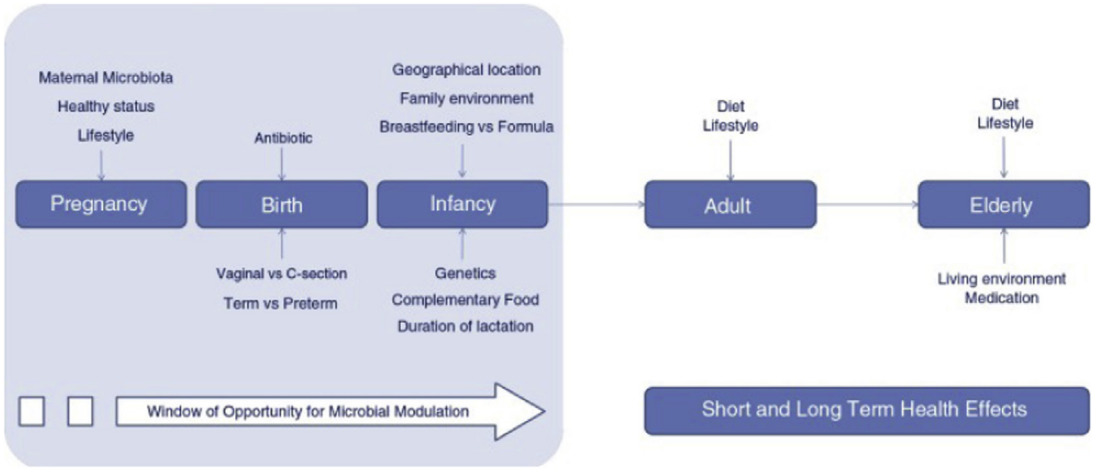


FIG. 8.2 Factors influencing the gut microbiota development during life. Reproduced from Rodríguez et al. (2015).

infections. This function of defense and protection against pathogens can be accomplished by different mechanisms, such as avoiding the pathogen adherence to the intestinal epithelium and use of specific nutrients favoring their development through the production of antimicrobial substances such as bacteriocins.

The process of formation of the colonic microbiota is continuous throughout human life (Fig. 8.2) and many factors influence it. Perinatal factors such as the mode of delivery, diet, genetics, or even the composition and structure of the mucin are factors that influence bacterial colonization. Once bacterial colonization is established it can be altered by bacterial infections, antibiotic treatment, style of life, surgeries, and changes in diet. Drifts in this complex ecosystem have been shown to increase the risk of disease.

An adequate establishment of the microbiota, as well as its maintenance throughout life, has been shown to reduce the risk of disease at early and late ages. This could include diseases directly related to the GIT such as inflammatory bowel diseases, but also others related to the immune system such as allergies and asthma.

Among the various factors that can alter the microbial composition of the GIT, diet plays a crucial role due to its direct impact on microorganisms. Numerous researchers have tried to understand how diet can modulate the intestinal microbiota, altering the abundance and metabolism of microorganisms inhabiting there and ultimately the health status of an individual.

There are numerous studies published about the effects of different diets (vegan, Mediterranean, gluten-free, ketogenic, etc.) on the intestinal microbiota and their consequence on the health of the individuals. However, all these studies have limitations since the microbiome response is very dependent on the individual with numerous influential factors (Rinniella et al., 2019). Therefore, what we eat will have direct effects on the composition of the resulting microbiota and its activity, with special reference to the ability to extract energy from specific constituents of the diet.

Due to its implication and influence on human health through different activities and functions, the manipulation of the intestinal microbiota arises as an emerging strategy to

intervene and modify human health. Diets targeting the gut microbiome to improve a function and or eliminate another thought the introduction of specific bacterial strains or ingredients could become a powerful tool in precision medicine. In addition, the gut microbiome itself can help to predict individual responses to foods (Brand-Miller & Buyken, 2020; Zeevi et al., 2015).

Thus, studies carrying out dietary interventions that specifically alter dietary fiber have been shown to exert a positive influence on the intestinal microbiota composition, but also on the improvement of different health markers of the participants (Duncan et al., 2007; Martínez et al., 2013; Walker et al., 2011). These findings together with the shortfall between fiber consumption in the average American diet (and also in all the countries following an occidental diet) under the recommended intakes suggest that the increase in fiber intake could be one of the best options to modulate the human immune system via the microbiota (Deehan & Walter, 2016) whenever possible and tolerated by individuals. Nowadays problems of intolerance to carbohydrates related to malabsorption are increased and different alternatives should be searched to modulate the microbiota.

8.2 Fermented foods and the beginning of probiotics

Fermented foods such as yogurt, kefir, tempeh, natto, kombucha, and kimchi have gained popularity in the last years due to the different findings related to their potential health benefits in animal models and humans (Dimidi, Cox, Rossi, & Whelan, 2019; Villarreal-Soto et al., 2020). Different clinical studies have linked the consumption of fermented foods to the prevention of different diseases such as obesity, diabetes, or cancer (Díaz-López, Bulló, Martínez-González, Corella, & Estruch, 2016; Gille, Schmid, Walther, & Vergères, 2018; Mozaffarian, Hao, Rimm, Willett, & Hu, 2011; Taylor et al., 2020). Fermented foods have an extended tradition in the diets of many cultures around the world, but their consumption was relegated in the substitution of processed foods typically consumed in occidental diets. The reintroduction of fermented foods into diets may provide higher benefits to human health through the microorganisms contained therein, compensating that lack of exposure due to the overconsumption of industrialized food. The results obtained in recent work with individuals following a diet rich in fermented foods have shown that these foods may be powerful modulators of the human microbiome, causing improvements in the immune system and be used for the prevention of non-communicable chronic diseases (NCCD) (Wastyk et al., 2021).

Ingredients such as non-digestible carbohydrates and probiotics (present in fermented food) are effective ingredients to modulate the microbiota. These fermented foods were the beginning of probiotics such as they are known today and whose definition has been included at the beginning of this book “Live microorganisms which when administered in adequate amounts confer a health benefit on the host” (Hill et al., 2014).

Thus, probiotics, precisely in the context of fermented foods have been always present in the human diet and their beneficial effects have been already known. In 1663, Robert Boyle said: “One who perfectly understands the nature of ferments and fermentation will be probably more capable than one who ignores to realize exact different phenomena of various diseases that may not be will never fully understand without entering the fermentation theory.” This scientist suggested that the origin of the fermentative processed and the origin

of diseases could be the same, suspecting that the agent causing both processes could be the same.

In 1853, Joseph Leidy published the book "A Flora and Fauna within Living Animals," a manuscript which was considered by several researchers as the origin of microbiota research. Then, the work of other important personalities such as Pasteur, Metchnikoff, Koch, Escherich, Kendall, among others, laid to establish the basis of e host–microorganism interactions. Pasteur developed the theory that germs could be the cause of disease, but also reinforced the idea that those non-pathogenic might have an important role in human physiology and health; Metchnikoff, in the same line of thinking, believed that composition and interactions of the human intestinal microbiota were essential for health; and Escherich deeply believed that understanding the functioning of the intestinal microbiota was essential for understanding the physiology of digestion as well as the pathology and, consequently, the therapy of intestinal disease (Savage, 2001).

The 19h century was a golden age for probiotics, not only because of the discovery that microorganisms can cause diseases but also because without them the human being would not exist. From this moment, the first probiotic microorganisms were isolated. In 1917, during the First World War, the bacteria *Shigella* caused a great number of cases of dysentery among the soldiers of bod sides causing a lot of deaths. Prof. Alfred Nissle isolated a strain of *Escherichia coli* (*Escherichia coli* strain Nissle 1917) from the feces of a German soldier who did not develop infectious diarrhea or any other intestinal disease (Nissle, 1918; Sonnenborn, 2016). This strain was shown to possess in vitro and in vivo antagonistic activities against different pathogenic enterobacteria (Nissle, 1925; Sonnenborn & Schulze, 2009). Since then, the *E. coli* strain Nissle, 1925 has been successfully used in the treatment of different gastrointestinal diseases (Montrose & Floch, 2005; Schultz, 2008) and continues, nowadays, to be commercialized.

In 1923, Henri Boulard, a French pharmacist and scientist, isolated the yeast *Sacharomyces boulardii* from lychee and mangosteen fruit after he observed that natives of Southeast Asia chewed on the skins of these fruits to lessen the symptoms of cholera (Na & Kelly, 2011). *S. boulardii* is recognized nowadays as a probiotic and its use is recommended in different clinical guides to prevent antibiotic-associate diarrhea (WGO, 2017).

With the discovering of the antibiotics, at the beginning of the century XX, the probiotics were relegated to a lower priority and although from a point of view existing some advances, these microorganisms did not attract attention.

At the beginning of century XXI when the changes of the lifestyle, the population aging, the increase of the non-non-transmissible diseases, and the growing interest by the population in their health and the diet together with the problem associated with the bacterial resistance to antibiotics when the interest is resurgent by the probiotics and another bioactive ingredient such as prebiotics and antioxidants. The OMS and the FAO establish then, their definition and the criteria of selection and used to provide the scientific support and veracity (see Fig. 8.3).

In addition, scientific societies on probiotics arise and the number of researchers interested in this investigation field started to increase. Research in the database Web of Science using the word "probiotic" in the search allow to observe asus it went from a publication in the year 1985 to 25 in 1991, passing by 223 in 2001 up to 3612 in 2021. In 1990 different publications reported about "probiotics, fact or fiction". In 2000 different publications reported on their effects on gastrointestinal diseases, and, currently, it is possible to find multiple publications of probiotics in different pathologies.

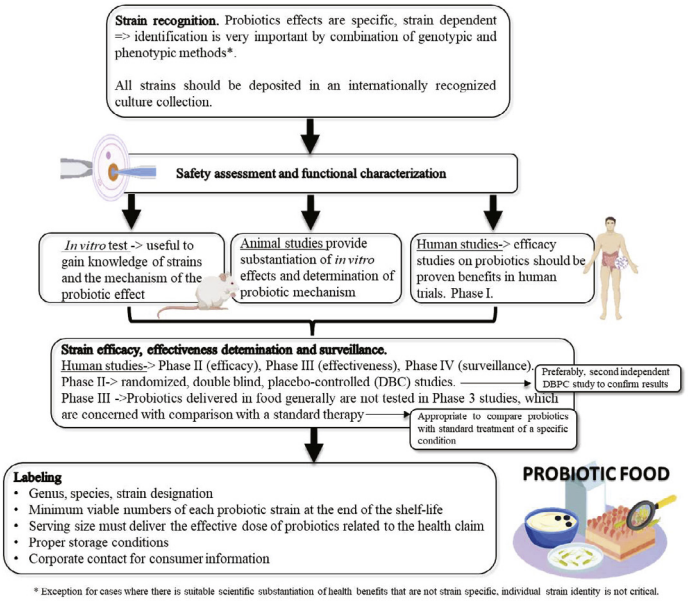


FIG. 8.3 Guidelines for the evaluation of probiotics for use, taken from FAO/WHO (2002).

Evidence-based scientific studies have shown the clinical potential of probiotics against many diseases (Liu, Tran, & Rhoads, 2018). Probiotics have been reported to suppress diarrhea (Kambale et al., 2021), alleviate lactose intolerance (Ibrahim et al., 2021) and postoperative complications (Stavrou et al., 2017), exert antimicrobial (Silva et al., 2020) and anti-colon cancer activities (Torres-Maravilla et al., 2021), reduce irritable bowel symptoms (Didari, Mozaffari, Nikfar, & Abdollahi, 2015), and prevent inflammatory bowel disease (Derwa, Gracie, Hamlin, & Ford, 2017). However, the health benefits associated with probiotics should not be generalized because the effects are strain-specific. This means that a specific effect attributed to one strain is not necessarily applicable to another strain even within one species (McFarland, Evans, & Goldstein, 2018).

Studies have shown that not all probiotics are equally effective. This efficacy depends, among other factors, on the manufacturing processes, the quality control of the product, and the rest of the ingredients within the product. In this sense, international associations recommend the strain designations when reporting outcomes in clinical trials so that strain-specific efficacy can be determined, but this recommendation has not been uniformly followed and there are a lot of published works where the strain have not been reported. The efficacy of a strain, when used in a commercial product with more substances, should be reported for the whole product and not only for the strain. Currently, different clinical guides exist on probiotic use, including applications, dosage forms, and clinical evidence, but there is still a long way to go (Álvarez-Calatayud & Mateos-Lardiés, 2018; Fernández, Cardelle-Cobas, Regal, Cepeda, & Fente, 2017; Skokovic-Sunjic, 2021a; 2021b).

An aspect that be elucidated is how the probiotics work. Since there are a lot of probiotic formulations, alone or combined with other functional ingredients (ex. prebiotics, vitamins,

and antioxidants), and it is known that the effects are strain-dependent, is important to establish why and how each strain exerts its effect. For example, *Lactobacillus reuteri* SD2112 (ATCC 55730) and *L. reuteri* RC-14 are genetically and functionally different (Cadieux et al., 2008), since the former produces reuterin, a potent antimicrobial agent capable of inhibiting the growth of pathogen microorganisms (Langa et al., 2014), whereas the latter produces biosurfactants capable of inhibiting the attachment of uropathogens (Velraeds, van der Belt, van der Mei, Reid, & Busscher, 1998).

Although there are general benefits associated with probiotics independently to the mechanism such as creating a more favorable gut environment, supporting a healthy digestive tract, and a healthy immune system, it is important to try not to describe the mechanisms in a general way for all the probiotics but to try doing it for each strain.

8.3 Probiotic mechanisms of action

In this section, the focus will be on the different mechanism through which probiotics can exert their activity within the intestine since each probiotic bacteria have multiple and diverse influences on the host through different mechanisms of action. The main mechanisms are indicated in Fig. 8.4.

8.3.1 Antimicrobial activity

Probiotic bacteria produce antimicrobial substances that can exert direct antimicrobial action toward competing enteropathogens that may lead to the pathogenic colonization of the gut.

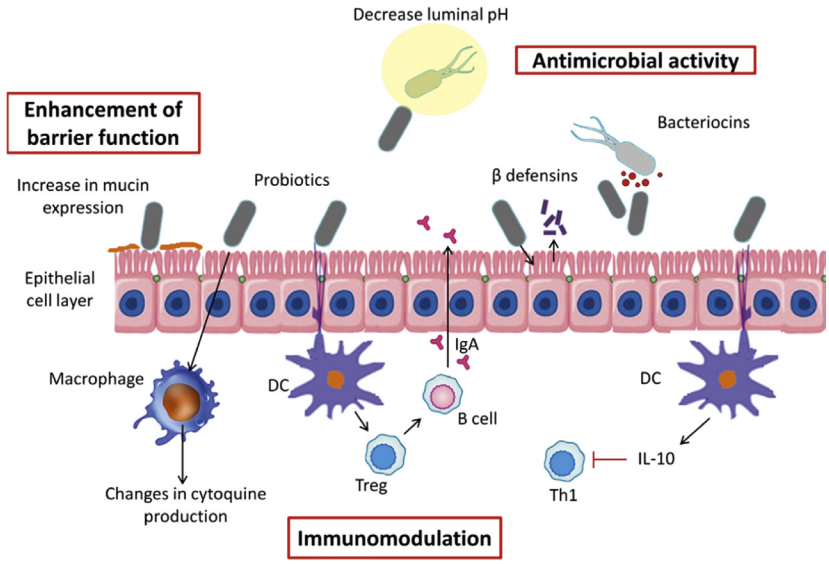


FIG. 8.4 A schematic diagram about probiotic mechanisms within the intestine. Reproduced from Cerdó, García-Santos, Bermúdez, and Campoy (2019).

Probiotics can exert their action through the production of different antimicrobial compounds: i) bacteriocins, ii) bacteriocin-like inhibitory substances, iii) lactic and other volatile acids, iv) other primary metabolites such as hydrogen peroxide, carbon dioxide, and diacetyl, v) siderophores, vi) biosurfactants, and vii) compounds inhibiting pathogen adhesion to intestinal cells.

Bacteriocins. Bacteriocins are a diverse group of antimicrobial cationic and hydrophobic peptides, composed of 20 to 60 amino acids. They are ribosomally synthesized by some bacteria that are active against other bacteria and to which the producer is immune. Activity of bacteriocins is such diverse as diverse are its structure and mechanisms

Both Gram-positive and Gram-negative bacteria and archaea release antimicrobial peptides extracellularly in the late-exponential to the early-stationary growth phases (Torres et al., 2013). These antimicrobial peptides possess activity against different bacteria, fungi, parasites, viruses, and even against natural resistant structures, such as bacterial biofilms, which reinforce the emergence of peptides as potential antimicrobial ingredients to be used in different food systems to prevent microbial growth and contaminations in the food industry (Hernández-González, Martínez-Tapia, Lazcano-Hernández, García-Pérez, & Castrejón-Jiménez, 2021).

Among the different bacteriocins, those produced by gram-positive bacteria are more abundant and more diverse than those produced by gram-negative bacteria. These abundant gram-positive bacteriocins are similar in structure to many peptides produced by eukaryotes; they are generally cationic, amphiphilic, membrane-permeabilizing peptides, and range in size from 2 to 6 kDa (Heng, Wescombe, Burton, Jack, & Tagg, 2007). Bacteriocins usually inhibit specific species that are related to the producer (Lajis, 2020; van der Donk & Nair, 2014), although there are some bacteriocins that possess a broader spectrum of action. This is the case of nisin and plantaricin, for example, produced for determining strains of *Lactococcus lactis* and *Lactobacillus plantarum*, respectively. But bacteriocins, as indicated above, can also exert their activity against fungi, parasites, viruses, and even against natural resistant structures, such as bacterial biofilms (Eyang, Elin, Sung, & Efang, 2014; Graham, Cruz, Garsin, & Lorenz, 2017; Martín-Escolano et al., 2019; Zheng, Gänzle, Lin, Ruan, & Sun, 2014).

The production of bacteriocins is a complex process since it is influenced by different factors as the pH, temperature, and growth medium. Thus, not all the bacteriocin-producing bacteria showing in vitro activities have shown in vivo activities. It is important to understand the complex environmental in the gut and for that all these strains should be tested in in vivo assays with the objective host (Fernandez, Le Lay, Jean, & Fliss, 2013; Turgis, Vu, Millette, Dupont, & Lacroix, 2016).

Traditionally, bacteriocin production has been an important criterion in the selection of a probiotic strain despite the fact that little is known about the impact of bacteriocin production on the ability of a strain to compete within the GIT and/or positively influence the health of the host (Corr et al., 2007). Different bacteriocin classification can be found in the literature. One of the possible classifications of these antimicrobial compounds is shown in Table 8.1.

Through the three following mechanisms probiotic-bacteriocins contribute to the functionality of the producer strain:

a. As colonizing peptides: bacteriocins aid the survival of the producing strain in the gut environment (Egan, Ross, & Hill, 2017);

TABLE 8.1 Classification of bacteriocins.

Class	Subclass	Features	Example	Mechanism of action	Receptors	Producing strain	Bacterial spectrum	References
I (modified, heat-stable), <15 kDa)	Ia (linear)	Lantibiotics (<5 kDa) lanthionine and β-methyl lanthionine	Nisin, lacticin 481	Membrane permeabilization by pore formation	Lipid II	*Lactococcus lactis* (strain NCDO 497)	Gram-positive	Dufour, Hindré, Haras, and Le Pennec (2007); Shin et al. (2016)
	Ib (globular)	Carbacyclic lantibiotics labyrinthin and labionin	Labyrinthopeptin A1	Perforation of lipid membranes by interacting with phosphatidylethanolamine (PE)	viral membrane lipid phosphatidylethanolamine (PE)	*Actinomadura namibiensis* (type *strain* HAG 010767T/ DSM 44197T)	HIV, HSV	Prochnow et al. (2020)
	Ic (multicomponent)	Sactibiotics (sulfur to alpha carbon-containing antibiotics)	Thuricin CD	Membrane permeabilization by pore formation	Not known	*Bacillus thuringiensis* DPC 6431	Gram-positive	Mathur et al. (2017); Reaa et al. (2010)
II (unmodified, heat-stable, <15 kDa))	IIa	Listeria-active peptides with YGNGV-C in the N-terminal (pediocin-like)	Pediocin PA-1, sakacins A and P, leucocin A.	Membrane permeabilization by pore formation	Mannose permease	*Pediococcus pentosaceus* (strains IE-3, FBB63…); *P. acidilactici* PAC-1.0, *Lactobacillus sakei* (strains 1151, 5…)	Gram-positive and Gram-negative	Rodríguez, Martínez, and Kok (2002); Chen et al. (2012) ; Trinetta et al. (2012) ; Balay, Dangeti, Kaur, and McMullen (2017)
	IIb	Two-peptide bacteriocins	Lactococcins G, plantaricin EF and plantaricin JK	Membrane permeabilization by pore formation	UppP (undecaprenyl pyrophosphate phosphatise)	*L. lactis* (LMGT-2081), *Lb. plantarum* (8PA3, NCFB 965)	Gram-positive	Oppegård, Rogne, Kristiansen, and Nissen-Meyer (2010);Nissen-Meyer, Oppegård, Rogne, Haugen, and Kristiansen (2011) ; Moll et al. (2020)

Class	Subclass	Features	Example	Mechanism of action	Receptors	Producing strain	Bacterial spectrum	References
	IIc	Circular bacteriocinsThiol activated peptides	Gassericin A, enterocin AS-48, garvicin ML	Membrane permeabilization by pore formation	ABC transporter	*Lactobacillus gasseri* (LA39) *Enterococcus faecalis* (S48. 7C5..), *L. garvieae* (DCC43)	Gram-positive	Borrero et al. (2011); Pandey, Malik, Kaushik, and Singroha (2013); Burgos et al. (2014)
	IId	linear, non-pediocin-like bacteriocins	Bactofencin A, LsbB	Membrane permeabilization by pore formation	Metallopeptidase	*L. salivarius* (DPC6502, PS7), *L. lactis* subsp. *Lactis* (BGMN1-5)	Gram-positive	O'Shea et al. (2013); Uzelac et al. (2013)
	IIe	microcin E492-like bacteriocins	Microcin E492	Depolarized and permeabilize the cytoplasmic membrane	Catecholate iron/siderophore receptors	*Klebsiella pneumoniae* (RYC492)	Gram-negative	Thomas et al. (2004)
III (Large, heat unstable, > 30 kDa)	IIIa	Bacteriolysins	Lysostaphin	Cell wall degradation, cell lysis	Peptidoglican	*Staphylococci S. simulans biovar staphylolyticus* (Lysostaphin)	Gram-positive (Staphylococci)	Bastos, Coutinho, and Coelho (2010)
	IIIb	Two-peptide bacteriocins (non-lytic)	Helveticin M, helveticin J and enterolysin A, caseicin 80	membrane-leakage and cell death	mannose permease	*Lb. crispatus* (K313), *L. helveticus* (481), *E. faecalis* (LMG 2333), *Lactobacillus casei* (B80).	Gram-positive and Gram-negative	Joerger and Klaenhammer (1986); Nilsen, Nes, and Holo (2003); Sun et al. (2018)
IV (circular peptides, heat stable, tail-head-peptide bonds)	-	Cell wall degradation cytosolic targets	Sublancin, Glycocin F (GccF)	Disruption of sugar-processing and regulatory activities	Binding to the transmembrane domain	*B. subtilis* (A52), *Lactobacillus plantarum* (KW30)	Gram-positive	Oman, Boettcher, Wang, Okalibe, and van der Donk (2011); Stepper et al. (2011)

Adapted from Kumariya et al. (2019).

b. By direct inhibition of the growth of pathogens;
c. As quorum-sensing molecules in the intestinal environment.

Bacteriocins may prevent the multiplication of pathogens (bacteriostatic effect) or may directly destroy the pathogens and providing to the probiotic producer the advantage to compete with the pathogen in gut colonization.

The way in which bacteriocins are able to exert this action is through their ability to adhere microbial cells and penetrate phospholipid membranes due to their small size and variations in hydrophobic and hydrophilic properties (Izadpanah & Gallo, 2005). Then, bacteriocins can induct the permeabilization of the cytoplasmic membrane of sensitive bacteria leading cell leakages, inhibition of DNA and RNA synthesis and/or cell wall protein synthesis and cause the death of pathogens (Cotter, Ross, & Hill, 2013; Islam et al., 2012; Nishie, Nagao, & Sonomoto, 2012).

Bacteriocin-like inhibitory substances. Bacteriocin-like inhibitory substances (BLIS) are substances with molecular weights and broad activity spectrum uncharacteristic of bacteriocins. All substances similar to bacteriocins but that do not match with bacteriocin definition and classification are named BLIS. For example, Corsetti, Settani, and Van Sinderen (2004) carried out the characterization of 5 compounds from five strains (two *L. plantarum*, two *L. pentosus*, and one unclassified *Lactobacillus* spp.) among 437 lactic acid bacteria from 70 sourdoughs. These isolates were inactivated by proteolytic enzymes, indicating them to be bacteriocins, but were classified as BLIS because they have not yet been characterized for amino acid and encoding nucleotide sequences. The BLIS 57 was previously isolated by the same research group from LAB isolates from sourdoughs (Corsetti & Gobbetti, 2002). *Lactobacillus paracesei* LA07, isolated from a fermented fish product known as "budu" typically consumed in Kelantan (Malaysia), as well as in southern Thailand was also studied for BLIS production (Abbasiliasi et al., 2011; Liasi et al., 2009).

Lactic and other volatile acids. Resulting from the metabolism of non-digestible carbohydrates by the commensal intestinal microbiota, different organic acids, most known as SCFAs, such as acetic, propionic, and butyric are produced. These organic acids exert and participate in different processes in the host, from the colon energy supply and trophic factors (Pascale et al., 2018), the regulation of T regulatory (Treg) cell colonies (Arpaia et al., 2013; Smith et al., 2013), and even their effect on several extern organs including the brain. SCFAs also inhibit the growth of pathogenic bacteria and may kill them by lowering the intracellular pH level (Russell & Diez-Gonzalez, 1998).

Examples of probiotic fatty acid-producing strains are *Lactobacillus rhamnosus* GG and *Lactobacillus gasseri* PA 16/8 (LeBlanc et al., 2017), which are propionic and lactic producers. *Bifidobacterium longum* SP 07/3 producing acetate and propionate (LeBlanc et al., 2017), and *L. acidophilus* CRL 1014 reported to produce acetate, butyrate, and propionate (Sivieri et al., 2013). Among the commensal bacteria, different species have been reported as SCFAs producers. This is the case of *Akkermansia municiphila* (Derrien, Vaughan, Plugge, & de Vos, 2004; Louis & Flint, 2017) that produces acetate and propionate, and *Faecalibacterium prautsnitzii*, or *Roseburia intestinalis* that produce butyrate, or some species of *Bifidobacterium* that produces acetate (Louis & Flint, 2009).

Other primary metabolites such as hydrogen peroxide, carbon dioxyl and diacetyl. Some commensal or probiotic lactic acid bacterial produce hydrogen peroxide. Hydrogen peroxide may cause a reduction in pathogen virulence and the invasion of epithelial cells, or may cause even the death of intestinal pathogens after epithelial intracellular diffusion, which alters gene transcription and signal transduction (Botteaux, Hoste, Dumont, Van Sande,

& Allaoui, 2009; Knaus, Hertzberger, Pircalabioru, Yousefi, & Branco Dos Santos, 2017). Strains such as *L. jonsonii* NCC533, *L. crispatus* F117, or *Lactobacillus paracasei* F2 and F28 have shown the capacity of inhibiting pathogenic growth in vitro and in vivo (van Zyl, Deane, & Dicks, 2020).

The ability to metabolize citrate to carbon dioxide (CO_2), acetate, diacetyl, acetoin, and eventually 2,3-butandiol is a common trait of many lactic acid bacteria, especially Lactococcus and Leuconostoc spp. (Hugenholtz, 1993). Diacetyl is responsible for the characteristic "buttery" aroma in milk products (Oberman & Libudzisz, 1998), and among their activities, it is the interference on arginine utilization (Jay, 1986).

Lactic acid bacteria also produce CO_2 during their fermentation process Thus, for example, *Leuconostoc* sp. is a microorganism with great importance in dairy fermentation due to its ability to produce CO_2 from lactose and citrate. During the fermentation of milk, the amount of CO_2 produced is critical as it affects not only the flavor and aroma of the fermented product but also the texture of blue cheese (Kihal, Prevost, Henni, Benmerchenene, & Diviès, 2007). But not only influences the organoleptic aspects of the fermented products, CO_2 also is able to produce an anaerobic environment and/or inhibition of enzyme decarboxylation and/or disruption of the cell membrane (Eklund, 1984). Thus CO_2 produced by heterofermentative lactobacilli also has a preservative effect in foods and at the intestinal level can be a metabolite with antimicrobial properties against pathogens.

Siderophores. Siderophores are low molecular weight, organic, and high-affinity iron-chelating compounds produced by bacteria and other microorganisms as fungi (Ahmed & Holmstrom, 2014). These substances are produced by these microorganisms because they need iron to survive and iron is not present in nature under the adequate form to be used by these microorganisms needing the siderophores to chelate it and be able to acquire it (Saha, Saha, Donofrio, & Bestervelt, 2013). Based on this, siderophore-producing bacteria sequester free iron available in their environment that is essential to other microorganisms vivo (van Zyl et al., 2020). For example, the growth of *Lactoccocus lactis*, *Clostridium difficile* and *Clostridium perfringens* was inhibited in the GIT by iron-binding *Bifidobacterium* strains that produce siderophores (O'Sullivan, 2014).

Biosurfactants. Some probiotics produce another kind of substances called biosurfactants that can avoid pathogenic growth in the GIT. Biosurfactants are a group of compounds that possess surface and emulsifying activities, and they are used in many different biomedical, environmental, food, and cosmetic applications (Rodrigues, Banat, Teixeira & Oliveira, 2016a; Nitschke & Costa, 2007; Sharma, Saharan, & Kapil, 2016). They are a group of molecules of microbial origin that are characterized by being amphipathic, that is, they have two different parts, one hydrophobic and the other hydrophilic. These biosurfactants alter the physical cell membrane structure and cause permeabilization through the disruption or lysis of the membranes (Heerklotz & Seeling, 2006). Biosurfactants from lactic acid bacteria have been shown to reduce adhesion of bacterial pathogen in glass, silicone rubber, surgical implants, and voice prostheses (Rodrigues, Moldes, Teixeira, & Oliveira, 2006b; Sharma et al., 2016).

Although there are many probiotic bacteria just a few species have been reported as siderophore producers. These include *Lactobacillus fermentum, Lactobacillus paracasei*, *Lactobacillus plantarum, Lactococcus lactis, Streptococcus thermophilus*, and *Propionibacterium freudenreichii* (Hajfarajollah, Eslami, Mokhtarani, & Akbari Naghabi, 2018). Specifically, the strains *Lactobacillus casei* CECT-5275, *Lactobacillus rhamnosus* CECT-288, *Lactobacillus pentosus*

CECT-4023and *Lactobacillus acidophilus* RC 14 have been reported to produce surfactin (Rodrigues et al., 2006a; Velraeds, Van Der Mei, Reid, & Busscher, 1997), a powerful surfactant commonly used as antibiotic. The strain *Lactococcus lactis* 53 produces a biosurfactant with a structure type protein (Rodrigues, Teixeira, van der Mei, & Oliveira, 2006c) and *Lactobacillus helveticus* MRTL91 produce a biosurfactant with a type glycolipid structure (Sharma, Saharan, Chauhan, Bansal, & Procha, 2014), among others.

Antiadhesive compounds and co-aggregation. The different metabolites with antimicrobial activity produced by certain bacterial strains have already been discussed, however, there are bacterial strains that non have a direct bactericidal effect, but possess capacities for inhibiting the pathogen adhesion through other mechanisms such as this own attachment to the intestinal mucosa. This property confers it the ability to compete with the pathogens for the host cell-binding site and although the adhesion ability of probiotic bacteria is not a synonym of health, it is true that offers the probiotic bacteria an opportunity to interact more with the host. Thus, for example, SCFAs produced by the probiotic bacteria can exert a local action and bacterial surface-located molecules can modulate the immune system through the induction of determined signaling pathways (Monteagudo-Mera, Rastall, Gibson, Charalampopoulos, & Chatzifragkou, 2019).

On the other hand, prebiotic oligosaccharides also may enhance the adhesion ability of probiotic strains, and their use together with the probiotics in a symbiotic product could be a potential tool to increase the time of residence of probiotic bacteria in the gut (Celebioglu et al., 2017). Fig. 8.5 shows the schematic representation of the antiadhesive properties of probiotics and prebiotics.

Autoaggregation has been correlated with adhesion since it is known to be a prerequisite for colonization and infection of the GIT by many pathogens. The coaggregation properties of probiotic strains with pathogens as well as their ability to displace pathogens are of importance for therapeutic manipulation and prevention of pathogens into the GIT.

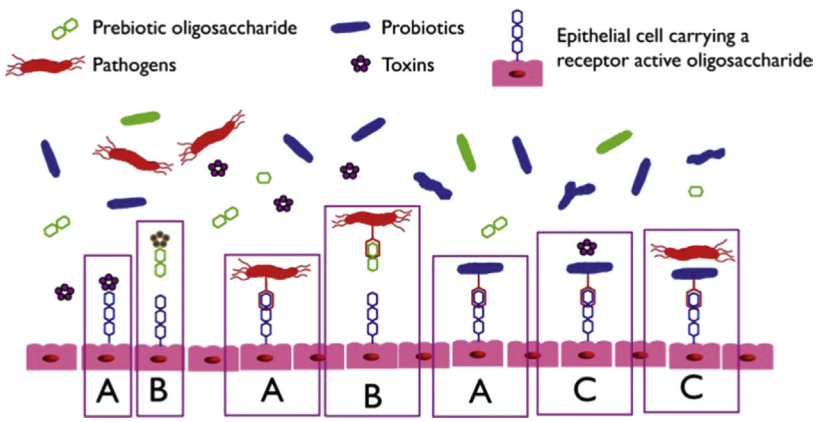


FIG. 8.5 Schematic representation of antiadhesive properties of probiotics and prebiotics. (A) Pathogens, probiotics, and bacterial toxins adhere to the cell surface through the oligosaccharides carried on glycolipids and glycoproteins; (B) prebiotic oligosaccharides with structural similarity to the cell surface glycoproteins inhibit adhesion of toxins and pathogens to cells; (C) The adhesion of probiotic bacteria to cell surface receptors is postulated to inhibit adhesion of toxins and pathogens to those receptors. Reproduced from Monteagudo-Mera et al. (2019).

The strain *L. reuteri* K7, isolated from cow colostrum, showed a high auto-aggregation (65.4%), hydrophobicity (30.6%), and good adhesion capacity to a mixed cell culture in a study carried out by Krausova, Hyrslova, and Hynstova (2019) indicating its great potential as probiotic based on these properties. In the same study, the strain *B. pseudolongum* S3Aisolated from feces from piglets showed a good adhesion capacity (29.5%), auto-aggregation (69.7%), and hydrophobicity (63.4%).

In another study, the ability of *L. fermentum* strain 4-17 to resist the GIT hostile environment was proved as well as its anti-bacterial effect, adhesion, and anti-infection abilities against *Escherichia coli* (ATCC 25922) causing urinary tract infection in humans. Other common food pathogens as *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aeruginosa* (PTCC 1707) were also tested with positive results (Falah et al., 2019).

Collado, Meriluoto, and Salminen (2008) evaluated commercial probiotic strains for their autoaggregation and coaggregation abilities with potentially pathogenic bacteria as *Listeria monocytogenes* ATCC 15313, *C. histolyticum* (DSM 627), *S. aureus* (*DSM 20231* Newman ATCC 25904) and *E. sakazakii* (ATCC 29544). *L. rhamnosus* LC-705 and *L. fermentum* ME-3 were the strains with higher percentages of coaggregation and also, higher auto aggregation abilities. Also, *L. acidophilus* NCFM and *L. plantarum* Lp-115 showed high coaggregation with *C. histolyticu*m DSM 627 and *S. aureus* DSM 20231.

In the scientific literature a great number of studies can be found related to the study of the antiadhesion and coaggregation properties of probiotic since the ability to autoaggregate together with cell-surface hydrophobicity and coaggregation abilities with pathogen could be used for preliminary screening in order to identify potentially probiotic bacteria suitable for human or animal use (Collado et al., 2008).

8.3.2 Stabilization of intestinal epithelial barrier and enhancement of barrier function

8.3.2.1 The mucosal immune system

To understand how probiotics can exert their action on the immune system, it is important to remind that the immune system is a complex network of cells and proteins that defends the body against any foreign substance (antigen). The place where these cells and proteins are synthesized differs and the component that is put in place to protect depends on whether this antigen is extracellular or intracellular. The immune system is typically divided into two categories, innate and adaptive, each one possesses specific characteristics, but both work in coordination to protect the host from the foreign.

Innate immunity refers to nonspecific defense mechanisms that come into play immediately or within hours of an antigen's appearance in the body. Being part of thus innate immunity are barriers such as skin, chemicals in the blood, and immune system cells that attack foreign cells in the body. The innate immune response is activated by chemical properties of the antigen. It is essential for the acquired immune response to start, but it also can generate autoimmunity.

The innate immunity provides a primary host response to microbial invasion, there is a recognition of the pathogens or substances undesirables by pattern-recognition receptors (PRRs). The PRRs include Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) among many others (Fukata & Arditi, 2013). What

occurs is that once recognized there is an inflammatory response with the main objective of localizing the infection of the strange agent and avoiding its dissemination.

Adaptive immunity refers to antigen-specific immune responses. The adaptive immune response is more complex than the innate. The antigen first must be processed and recognized. Once an antigen has been recognized, the adaptive immune system creates an army of immune cells specifically designed to attack that antigen. Adaptive immunity also includes a "memory" that makes future responses against a specific antigen more efficient (Monteagudo-Mera et al., 2019).

The mucosal immune system is the largest component of the entire immune system, having evolved to provide protection at the main sites of infectious threat: the mucosae. This system is functionally and anatomically complex. A difference of the immune system is permanently in touch with foreign antigens (ex. intestinal bacteria and food components) and unlike the first not only it must recognize the stranger and respond to it but also recognize the own and respect it. Namely, the mucosal immune system has three main functions (Holmgrem & Czerkinsky, 2005):

To protect the mucous membranes against colonization and invasion by potentially dangerous microbes;
To prevent uptake of undegraded antigens from different origin (ex. ingested food and microorganism);
To prevent the development of potentially harmful immune responses to these antigens if they do reach the body interior.
The mucosal immune system must tolerate the presence of certain antigens such as those provided from foods or from the own commensal microbiota.

Different cells act depending on the antigens are intra- or extracellular. Examples of cells of innate immunity that act against extracellular antigens are macrophages, neutrophils, eosinophils, and against intracellular, natural-killer (NK). The adaptive immune system is made up of T lymphocytes in the tissue between the body's cells. B lymphocytes are also found in the tissue between the body's cells. Antibodies are found in the blood and other bodily fluids.

The immune system of the intestinal mucosa is special because it has continuous exposure to antigens and only a layer of intestinal cells separates these antigens from the immune cells. The lymphoid tissue of the mucosa is called MALT (mucose-associated lymphoid tissue) and depending on where is locate this name change. Thus, GALT is the name for the lymphoid tissue located in the intestine, the "gut-associated lymphoid tissue" is divided into two different ways: i) the organized gut lymphoid tissue (organized MALT) and ii) the diffuse gut lymphoid tissue (diffuse MALT).

In the organized-GALT is where the encounter with the antigen takes place, its main function is to induce the immune reaction. It is composed of Peyer's patches which are small masses (cells) of lymphatic tissue found throughout the ileum region of the small intestine and contain CD4, CD8, T cells and B cells. The M cells that cover the epithelium are the responsible to transport those antigens to the Peyer's patches which will initiate the adaptive immune response. Once initiated the response, the production of secretory IgA (sIgA) occurs and other immune cells enter the, systemically, through the Peyer's patches and into the mesenteric lymph system. The function of sIgA cells prevents pathogen adhesion to the intestinal surface, preventing the cell damage (Mantis, Rol, & Corthésy, 2011).

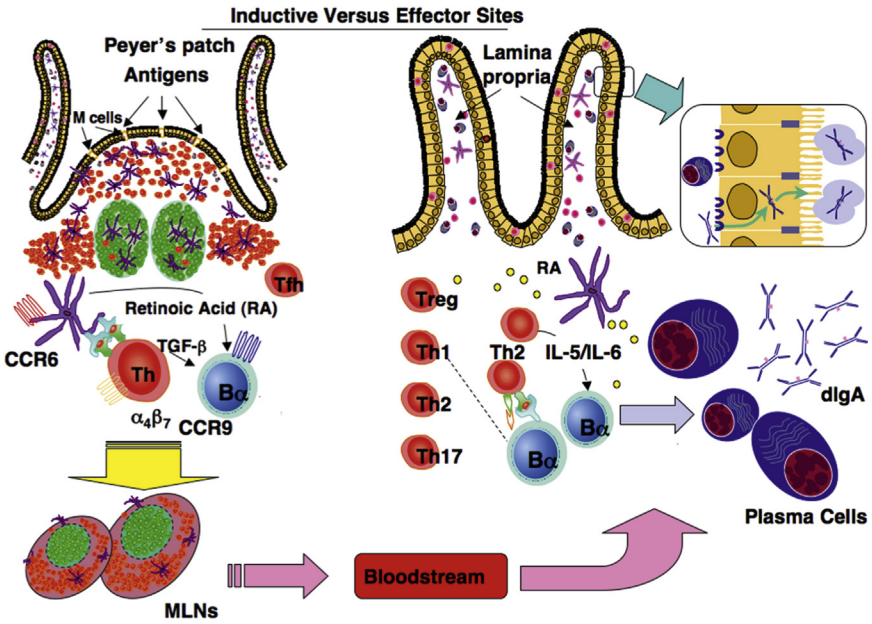


FIG. 8.6 The gut-associated lymphoid tissue, GALT. Reproduced from MacGee and Fujihashi (2012).

On the other hand, diffuse GALT is composed of scattered cells, made up of intraepithelial, sparse lymphocytes, lamina propria cells, and epithelial cells which acts as a physical barrier and are also important in the immune response. This part of GALT is made up from mature effector cells ready to perform their actions. Is the effector site.

In brief, the GALT can be divided into two sites, the inductive and the effector based upon their anatomical and functional properties. The migration of immune cells from the inductive to the effector sites is produced through the lymphatic system. The communication between these two sites is the basis of the immune response in the GI tract. Fig. 8.6 shows in a schematic way, this mechanism (MacGee & Fujihashi, 2012).

Intestinal epithelial cells (IECs) until a few years ago were not considered very relevant from the point of view of immunity since they were only understood as a physical barrier against antigens. Currently, it is known that they are relevant from an immunocompetent point of view and gained importance since they are capable of detecting Toll-like receptor antigens and producing cytokines (coordination immune response), besides having immunocompetent cells in their proximity (Fig. 8.7).

IECs are fundamental in the maintenance of intestinal mucosa integrity (Rooks & Garret, 2016). From a structural point of view, the monolayer of IECs separates the mucus and the commensal microbiota from the immune cells (Ohland & Jobin, 2014). The mucus produced by the goblet cells, and the IECs conform thus a barrier that is the main defense mechanism against infection and inflammation, and the disruption of its integrity is one of the primary causes, ahead of other factors such as genetics, of several intestinal disorders such as the inflammatory bowel diseases (Gareau, Sherman, & Walker, 2010; Sommer, Anderson, Bharti, Raes, & Rosenstiel, 2017).

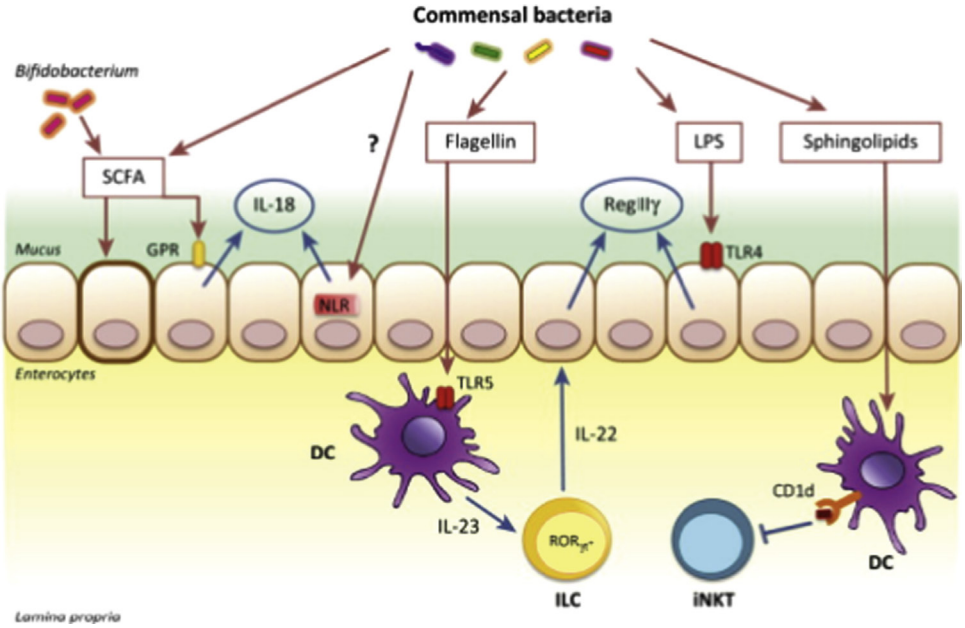


FIG. 8.7 Microbiota influence on the innate immune responses. *Reproduced from Kabat, Srinivasan, and Maloy (2014).*

8.3.2.2 Influence of the intestinal microbiota on the immune system

The intestinal microbiota takes part of the mucous ecosystem and the factors that influence their correct training and their functions have already been discussed before. However, the intestinal microbiota also influences the immune response and is a key piece in the maintaining of the intestinal homeostasis. Fig. 8.8 shows the schematic way of the effects of some metabolites produced by the commensal microbiota on the innate immune system. Intestinal microbiota can generate tolerogenic substances, as the T-regulatory cells (Tregs) (FOXP3+), which limit the immune response. They are permanently in the human immune system and their function is to tolerate the substances that produce the microbiota, suppress immune responses, and modulate, in consequence, chronic tissue inflammation and autoimmune diseases. Thus, for example, when SCFAs are produced by the gut bacteria the FOXP3+ accept and do not respond to them. Among the substances that these cells produce is interleukin-10.

The Tregs possesses certain plasticity but little is known about it, being of great interest for therapeutic purposes. What it is known is that dietary factors, via direct effects on immune cells or by acting indirectly through modulation of the gut microbiota, may regulate Tregs' plasticity and function and, therefore, may have the potential to control disease outcomes (Arroyo Hornero, Hamad, Côrte-Real, & Kleinewietfeld, 2020). Although more research aid is necessary, this opens new opportunities for the use of specific dietary nutrients as prebiotics and probiotics and assess their influence on Tregs, autoimmunity, and human health.

Other compounds produced by the microbiota, as the bacterial lipopolysaccharide (LPS), have an important influence on the immune system. The LPS is the major component of the outer surface membrane in Gram-negative bacteria and acts as extremely strong stimulator

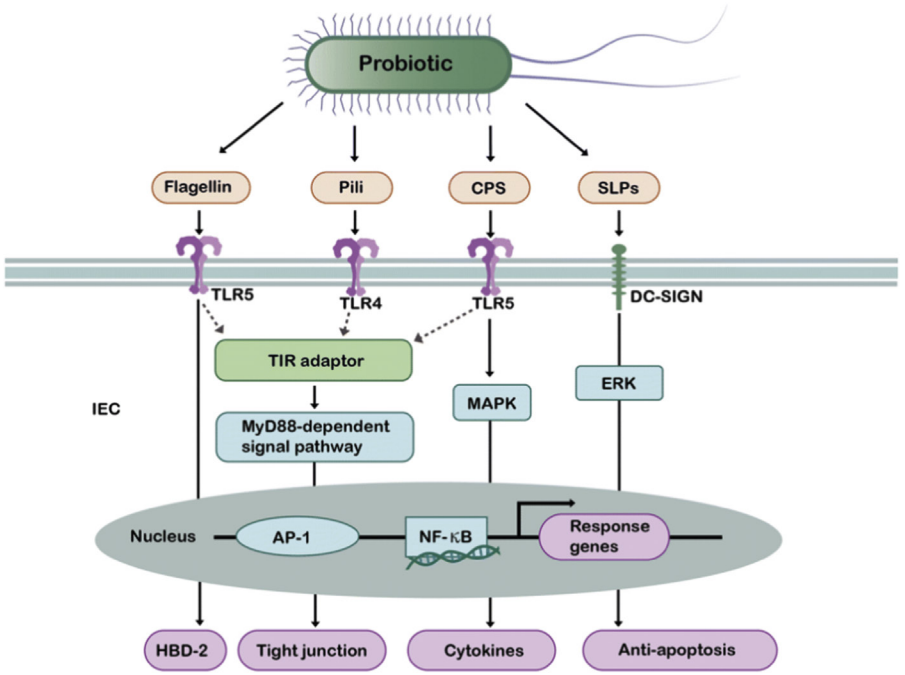


FIG. 8.8 Effects of surface molecular of probiotics on intestinal epithelial barrier. Reproduced from Liu et al. (2020).

of innate immunity. It is anchored in the outer bacterial membrane by a specific carbohydrate lipid moiety termed lipid A. The lipid A component is the primary immunostimulatory center of LPS. Agonists forms of LPS or lipid A trigger numerous physiological immunostimulatory effects in mammalian organisms, but in higher doses can also lead to pathological reactions such as the induction of septic shock (Alexander & Rietschel, 2001). An increased exposure to this bacterial component is associated with low-grade inflammation, insulin resistance, and enhanced cardiovascular risk (Cani et al., 2008; Creely et al., 2007).

Flagellin also produced by motile bacteria is another substance influencing immunity. Among the different factors associated with the inflammation due to a gut microbiota dysbiosis, is the levels of flagellin. Flagellin possess the ability to activate pro-inflammatory gene expression via TLR5 and the NLRC4 inflammasome. When the intestinal microbiota is enriched in motile bacteria of bacteria, the mucosa can be easily invaded and activate the expression of proinflammatory genes (Tran, Ley, Gewirtz, & Chassain, 2019). A study carried out in 2019 showed as administration of purified flagellin with the aim of generating mucosal antigens (flagellin-specific IGA) might help keep flagellated bacteria in check and, consequently protect against the development of chronic gut inflammation (Tran et al., 2019).

Sphingolipids are another set of molecules influencing notably the homeostasis intestinal. They are structural membrane components and important eukaryotic signaling molecules. They regulate inflammation and immunity and have been recently identified as the most differently abundant metabolite in stool from inflammatory bowel disease (IBD) patients. However, not all sphingolipids are detrimental for intestinal health. Recently, a study has

shown as Bacteroides-derived sphingolipids are crucial in the maintaining of homeostasis in the gut and that their deficiency causes inflammation (Brown et al., 2019).

One of the most known and studied metabolites produced by the intestinal microbiota are the SCFAs, and those that have already been discussed previously in the chapter. SCFAs, in addition to be the main energy source for colon and ileum cells, improving the intestinal epithelial barrier and in consequence, the defense functions by regulating the related gene expression, they also possess other functions. They regulate the function of innate immune cells such as macrophages, neutrophils, and dendritic cells. Third, SCFAs can also regulate the differentiation of T cells and B cells and the antigen-specific adaptive immunity mediated by them (Yao et al., 2022).

These examples show as commensal bacteria can influence the immune responses and as depending of their equilibrium can be the difference among health of disease. Without the presence of the microbiota, an adequate immune system does not develop, and alterations in the immune system lead to changes in the composition of the microbiota (dysbiosis), with the consequent development of pathological situations and effects in areas far away of the intestine such as the brain or the respiratory system, which are aspect discussed in other chapters of this book.

8.3.2.3 Probiotics as a strategy for the immune system modulation

In the same way that commensal microbiota can influence and modulate the mucosal immune system, probiotics also can. Thus, the surface components of probiotics, such as flagella, pili, surface layer proteins (SLPs), capsular polysaccharide (CPS), lipoteichoic acid, and LPS, which constitute the known as microbial-associated molecular patterns (MAMPs) can be specifically recognized by pattern recognition receptors (PRRs) and play a role in maintaining intestinal homeostasis and promoting gut health (Lebeer et al., 2018; Liu et al., 2020).

These MAMPs regulate a cellular protease-dependent signaling cascade to produce a variety of cytokines and chemokines that alleviate inflammation and enhance intestinal epithelial function (Artis, 2008; Sharma, Young, & Neu, 2010). In addition, some metabolites produced by probiotics, already mentioned, such as SCFAs and bacteriocins, protect the gut epithelial barrier by increasing mucus secretion, acting against pathogens, etc.

The action of probiotics may also possess effects far away of the intestine, directly through the transfer of the bacteria, as occurs in the case of the translocation of bacteria from the intestine to the mammary glands of lactating women, by the absorption of different molecules through the intestine or through the different direct or indirect mechanisms already mentioned in the previous sections (Arroyo et al., 2010). The ability of probiotic strains to reduce the severity and duration of respiratory tract infections illustrates a distant site effect likely mediated by enhancement in immune mediators (Hao, Dong, & Wu, 2015).

The interest about the possible antiviral activity of probiotics has increased in recent years (Chauhan & Singh, 2019), but the mechanism of action through which these probiotic bacteria produce their antiviral effects remains still unknown. Some in vitro studies seams indicate that one possibility is that this antiviral effect takes place through the action of extracellular enzymes secreted by the bacteria.

However, despite the fact that is clear the fact that probiotics and commensal microbiota can modulate the immune system through their surface components and metabolites, the control of this modulation is complex and very difficult to evaluate (Reid, 2015). The same

strain might be capable to exert different effects depending on the host, the composition and activity of the gut microbiota, the status of the intestinal epithelia, the number of cells, and perhaps the delivery method. A clear example is the probiotic bacteria *L. rhamnosus* GG which can interact with the host immune system through different mechanisms and likewise, there are many ways for the host to respond (Segers & Liber, 2014).

The Table 8.2 shows some of the clinical studies carried out to date with different probiotic strains administered as a supplement or as functional food showing the effects on different immunological parameters. Although it is necessary to develop more consistent studies, including a major number of volunteers and controlling parameters as the diet, the results obtained to date indicate a clear beneficial effect of probiotics in the immune system. Patients with immune-related diseases, resulting from either deficiency or oversensitivity of this system, may benefit from probiotics. It is noteworthy that this effect is largely dependent on the strain and dosage of probiotics, as well as the investigated conditions.

TABLE 8.2 Different probiotic strains evaluated in clinical studies for immunomodulation.

Bacterial strain	Study conditions	Main results	References
Yogurt containing *Lactobacillus gasseri* SBT2055 and *Bifidobacterium longum* SBT2928	Placebo-controlled, randomized, double-blind clinical trial224 healthy Japanese adult volunteers (males n = 69, females n = 155; with ages from 32 to 76 years); 109 placebo subjects and 115 test subjects 12 weeks, 100 g yogurt/day	NK cell activities of the test yogurt group placebo yogurt group adrenocorticotrophic hormone (ACTH) in test group placebo yogurt group	Nishihira et al. (2014)
Fermented milk containing *Lactobacillus casei* Shirota vs unfermented milk	17 healthy volunteers (7 males, 10 females; 20–40 years old) 4×10^{10} live cells/daily for 3 weeks	NK cells activity in the test group during the intake. NK cell activity remained elevated during 3 weeks after finishing the intake. Then returned to initial values. No changes were observed in the control group.	Nagao, Nakayama, Muto, and Okumura (2000)
Lactobacillus casei Shirota	20 healthy male volunteers (40–65 years old); 10 placebo, 10 control. Placebo-controlled, randomized, clinical trial 8 weeks. The first and two last weeks, the subjects received a strictly controlled diet without fermented products. The intermediate 4 weeks the test group received, three times daily, 100 ml of fermented milk containing 10^9 CFU *L. casei* Shirota/mL. The control group received the same amount of an unfermented milk	Test group: *Lactobacillus* count in feces being *L. casei* Shirota predominant (10^7 cfu/g wet feces), *Bifidobacterium* counts *Clostridium*. No effects were observed for any of the immune parameters measured (including natural killer (NK) cell activity, phagocytosis and cytokine production	Spanhaak, Havenaar, and Schaafsma (1998)
Fermented product with *L. gasseri* CECT 5714 and *L. coryniformis* CECT 5711 *vs* standard yogurt (control)	Healthy adult human volunteers (n = 30; 15 males and 15 females) 4 weeks, diet without fermented products	Both probiotic products induced immune effects, been more remarkable in the new fermented product. NK cells, IgA concentration and IL-10 IgE and Il-4 The best results were obtained after 2 weeks of treatment	Olivares et al. (2006)

(*Continued*)

TABLE 8.2 *(Cont'd)*

Bacterial strain	Study conditions	Main results	References
Lactobacillus salivarius CECT5713	40 healthy adult volunteers (20 females and 20 males) between 23- and 43-year-old; 20 control, 20 test 6 weeks. Control group: two capsules containing maltodextrins as placebo (Glucidex 39, La Roquette), daily, for 4 weeks. Test group: two capsules with 10^8 cfu/capsule of the strain L. salivarius CECT5713 (2×10^8 cfu/day) with maltodextrins	Test group: fecal lactobacilli counts. Improvement in the frequency of defecation ($p < 0.05$). % NK cells and monocytes as well as the plasmatic levels of immunoglobulins M, A, and G, and the regulatory cytokine IL-10 ($p < 0.05$).	Sierra et al. (2010)
Bifidobacterium animalis ssp. *lactis* (BB-12) *Lactobacillus paracasei* ssp. *paracasei* (L. casei 431)	Randomized, double-blind, placebo-controlled study 211 healthy volunteers (118 females and 93 males completed the study) 6 weeks. After 2 weeks, a seasonal influenza vaccination was given. Four groups of study =>1) a capsule containing *Bifidobacterium animalis* ssp. *lactis* (BB-12) 2) a placebo capsule3) an acidified dairy drink (110 mL) containing the probiotic strain *L. casei* 431 (ATCC55544)4) placebo acidified dairy drink (110 mL)	IgG, IgG1 and IgG3 in both probiotic groups v. the corresponding placebo group ($p < 0.05$) for vaccine-specific secretory IgA in saliva in both probiotic groups v. placebo ($p < 0.05$) No differences were found for plasma cytokines or innate immune parameters	Rizzardini et al. (2012)
Lactobacillus fermentum CECT5716	Randomized, double-blinded, placebo-controlled human clinical trial 50 volunteers (31 male and 19 female; 33.0 ± 7.7 y old) Control group: oral daily dose of methylcellulose (placebo); 25 subjects. Test group: probiotic bacteria (1×10^{10} ufc/day 2 weeks before vaccination and 2 weeks after vaccination).	2 wk after vaccination, in % NK cells in the probiotic group but not in the placebo groupanti-influenza specific-IgA ($p < 0.05$) total IgM and IgG in plasma in the test group No changes were observed in anti-influantigen-specific immunoglobulin M.	Olivares et al. (2007)
Yogurt containing *Lactobacillus paracasei*, *L. casei* 431 and *Lactobacillus fermentum* PCC	randomized, double-blind, placebo-controlled, prospective trial 134 healthy volunteers with at least 4-6 common cold in the past year 867 placebo group; 67 test group)12-week probiotics intervention. Placebo group traditional yogurt drink: The starter culture contained 1×10^5 CFU/mL *Lactobacillus bulgaricus* and 1 × 1010 CFU/mL *Streptococcus thermophilus*. Test group: The probiotic yogurt contained starter culture at 2×10^7 CFU/mL plus 1×10^8 CFU/mL, L. casei 431 at 1×10^8 CFU/mL and Lactobacillus fermentum PCC at 6×10^7 CFU/mL Probiotic drink (150 mL) or placebo drink (150 mL) was administered after lunch for a total of 12 weeks.	The consumption of probiotics significantly reduced the incidence of upper respiratory infection ($p < 0.023$) and flu-like symptoms with an oral temperature higher than 38 °C ($p < 0.034$) as compared to the placebo group. IFN-γ in the serum ($p < 0.001$) and sIgA in the gut ($p < 0.010$) in the test group *vs* the placebo group, and the baseline data. No significant differences in the serum IL-4, IL-10, IgA, IgG, or IgM between groups	Zhang et al. (2018)

8.4 Conclusions

Probiotics can modulate the composition of the gut microbiota through different mechanisms which are related to their metabolite production and membrane components. As the commensal microbiota, probiotics also influence the immune system response. This chapter includes a review of the different mechanisms through probiotics may exert this action, but due to the complexity of the GIT ecosystem, to establish how a probiotic strain can be acting, result in a very complex task. Different mechanisms can be taken place at the same place and factors as the composition of the intestinal microbiota, the status of the intestinal barrier are of great influence in the probiotic functionality.

Which is clear is that the beneficial effect associated to a specific strain and pathology cannot be applied to another strain, even within the same species and that the effect of that strain can be altered if that strain is given together with other substances (ex. prebiotics, vitamins, and antioxidants). Even the effect showed for a probiotic may vary among individuals. In this sense more investigations elucidating the mechanisms of probiotics are necessary to understand the full potential of these beneficial microorganisms in the therapeutic treatment of different diseases. In the near future, a specific intervention with a specific probiotic for a specific individual with a concrete pathology may be carried out. Clinical parameters, the pre-intervention status of the intestinal microbiota, the genetics and other may be evaluated by means machine learning to evaluate the best intervention option.

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CHAPTER

9

Interplay between probiotics and prebiotics for human nutrition and health

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9.1 Introduction

The increasing knowledge in the field of human nutrition and health has demonstrated the pivotal role of probiotics, prebiotics, and synbiotics in achieving health beneficial effects and improving people's welfare and quality of life. The word "probiotic," which comes from Greek and has the connotation "for life," is currently used as a broad concept to designate microorganisms with beneficial effects in humans and animals (Hill et al., 2014; Markowiak & Ślizewska, 2017). The perception of the benefits of lactic acid fermentation on human health goes back to ancient times, with reports of the consumption of different fermented milk types by ancient Roman, Greek, and Egyptian communities (Markowiak & Ślizewska, 2017). However, scientific knowledge regarding this matter was only obtained by the observations of the Russian Nobel Laureate Élie Metchnikoff, with his work at the Pasteur Institute, at the beginning of the 20th century. Metchnikoff suggested that the regular intake of fermented dairy products with lactic acid bacteria could be related to improved health and longevity in Bulgarian and Russian Steppes nomads. In his work, Metchnikoff proposed that it is possible to adopt measures to change the intestinal flora of the human body, substituting the harmful microorganisms with beneficial ones (Metchnikoff, 1907). Since then, the probiotic concept has been related to beneficial microorganisms for host health (Martín & Langella, 2019). Regarding prebiotics, they may be used as substitutes of probiotics or to provide them additional support in improving human health, due to their high potential to modulate the intestinal microbiota (Markowiak & Ślizewska, 2017). The combination of probiotics and prebiotics in a single product is commonly referred to as synbiotic (Martín & Langella, 2019).

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This chapter describes the current knowledge regarding probiotics and prebiotics, exploring their beneficial effects on improving human health, underlying mechanisms of action, the possible sources for their purification/production, and the criteria involved in their selection. Also, the interaction between probiotics and prebiotics is highlighted, as well as their emergent combination as synbiotics.

9.1.1 Probiotics

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). In the last years, the scientific community proposed probiotics as therapeutic and prevention options for a wide panoply of human diseases (Zommiti, Feuilloley, & Connil, 2020). In this scope, several clinical trials emphasizing the beneficial effects of probiotics on human health are available in the literature (see Table 9.1).

TABLE 9.1 Examples of clinical trials involving probiotics and their human health benefits, published in 2021.

Target condition	Probiotic	Key findings	References
Anxiety and depression	*Lactobacillus plantarum* PS128	Daily administration of this probiotic may reduce depressive symptoms, fatigue level, cortical excitation, and an enhancement in sleep quality during the deep sleep stage among insomniacs.	Ho, Tsai, Kuo, and Yang (2021)
Antibiotic-induced fecal short-chain fatty acid (SCFA) and microbiota composition disruptions	*Bifidobacterium animalis* subsp. *lactis* BB-12	Simultaneous administration of amoxicillin/clavulanate and BB-12 yogurt to healthy participants was related to a significantly smaller diminution in the fecal SCFA levels and a more stable taxonomic profile of the microbiota over time than the control group.	Merenstein et al. (2021)
Type 2 diabetes	*Lactobacillus plantarum* HAC01	An eight-week course of *L. plantarum* HAC01 supplementation significantly improved certain metabolic parameters (HbA1c and 2h-postprandial glucose levels) relative to placebo in prediabetic individuals and there were no reports of the serious adverse effects.	Oh et al. (2021)
Constipation	*Lacticaseibacillus paracasei* strain Shirota	Daily consumption of this probiotic for 9 weeks seemed to alleviate constipation and improve the potentially depressive symptoms in patients with depression and significantly reduce IL-6 levels. Furthermore, this probiotic supplementation appeared to regulate the gut microbiota associated with mental disease.	Zhang et al. (2021)
Rheumatoid arthritis	*Lactobacillus acidophilus* La-14, *Lactobacillus casei* Lc-11, *Lactococcus lactis* Ll-23, *Bifidobacterium lactis* Bl-04 and *Bifidobacterium bifidum* Bb-06	The mixture of these probiotic strains reduced inflammatory biomarkers and enhanced oxidative/nitrosative profile in patients with rheumatoid arthritis.	Cannarella et al. (2021)

Target condition	Probiotic	Key findings	References
Stress	*Lactiplantibacillus plantarum*, *Lacticaseibacillus paracasei*, *Levilactobacillus brevis*	The intake of beverages fermented with these probiotic bacteria may reduce the stress and modulate the intestinal microbiota (correcting dysbiosis) in university students, without interfering with their lifestyle or diet.	Márquez-Morales et al. (2021)
Allergic rhinitis	*Bifidobacterium animalis* subsp. *lactis* BB12 and *Enterococcus faecium* L3	When administered as a prophylactic treatment, a mixture of these probiotic strains reduced signs and symptoms of allergic rhinitis and diminished the need for conventional therapy.	Anania et al. (2021)
Atopic dermatitis and cow's milk protein allergy	*Lactobacillus rhamnosus* ŁOCK 0900, *Lactobacillus rhamnosus* ŁOCK 0908, and *Lactobacillus casei* ŁOCK 0918	Supplementation of the children's diet with the probiotic preparation for three months led to a significant improvement in atopic dermatitis symptom severity. Furthermore, this probiotic preparation is safe, and triggers benefits, especially in allergen sensitized patients.	Cukrowska et al. (2021)
Fibromyalgia syndrome	*Lactobacillus rhamnosus* GG, *Lactobacillus paracasei*, *Lactobacillus acidophilus* and *Bifidobacterium bifidus*	The probiotic treatment produced an improvement in attention by reducing errors on an attention task, but it did not affect memory, in patients with fibromyalgia syndrome.	Cardona, Roman, Cañadas, and Sánchez-Labraca (2021)
Irritable bowel syndrome	*Bacillus coagulans* LBSC	The probiotic intervention was safe for human consumption and efficacious in relieving overall pathophysiological symptoms of irritable bowel syndrome thereby improving quality of life.	Gupta and Maity (2021)
Halitosis	*Weissella cibaria* CMU	Oral ingestion of *W. cibaria* CMU may aid in reducing subjective halitosis and improve oral-health-related quality of life, being reported the possibility of oral-probiotic use by college students without any difficulties or adverse effects.	Lee, Kim, Nam, Kang, and Lee (2021)
Pancreatitis	*Bacillus subtilis* and *Enterococcus faecium*	Probiotic supplementation seemed to be safe and effective in reducing the duration of hospitalization in patients with mild acute pancreatitis.	Wan, Zhu, Bian, and Sun (2021)
Community-acquired common colds	*Lactiplantibacillus plantarum* HEAL9 and *Lacticaseibacillus paracasei* 8700:2	Intake of this probiotic product may be protective against multiple colds in subjects prone to getting colds.	Ahrén, Hillman, Nordström, Larsson, and Niskanen (2021)
Constipation, diarrhea, acute respiratory infections and nutritional status	*Lactobacillus casei* strain Shirota	Daily intake of fermented milk containing this probiotic bacterium strongly prevented the incidence of constipation and acute respiratory infections in Vietnamese children. Furthermore, this probiotic drink may contribute to diarrhea prevention and nutritional status improvement.	Mai et al. (2021)

The exact mechanisms by which probiotics accomplish their benefits are diverse, heterogeneous, and strain-specific, remaining not fully clarified (Plaza-Diaz, Ruiz-Ojeda, Gil-Campos, & Gil, 2019). Nevertheless, four main mechanisms of action have been pointed, as depicted in Fig. 9.1: i) interference with pathogens; ii) strengthening of the barrier function of the epithelial lining; iii) modulation of the immune system; and iv) gut-brain axis interaction (Sánchez et al., 2017). For instance, probiotics play a key role in the prevention of pathogenic colonization. This can be mediated by binding of probiotics to the host adhesion sites, competition for available nutrients, coaggregation ability, production of antimicrobial compounds (such as bacteriocins, biosurfactants, hydrogen peroxide, and organic acids), and/or the inhibition of flagella motility of pathogens (van Zyl, Deane, & Dicks, 2020).

Probiotics also contribute to the maintenance of the epithelial barrier integrity, through the stimulation of mucin glycoproteins secretion by the goblet cells that assemble into a thick mucus layer. Probiotics increase the secretion of antimicrobial proteins (defensins) by the

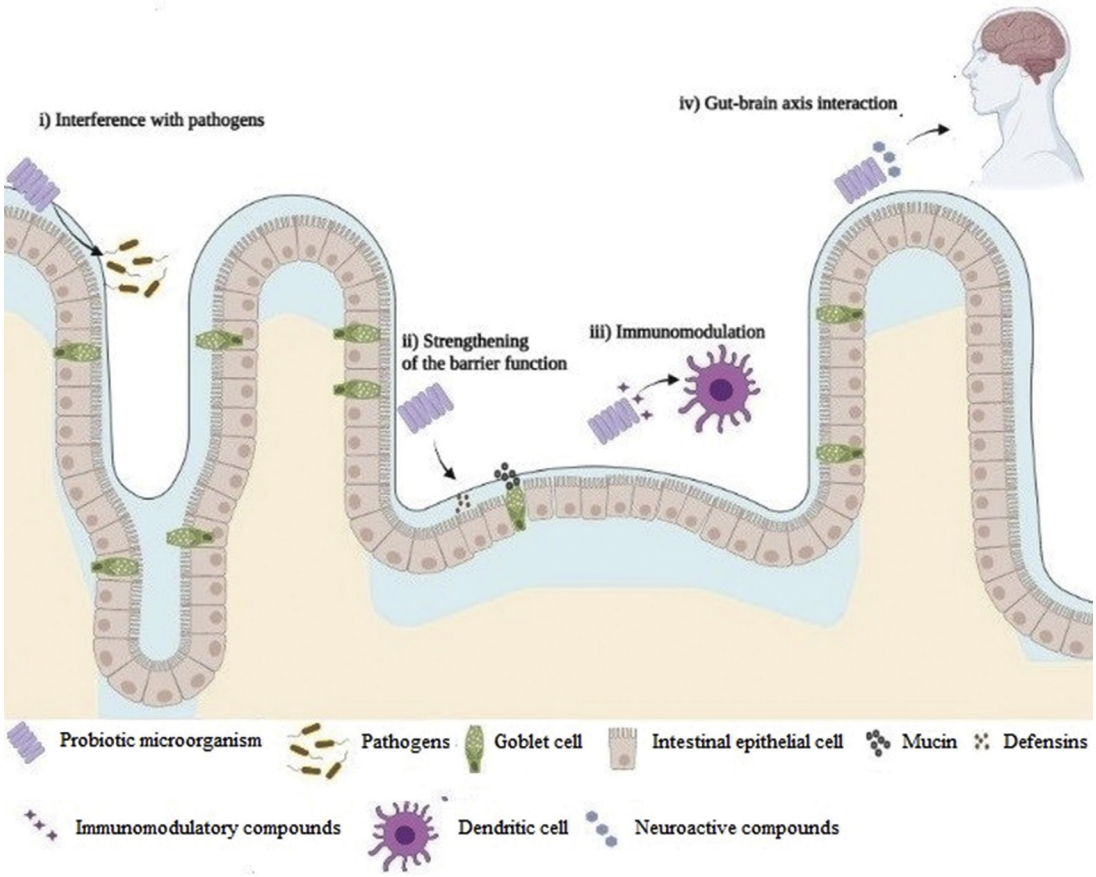


FIG. 9.1 Probiotic mechanisms of action. This graphical representation illustrates the four main mechanisms underlying health benefits exerted by probiotics namely: i) interference with pathogens; ii) strengthening of the barrier function; iii) immunomodulation, and iv) gut-brain axis interaction. *Figure created in BioRender.com.*

intestinal epithelial cells, which help the clearance of pathogens that penetrate the mucus layer, and improve the stability of intercellular junctional complexes, which reduces the intestinal epithelial cells intercellular permeability to pathogens (van Zyl et al., 2020). Moreover, it is often described that probiotics may exert regulatory effects on the host's innate and adaptive immune responses, since these beneficial microorganisms may modulate the functions of dendritic cells, monocytes/macrophages, and T and B lymphocytes (Plaza-Diaz et al., 2019; van Zyl et al., 2020). In addition, probiotics may interact with the central nervous system (gut-brain axis), producing microbial metabolites, such as short-chain fatty acids, which can alter neuronal excitability, and neuroactive compounds (namely dopamine, gamma-aminobutyric acid, histamine, acetylcholine, and tryptophan) (Plaza-Diaz et al., 2019).

Probiotic microorganisms can be isolated from many sources including the gastrointestinal tract, breast milk, and fermented food products (Shokryazdan, Faseleh-Jahromi, Liang, & Ho, 2017; Zommiti et al., 2020). In addition, these microbes must meet certain criteria in terms of safety, functionality, and technological utility to be qualified as a probiotic (Binda et al., 2020; Zommiti et al., 2020). Considering Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) guidelines, recently reiterated by International Scientific Association for Probiotics and Prebiotics (ISAPP), a probiotic microorganism should be sufficiently characterized at a genus, species, and strain level. Furthermore, probiotics should be named according to scientifically valid nomenclature (including a strain designation) and deposited in an internationally recognized culture collection. In addition, a probiotic strain should be safe for the intended use; its health benefit must be proven from at least one study involving humans; and the survival of adequate levels of the probiotic strains throughout the products' shelf life to deliver a health benefit must be guaranteed (Food and Agriculture Organization of the United Nations; World Health Organization, 2002; International Scientific Association for Probiotics and Prebiotics, 2018).

The major commercialized probiotic microorganisms are found within the *Lactobacillus*, recently reclassified into 25 genera (Zheng et al., 2020), and *Bifidobacterium* genera. Nonetheless, some members of *Bacillus* (*B. coagulans* and *B. subtilis*), *Streptococcus thermophiles*, and yeast *Saccharomyces cerevisiae* variant *boulardii* have also been marketed (Gomes, Andrade, & Freitas, 2017). These probiotics, commonly designated as traditional probiotics, have been considered as Generally Regarded as Safe (GRAS) by the Food and Drug Administration (FDA) or as Qualified Presumption of Safety (QPS) by the European Food Safety Authority (EFSA), due to their long history of safe use (Martín & Langella, 2019). Nevertheless, the probiotic market demands the implementation and diversification of the available products. For this purpose, several studies targeting the selection of probiotic microorganisms with diverse functional properties have been performed (de Melo Pereira, de Oliveira Coelho, Magalhães Júnior, Thomaz-Soccol, & Soccol, 2018).

In this alignment, various intestinal commensals, including *Akkermansia muciniphila*, *Faecalibacterium prausnitzii*, *Anaerobutyricum hallii*, *Prevotella copri*, and *Christensenella minuta*, have emerged as potential novel probiotics, also termed as next-generation probiotic candidates (Andrade et al., 2020). However, the novelty linked to such bacterial strains entails the need to clarify several key parameters regarding their effectiveness, safety, physiological, genomic, and metabolomic features, before their practical application. In addition, several technological aspects require substantial improvement, namely in terms of production, storage stability, and delivery (Almeida et al., 2020; Andrade et al., 2020).

9.1.2 Prebiotic

In its first definition, a prebiotic was described by Gibson and Roberfroid (1995) as a "non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health." More recently, this definition has been modified to "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017), which broadens the definition to all ecosystems where a selective targeting of particular microorganisms is required for host well-being. Prebiotics are present in natural products and may be extracted and applied to more common food preparations to improve their nutritional and health values. Acting as fermentable substrates for specific bacteria, prebiotics will lead to the stimulation of their growth and metabolic activity, which are linked to different health outcomes (Markowiak & Ślizewska, 2017; Voss, Campos, & Pintado, 2021).

Until recently, the main recognized prebiotics are carbohydrate-based, however, the new definition enables other substances, such as polyphenols, peptides, and polyunsaturated fatty acids, to be included whenever these reveal proven prebiotic effects (Gibson et al., 2017).

Among the carbohydrate-based prebiotic substances, short and long-chain β-fructans (fructooligosaccharides and inulin, respectively), lactulose, galactooligosaccharides, and β-glucans, are the most well-established (Markowiak & Ślizewska, 2017), and their supplementation in the diet has shown numerous health benefits. Thus, considering the importance of prebiotics on the human gut microbiota balance and their potential role on human health, the search for new prebiotics is continuous, and fruit, vegetables, cereals, and other edible plants are sources of carbohydrates constituting potential prebiotics (Markowiak & Ślizewska, 2017; Roberfroid et al., 2010; Voss et al., 2021).

9.1.2.1 Prebiotic substances and their selection criteria

Several food oligosaccharides and polysaccharides (including dietary fiber) have been claimed to have prebiotic activity, but not all dietary carbohydrates are prebiotics (Gibson, Probert, Loo, Rastall, & Roberfroid, 2004). Furthermore, not all prebiotics are carbohydrates, and two criteria can be used to differentiate fiber from carbohydrate-derived prebiotics: (i) fibers are carbohydrates with a degree of polymerization equal to or higher than 3 and (ii) endogenous enzymes in the small intestine cannot hydrolyze them (Davani-Davari et al., 2019). In addition, since other substances might fit the updated definition, such as the previously mentioned polyphenols and polyunsaturated fatty acids converted to respective conjugated fatty acids (Gibson et al., 2017), there is a need to establish clear criteria for classifying a food ingredient as a prebiotic. According to Gibson et al. (2004) and Wang (2009), there are four criteria for the classification of food ingredients as prebiotics, as shown in Fig. 9.2.

Prebiotic substances may serve as a medium for probiotics, stimulating their growth. Previous studies have demonstrated that prebiotics increase beneficial bacteria in the human gut, and inhibit pathogenic bacteria, mainly promoting the production of short-chain fatty acids (SCFAs) and lactic acid during their metabolic degradation, which confers a health benefit to the host (Roberfroid et al., 2010; Voss et al., 2021).

9.1.2.2 Novel prebiotic substances and sources

There are many natural whole sources of compounds with prebiotic activity. The most common include chicory, leeks, Jerusalem artichokes, garlic, onions, barley, and oats, which

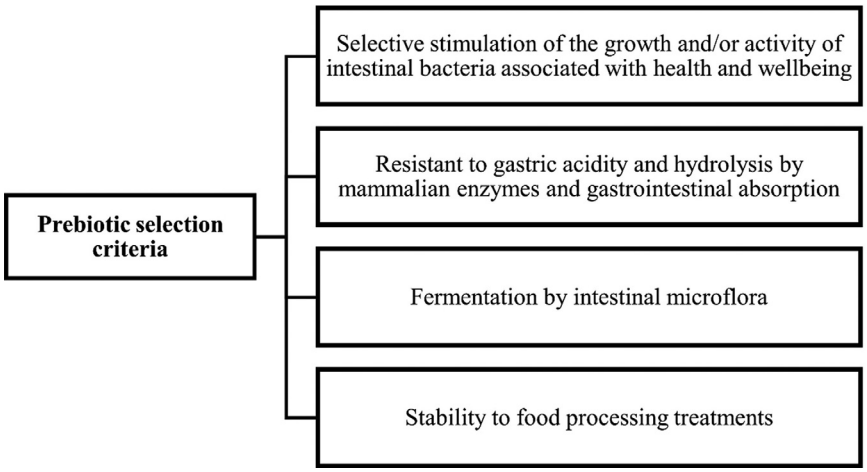


FIG. 9.2 Criteria required for classification of a food ingredient as prebiotic (Gibson et al., 2004; Wang, 2009).

are sources of the well-established prebiotic fructans and β-glucans. As functional ingredients to be used in the fortification of specific foods, these prebiotic compounds may be simply extracted from their natural sources or commercially produced via hydrolysis of polysaccharides or catabolic enzymatic reactions from lower molecular weight sugars (Scott et al., 2020). More recently, other natural products, including different fruits, seeds, vegetables, flowers, leaves, and roots have been described as candidate prebiotics due to the presence in their constitution of some recognized prebiotics or even synergetic behaviors between them.

Several fruits have been largely studied for their prebiotic potential because of their richness in nondigestible carbohydrate compounds, such as the case of pineapple, bananas, apple, pear, and even citrus fruits (Gómez-García et al., 2022). Generally, fruits have a high content of pectin, medium to high molecular weight sugars, or inulin, all of which may be considered prebiotic substances. For instance, bananas are a good source of molecule mixtures with prebiotic potential, given their constitution rich in vitamins, minerals, and dietetic fibers, containing small quantities of inulin. Moreover, when consumed in an unripened form, the fruit contains high content of resistant starch, which is also known to promote a prebiotic effect. On the other hand, fruits containing high content of pectin and a high content of polyphenols, in free or complexed form, may also enable potential prebiotic effects. For instance, all varieties of apples and pears, as well as oranges and all of the citrus family are recognized for the improvement of the digestive tract health through enhancement of the human gut microbiota due to the synergies promoted by the pectins and polyphenols (Akter & Rabeta, 2021). Recent studies have demonstrated the potential prebiotic activity of pineapple by-products juice, associated with the presence of oligosaccharides and galactomannans, which were able to promote the growth of both *Bifidobacterium* and *Lactobacillus* genera, contributing towards the improvement of the fermentation conditions (Gómez-García et al., 2022). Further details will be presented in Section 9.2.2.4 of this chapter.

In what concerns tubers, Jerusalem artichoke (*Helianthus tuberosus* L.), coming from a sunflower species has been described for its composition high in water and inulin, unlike other tuber-like foods that contain high amounts of starch and sucrose; such composition is

associated with an important prebiotic potential of artichoke (Brkljača et al., 2014). The Konjac root (*Amorphophallus konjac*) is another tuber recognized for its health-promoting effects directly associated with a prebiotic potential. Unlike artichoke, konjac root and derived flours contain between 70 and 90% of glucomannan fiber. The glucomannan fiber contains polysaccharides of high molecular weight and water-soluble, consisting mainly of units of D-glucose and D-mannose under a ratio of 1.6:1.0 connected by β(1-4)-glycosidic bonds. This structure enables the fiber to have a highly viscous nature, which improves the carbohydrates metabolism and the growth promotion of probiotic bacteria EFSA Panel on Food Additives and Nutrient Sources added to Food ANS et al., 2017.

Besides roots and tubers, some types of seeds have been recognized for their potential as a prebiotic food, the most well-known being cocoa beans (the basis for chocolate production). Fresh cocoa beans contain a high variety of metabolites mainly polyphenols, but flavanols and procyanidins are the most representative. Several studies have shown the positive interaction between these molecules with the gut microbiota since flavanols influence positively the *Bifidobacterium* and *Lactobacillus* growth, while the growth of nonprobiotic bacteria is not positively affected (Tzounis et al., 2008).

Flax seeds (*Linum usitatissimum*) are also recognized to be a health-promoting food. These seeds are small oil seeds originally from the Middle East with high content of α-linolenic acid (ALA), omega-3 fatty acids, lignans phytoestrogens, soluble fiber, digestible proteins, and phenolic compounds, and, in combination, these ingredients improve digestion and bowel mobility (Prasad, 2009).

While the cocoa beans' prebiotic potential is associated with specific polyphenols, in the case of flax seeds it is the overall impact of the food that potentiates the positive environment within the gut microbiota. So, prebiotic foods show high potential to improve health and well-being and even for preventing illness.

9.1.2.3 Mechanism of action and health benefits associated with prebiotics

The presence of prebiotics in the diet may lead to various health benefits. Over the last few years, clinical evidence has demonstrated positive effects on the gastrointestinal tract (Guarino et al., 2020; Liong, 2008; Moreno, Corzo, Montilla, Villamiel, & Olano, 2017), cardiovascular system (Aliasgharzadeh et al., 2015; Wu & Chiou, 2021), mental health (Chen et al., 2017; Johnstone et al., 2021; Pourjafar, Ansari, & Vaccine, 2020; Schmidt et al., 2015), and bone (Mtasher, Abdulhussein, & Mutlag, 2018; Pandey, Naik, & Vakil, 2015; Whisner et al., 2013). Fig. 9.3 presents the principal postulated mechanisms of prebiotic action and some of the possible health benefits on the host's health.

9.1.2.3.1 Modulation of the gut microbiota

The mechanism of a beneficial effect of prebiotics on immunological functions in the gut has not been established and possible hypotheses have been proposed. Currently, some studies have been indicated that the gut microbiota is deeply involved in the metabolic functions extending beyond the gut and prebiotics may play an important role in this system (Moreno et al., 2017). In addition, human clinical evidence has reported that prebiotics affects the composition of the gut microbiota. The nondigestible carbohydrate-based prebiotics can resist small-intestinal digestion and reach the colon intact, where they are efficiently metabolized leading to an increase in health-promoting organisms, such as *Lactobacillus* and

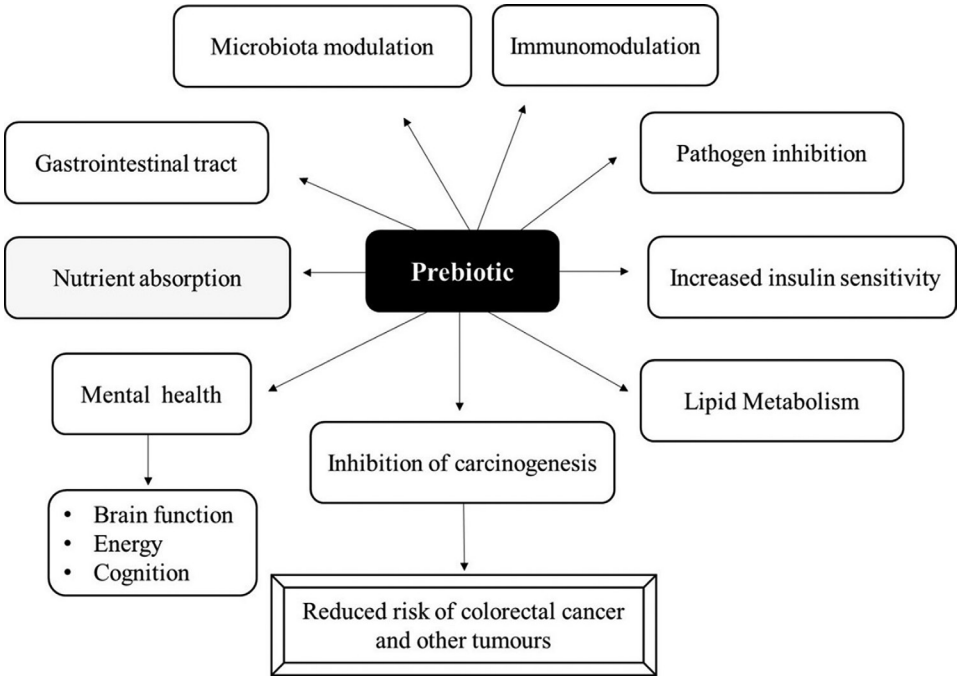


FIG. 9.3 The possible health benefits and postulated mechanisms of action of prebiotics.

Bifidobacterium species (Guarino et al., 2020; Mtasher et al., 2018). In addition, some species are also able to ferment prebiotic substances and produce SCFAs (including acetate, butyrate, and propionate), which are important sources of energy for the host that can act to improve barrier function besides other benefits (Guarino et al., 2020). The chain length of the prebiotic can be considered an important factor for distinguishing between species, since although a few species can ferment substances with a high polymerization degree, such as inulin, several microorganisms are only able to degrade prebiotics with polymerization degree below 10, such as FOS (Davani-Davari et al., 2019).

Concerning the metabolites produced during fermentation, butyrate is associated with different biological properties. However, this metabolite is not a usual end-product from the fermentation of *Lactobacillus* and *Bifidobacterium* species. Although *Bifidobacterium* species do not produce butyrate, they have been shown to stimulate butyrate-producing bacterial species, such as Eubacteria in the gut (Liong, 2008; Mtasher et al., 2018). Butyrate produced in the colon is related to the type of prebiotic compounds used (Liong, 2008). Gut microbial opportunists and pathogens are involved in the pathogenesis of several gastrointestinal disorders and different therapeutic strategies can help modify the negative effects caused by the gut microbiota imbalance. In this respect, the production of organic acids and the proliferation of beneficial bacteria through prebiotic modulation will result in a reduction in luminal pH, inhibiting the growth of pathogens. Furthermore, nutrient availability as well adhesion receptors for pathogens are significantly reduced contributing to reduced colonization and infective capacity. Finally, the increase of beneficial bacteria will promote

immunomodulation with the increase of secretory IgA that will reduce pathogens in the colon. Currently, there is an increasing interest in dietary strategies to modulate the composition and metabolic activity of gut microbiota, being dietary interventions using prebiotics among the most applied (Moreno et al., 2017). Nevertheless, the prebiotics action on microbial diversity in the colon is still under debate (Guarino et al., 2020).

9.1.2.3.2 Inhibition of colorectal cancer

Evidence suggested that prebiotics can be protective against the development of cancer. Investigations have demonstrated that colorectal carcinoma occurs less commonly in individuals who often eat vegetables and fruit, and these effects can be commonly associated with prebiotics (Markowiak & Ślizewska, 2017). As already mentioned before, prebiotic compounds modify the microbiota by increasing the numbers of beneficial bacteria, such as *Lactobacillus* and or *Bifidobacterium* species in the colon, and often generate SCFAs, such as acetate, propionate, and butyrate, that have been shown to inhibit the development of the colon tumor cells (Mtasher et al., 2018). Furthermore, although the butyrate is produced in less amount (approximately 5% of total SCFAs), this metabolite has a particular interest, owing to its association with many biological properties in the colon and could have protective effects against the risk of colorectal cancer, as well as its progression (Davani-Davari et al., 2019; Liong, 2008). In general, the products resulting from the prebiotics fermentation are mainly organic acids, leading to a decrease in the gut pH value. This alteration can modify the population of acid-sensitive species, such as *Bacteroides*, promoting the butyrogenic effect, through butyrate formation by *Firmicutes* (Davani-Davari et al., 2019). Moreover, when butyrate is present, apoptosis may be increased in transformed cells but inhibited in normal cells. Thus, an important mechanism of protection against carcinogen-enhanced colon cancer may be an enzyme induction by butyrate, or by the microbiota, and increased activity by prebiotics (Wollowski, Rechkemmer, & Pool-Zobel, 2001).

9.1.2.3.3 Cardiovascular diseases

Some investigations have suggested that people consuming high amounts of prebiotics exhibited beneficial effects on cardiovascular diseases. Some possible explanations for these potential mechanisms of prebiotics have been suggested, such as modulation of gut microbiota and inflammatory responses (Wu & Chiou, 2021). In addition, the metabolites of intestinal microbiota, such as SCFAs have been demonstrated to be inversely associated with some risk factors for cardiovascular diseases. The production of SCFAs can improve cardiovascular homeostasis, indicating a potential target for reducing cardiovascular risk (Wu, Xu, Tu, & Gao, 2021). Previous studies using animal models showed that acetate and propionate can regulate blood pressure (Natarajan et al., 2016), while butyrate compounds attenuated blood pressure in angiotensin II-induced hypertensive rats, mainly via reducing the expression of renal protein receptors and renin (Wang et al., 2017). Therefore, prebiotic compounds and their metabolites (SCFAs) in the gut exhibit beneficial effects on cardiovascular diseases through several mechanisms involved in alteration of the gut environment, histone deacetylation, improvement of gut epithelial permeability, contributing to reduced total and LDL cholesterol and hs-CRP, and lowering the incidence of risk factors in cardiovascular diseases such as hypertension, obesity, and diabetes (Wu & Chiou, 2021).

Additionally, in a previous clinical trial performed by Aliasgharzadeh et al. (2015), the effects of oligofructose-enriched inulin were evaluated on some cardiovascular disease risk factors in women with type 2 diabetes. Based on these results, oligofructose-enriched inulin supplementation may improve glycemic indices, lipid profiles, antioxidant status, and decrease the malondialdehyde (a marker of lipid peroxidation and oxidative stress) concentrations in type 2 diabetic patients. Many studies have established the association between inflammation and cardiovascular risks. Thus, prebiotics have positive effects on the preventing the development of these diseases as a result of their anti-inflammatory action (Wu & Chiou, 2021).

9.1.2.3.4 Mental health

In recent years, studies have been shown that prebiotics can also have an important role in neuroimmune processes (Pourjafar et al., 2020). The gut microbiota plays a role to understand the factors that influence brain function and behavior, and the effects of prebiotics on brain health are related to the interactions between gastrointestinal microbiota and immune and nervous systems (Johnstone et al., 2021; Pourjafar et al., 2020). The gut and the brain are deeply connected via the gut-brain axis, which involves bidirectional communication via neural, endocrine, and immune pathways, and the study of their role in neuropsychiatric diseases is rapidly advancing (Pourjafar et al., 2020). Thus, fostering a beneficial gut microbiome with prebiotics, such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), and inulin, is a viable approach, but under-investigated (Chen et al., 2017). In addition, nutrition has been increasingly recognized as an important factor that intermediates the composition and metabolic function of the gastrointestinal microbiota, and the ingestion of prebiotics is a path that modulates the microorganisms in the gastrointestinal tract (Pourjafar et al., 2020).

Previous findings have demonstrated that prebiotic treatment can change the behavior across domains related to depression, anxiety, stress reaction, cognition, and social behavior, thereby opening new prospects in the field of nutritional neuropsychopharmacology (Chen et al., 2017; Pourjafar et al., 2020). The effects of two prebiotics (FOS and Bimuno-galactooligosaccharides [B-GOS]) on the secretion of the stress hormone, cortisol, and emotional processing in healthy adults were investigated over 3 weeks by Schmidt et al. (2015). The results showed a lower secretion of the stress hormone in the presence of the prebiotic compound in comparison to the placebo. The volunteers also exhibited decreased attentional vigilance to negative versus positive information in a dot-probe task after intake of B-GOS compared to placebo. However, no effects were found in volunteers after intake of FOS. In another study using a rodents model, Burokas et al. (2017) also studied FOS and GOS, and tested whether chronic prebiotic treatment modifies behavior across domains relevant to anxiety, depression, cognition, stress response, and social behavior. The results exhibited antidepressant and anxiolytic effects. In addition, the administration of GOS and the combination of FOS and GOS reduced stress-induced corticosterone release. Thus, these data suggest a beneficial role of prebiotic treatment for stress-related behaviors. But, although prebiotics and probiotics can be offered as new potent antidepressants, more investigations are needed for confirmation.

9.1.2.3.5 Mineral absorption

Minerals are the micronutrients required for the smooth functioning of the body (Pandey et al., 2015). Previous works have demonstrated that the most significant health effects of

prebiotics on mammalian physiology are the capacity to improve calcium, magnesium, iron, and zinc absorption, and the attendant enhancement of bone mineralization (Mtasher et al., 2018). There are clinical trials on the impact of different prebiotics on calcium absorption, but the results are conflicting. In a previous study, Abrams et al. (2005) showed that prebiotic short- and long-chain inulin-type fructans increase calcium absorption and enhance bone mineralization during pubertal growth. However, in another study using milk enriched with FOS by López-Huertas et al. (2006) no significantly increased calcium absorption was observed. However, in another study, Whisner et al. (2013) investigated the dose-response relationship of GOS supplementation on calcium absorption in adolescent girls, and the results indicated that daily consumption of 5 g of GOS improved calcium absorption, which may be mediated by the gut microbiota. Many investigations have suggested that a fraction of calcium is absorbed from the colon, as a consequence of the fermentation of prebiotics by the intestinal microbiota. The fermentation leads to the production of SCFAs which lower the intraluminal pH generating an acidic environment where the insoluble and unabsorbed calcium is converted to the ionic soluble form. So, low intraluminal pH and SCFAs lead to an expansion of the surface area of the intestine enabling improved calcium absorption (Cashman, 2003; Pandey et al., 2015).

9.1.2.4 *Effects of prebiotics on the growth/survival and metabolism of probiotics*

As previously described in Section 9.2.2.1 and Fig. 9.2, a compound can be considered a prebiotic if it follows the established requirements of being fermentable by the intestinal microbiota and stimulating bacteria in a selective way (only probiotics). In other words, a prebiotic must be capable of being fermented by saccharolytic bacteria (e.g. *Bifidobacterium* genus).

Saccharolytic bacteria are in general present in the bowel and can metabolize complex carbohydrates leading to bacterial multiplication, associated energy production, and the production of specific metabolites such as the previously mentioned SCFAs (formic, acetic, propionic, butyric, isobutyric, valeric, isovaleric, and 2-methylbutyric acids) besides other organic acids (lactic, pyruvic, and succinic acids) and ethanol (Loo, Clune, Bennett, & Collins, 2005; Miller & Wolin, 1979). These metabolites are of high importance in the regulation of gut health and functions including mucus barrier function of the gut epithelia, regulation of glucose and lipid metabolism, satiety, and homeostasis (Cunningham et al., 2021). All of the aforementioned metabolites are essential for the interconnection of glycolysis pathways, more specifically the tricarboxylic acid/Krebs cycle, oxidative phosphorylation (OXPHOS), as well as, amino acids and fatty acids metabolisms, leading to a direct impact on the immune cells, mainly macrophages, which respond to different physiological and pathological stimulations, acquiring at the end, specific functions. The two most important types of macrophages are controlled by the different metabolism consumption of glucose (Belizário, Faintuch, & Garay-Malpartida, 2018). Thus, it is of high importance to understand the glucose metabolisms promoted by the fermentation of both *Bifidobacterium* and *Lactobacillus* genera.

Novel sources of prebiotic compounds also play a supportive role in what concerns the metabolic activity of probiotic strains. As previously described pineapple peel juice played an important role in the fermentation capacity of three types of *Bifidobacterium* species and three types of *Lactobacillus* species (Gómez-García et al., 2022). The pineapple peel juice was

characterized in terms of polyphenols where chlorogenic, ferulic, and caffeic acids were the main compounds found, and, in the case of carbohydrates, trisaccharides, and oligosaccharides were the most common. *Bifidobacterium* species were able to ferment the pineapple peel juice and promote the production of significant levels of lactic and acetic acids in the medium. Furthermore, the samples also promoted the growth of *Lactobacillus* strains tested, which was also proven by the production of lactic acid and reduction of glucose in the medium. Although the fermentations were performed in an isolated single-strain form and the impact for the combined fermentation of strain consortia was not assessed, other studies have demonstrated the positive impact of prebiotic foods on the combined fermentation of *Lactobacillus* and *Bifidobacterium* genera (Campos, Ribeiro, Teixeira, Pastrana, & Pintado, 2020).

Schley and Field (2002) described that the direct immunological impact was not so clear as published by other authors, but described several possible models that empower the beneficial effect of prebiotics: i) regulation action upon hepatic lipogenic enzymes promoting the production of SCFAs; ii) butyric acid (from SCFAs) as a modulator of histone acetylation; iii) modulation of mucin production; iv) promotion of an increased amount of lymphocytes and/or leukocytes in the gut-associated lymphoid tissues (GALTs)[1] and peripheral blood; v) increase secretion of IgA by GALTs can stimulate the function of phagocytosis of the intra-inflammatory macrophages (Markowiak & Ślizewska, 2017).

But further work is required to enable full comprehension of the prebiotic metabolism promotion and consequential impact in the associated cascade of signals and activation within the gut epithelia and immunological system. Nevertheless, it has been shown that the different glucose metabolisms are important and that prebiotics can promote the proliferation and metabolic modulation of positive bacteria in the human gut, as well as the impairment of nonprobiotic bacteria.

9.2 Synbiotic emergent association between probiotics and prebiotics

Considering the recognized beneficial effects of both probiotics and prebiotics, their combination in a single product comes as a natural step, in an attempt to potentiate their impact on human health. Since the introduction of the term "synbiotic" by Gibson and Roberfroid, in 1995, the definition has evolved to designate these combined products, which are now defined as "a mixture comprising live microorganisms and substrate(s) selectively utilized by the host microorganisms that confer a health benefit on the host" (Fig. 9.4; Gibson & Roberfroid, 1995; Swanson et al., 2020). In this context, "host microorganisms" include both the resident/colonizing microorganisms (autochthonous) and the externally applied (allochthonous), such as probiotics, that may be transient in the host-microbiota (Swanson et al., 2020). Several types of probiotic and prebiotic combinations fall within this definition of synbiotics, which can be divided into two main groups: complementary synbiotics and synergistic synbiotics (Fig. 9.4; Cunningham et al., 2021; Swanson et al., 2020). Complementary synbiotics result from the combination of accepted prebiotics and probiotics, meaning that

[1] GALTs (Gut Associated lymphoid tissues) it is a very important structure present at the gut, being the biggest in size at the human body, responsible for the management of the immune response to the massive antigen exposure experienced, while maintaining a potent adaptive immune response to protect the host from mucosal pathogens.

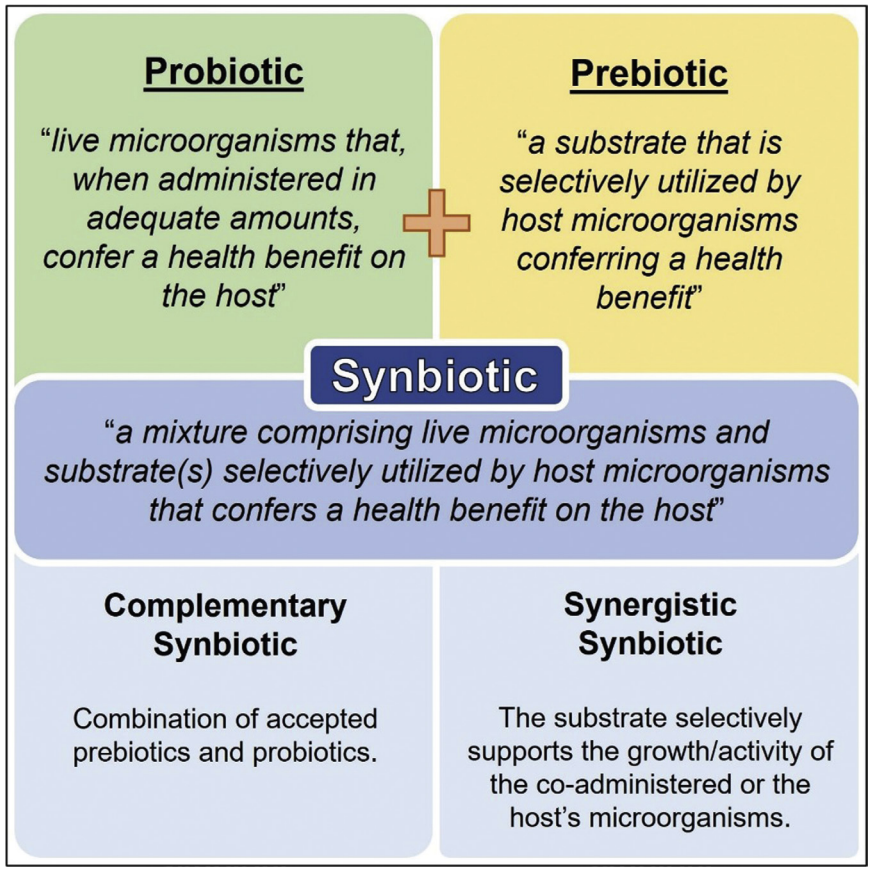


FIG. 9.4 Definition of synbiotics. The production of synbiotic products appears as a natural evolution upon the establishment of probiotics and prebiotics (on the top). Two types of synbiotics were defined: complementary and synergistic synbiotics (on the bottom). Complementary synbiotics result from the direct combination of pro- and prebiotics, while synergistic synbiotics' components might not correspond to previously defined probiotics and prebiotics.

each component must meet the criteria established for its category; their mechanisms of action can be independent of each other, and each part must have its specific demonstrated health benefits. Despite that, evidence that the combination of both provides a health benefit in the target host is also required for the categorization as a synbiotic (Cunningham et al., 2021; Swanson et al., 2020). Concerning the synergistic synbiotics, the substrate is designed to selectively support the growth or activity of the coadministered live microorganism(s), which, in turn, is selected based on its ability to provide a health benefit. Although their combination must have a demonstrated health benefit, the substrate and the live microorganism might not be able to elicit a health benefit independently from each other, which means that their previous establishment as probiotics or prebiotics is not required (Cunningham et al., 2021; Swanson et al., 2020).

Regarding the characterization of a synbiotic, it is required to ensure its safety and stability, starting with the characterization of both the live microorganism and the substrate components. Even if not categorized as so, the live microorganism counterpart must meet most of the criteria required for a probiotic strain to be used for human consumption: it should be identified, to the strain level; it should be deposited into at least a recognized international culture collection and its genome sequence and respective annotation should be available in public databases. In parallel, it should be assessed in terms of safety, both phenotypic and genotypic, concerning the risk of toxin production and resistance genes transferability. Also, the purity of the strain and its potency, and specific attributed benefits must be assessed (Sanders et al., 2014; Swanson et al., 2020). In what concerns the substrates, their characterization must include the appropriate chemical analysis to determine their structure and purity, including for the presence of contaminants of microbial origin, among others.

Regarding substrates that might be digested and absorbed during the oro-gastro-intestinal tract, the actual amount of product that is delivered to the target should also be assessed and considered when calculating the dosage required to yield a given effect. Finally, the components of the synbiotic must be stable to ensure that the right dosage of live microorganisms and substrate (if that is the case) reach the target in the gastrointestinal tract and exert their expected health benefit. This must consider the specific requirements of the components and also the production constraints, distribution chain, and intended shelf-life of the final product, and ultimately, its passage through the gastrointestinal tract itself. This is particularly relevant for the live microorganisms, whose viability is dependent on several factors, including the matrix nature and water content, storage temperature, pH, and oxygen levels. The safety of the synbiotic should also be assessed according to the legislation required for the intended use and country of commercialization (Swanson et al., 2020).

As mentioned, even if the beneficial effects of the substrate and/or live components were already demonstrated, the effect of their combination must also be assessed, through specifically designed trials to demonstrate their health benefit in the host (Klurfeld et al., 2018; Moher et al., 2012). In an ideal synbiotic, the health benefit should be increased when compared to the sum of its individual components. However, this might not happen with some synergistic synbiotics, since the observed effect might be delivered by the live microorganism, whose survival and growth were, in turn, supported by the substrate. For synergistic probiotics, besides the health benefit on the host, appropriate studies must be conducted to demonstrate the utilization of the substrate, either by the host microbiota or by the probiotic counterpart of the synbiotic itself (Swanson et al., 2020). The assessment of the synbiotic's activity is also essential to exclude potential – although improbable – antagonistic effects of the combination, which may decrease the expected health outcomes (Swanson et al., 2020). For instance, the metabolization of some carbohydrate substrates can increase the production of antimicrobial compounds by the probiotic strains, which might harm the commensal microbiota upon delivery to the gut (Tzortzis, Baillon, Gibson, & Rastall, 2004).

Several randomized controlled trials were conducted in humans in recent years to assess the health benefits of emerging synbiotics. These trials focus on a vast range of pathologies, which include overweight and obesity (Hadi, Alizadeh, Hajianfar, Mohammadi, & Miraghajani, 2018), type 2 diabetes (Mahboobi, Rahimi, & Jafarnejad, 2018; Nikbakht et al., 2018), nonalcoholic fatty liver disease (Hadi, Mohammadi, Miraghajani, & Ghaedi, 2019; Sharpton, Maraj, Harding-Theobald, Vittinghoff, & Terrault, 2019), irritable bowel syndrome (Chlebicz-Wójcik

& Śliżewska, 2021; Ford, Harris, Lacy, Quigley, & Moayyedi, 2018), surgical infections (Kasatpibal et al., 2017; Skonieczna-Żydecka et al., 2018), chronic kidney disease (McFarlane, Ramos, Johnson, & Campbell, 2019; Pisano, D'Arrigo, Coppolino, & Bolignano, 2018), and atopic dermatitis (Chang et al., 2016). The most used bacterial strains belong to the classical genera *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, and are usually applied as consortia instead of single-strain applications (Gyawali et al., 2019; Swanson et al., 2020). However, some next-generation probiotics are also being employed, namely *Akkermansia muciniphila* and *Anaerobutyricum hallii* (Perraudeau et al., 2020). Regarding the substrates, the most used are usually galactooligosaccharides, inulin, or fructooligosaccharides, administered in variable doses (Table 9.2; Gyawali et al., 2019; Swanson et al., 2020). A case-by-case optimization is expectedly required, considering not only the demands of the microorganisms to be administered or enhanced in the host's gut but mainly because each individual has its unique gut microbiome and even its genetic predispositions can impact the viability and effectiveness of the administered probiotics, prebiotics and synbiotics (Peng et al., 2019). Additionally, diverse combinations of bacterial strains and substrates will work differently on diverse dysbiotic states, considering their specific characteristics and those of their resulting by-products and metabolites (Cunningham et al., 2021).

The synbiotic formulations can be delivered to the consumer as food products, dietary supplements or drugs (Andrade et al., 2020). Currently, there are several products in the market, sold as dietary supplements under the label "synbiotics". However, in many of them, the following disclaimer may be read: "Statements regarding dietary supplements have not been evaluated by the FDA and are not intended to diagnose, treat, cure, or prevent any disease or health condition." In fact, the FDA is the authority that regulates both the food and drug markets in the United States of America, whereas in the European Union, the EFSA establishes the rules for the introduction of novel foods, while drugs are regulated by the European Medicines Agency (Cordaillat-Simmons, Rouanet, & Pot, 2020; O'Toole, Marchesi, & Hill, 2017). There are clear and well-established definitions for both food supplements – which are intended to maintain or improve a healthy state in a healthy or at-risk population – and drugs, with an expected therapeutic or prophylactic effect in human diseases (Cordaillat-Simmons et al., 2020). In addition, together with a distinct definition, specific requirements are established for each product category. However, concerning dietary supplements, other regulations apply.

There is no global consensus on what should or should not be part of this category; a substance might be considered a dietary supplement in a certain region but fall into another class of products if considering other jurisdictions, with consequent changes in the legislation to be applied. Although the safety concerns must always be addressed, some of the claimed health benefits might result mostly from empirical observation than from exact scientific evidence (Dwyer, Coates, & Smith, 2018). It is also important to mention a novel product category, proposed by the FDA in 2012, and accepted in 2019 in Europe, designated as Live Biotherapeutic Product (LBP): "a biological product that 1) contains live organisms, such as bacteria; 2) is applicable to the prevention, treatment, or cure of a disease or condition of human beings; and 3) is not a vaccine" (European Pharmacopoeia Commission, 2019; FDA, 2016). This definition, and respective evaluation criteria, can be applied to probiotics and synbiotics – but not to prebiotics. Regardless of the final formulation and application, products with the claim "synbiotics" should be attentively considered, as some lack a proper

TABLE 9.2 Examples of clinical trials involving synbiotic mixtures in the amelioration of several conditions, run in the last 4 years (2018–2021).

Microorganism(s) [concentration]	Substrate(s)	Conditions	Main conclusions	Type of study	Other info	References
Lactobacillus acidophilus, Bifidobacterium lactis, Bifidobacterium bifidum, Bifidobacterium longum [10^9 CFU each]	Inulin-based prebiotic	Type 2 diabetes	Significant decrease in glycemic biomarkers; decreased insulin resistance	Randomized, double-blind clinical trial	24 weeks; 120 participants from Iran	Nasri et al. (2018)
Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus bulgaricus, Bifidobacterium breve, Bifidobacterium longum, Streptococcus thermophilus [10^9 CFU]	Fructo-oligosaccharide	Nonalcoholic fatty liver disease (NAFLD)	Significant improvement in liver enzymes (in combination with sitagliptin)	Randomized, double-blind trial	16 weeks; 138 patients from Iran	Sayari, Neishaboori, and Jameshorani (2018)
Bifidobacterium animalis subsp. *lactis* (strain BB-12) [10^8 CFU/mL] + yogurt starter cultures (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*)	Inulin (1.5g)	NAFLD	Synbiotic yogurt improved hepatic steatosis and liver enzyme concentrations	Open-label randomized controlled clinical trial	24 weeks; 90 patients from Iran	Bakhshimoghaddam, Shateri, Sina, Hashemian, and Alizadeh (2018)
Lactobacillus paracasei, Lactobacillus rhamnosus, Lactobacillus acidophilus, Bifidobacterium lactis [10^9 –10^8 CFU each]	Fructo-oligosaccharide (6 g)	Brain disorders	Weak effects in the management of depressive symptoms or cognitive decline	Secondary study from a broader randomized, double-blind controlled clinical trial	24 weeks; 49 elders from Brazil	Louzada and Ribeiro (2018)
Lactobacillus acidophilus (strain T16), *Lactobacillus casei* (strain T2), *Bifidobacterium bifidum* (strain T1) [2×10^9 CFU/g each]	Inulin (800mg)	Polycystic ovary syndrome (PCOS)	Improved PCOS-associated insulin resistance; demonstrated synbiotic activity higher than probiotics themselves	Randomized, double-blind, placebo-controlled clinical trial	12 weeks; 60 patients from Iran	Samimi et al. (2019)
Lactobacillus rhamnosus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus salivarius, Bifidobacterium bifidum, Bifidobacterium longum [1×10^{13} CFU]	Fructo-oligosaccharide (175 mg), *Ulmus davidiana* (150 mg), *Geum urbanum* (10 mg), Inulin (100 mg)	Irritable bowel Syndrome (IBS)	Decrease the fatigue in IBS individuals; relief in abdominal pain/discomfort, bloating, stool patterns	Randomized, double-blind, placebo-controlled clinical trial	8 weeks; 28 patients from Korea	Lee et al. (2018)
Bifidobacterium lactis (DSMZ 32269), *Bifidobacterium longum* (DSMZ 32946), *Bifidobacterium bifidum* (DSMZ 32403), *Lactobacillus rhamnosus* (FloraActive 19070-2), *Lactobacillus acidophilus* (DSMZ 32418) [5.0×10^9 CFU]	Short-chain Fructooligosaccharide (947 mg)	IBS	Attenuation of IBS symptoms such as pain, flatulence, stool pressure, feeling of incomplete bowel movements; decreased severity of IBS symptoms	Randomized, double-blind, placebo-controlled, parallel group trial	8 weeks; 68 patients from Poland	Skrzydło-Radomańska et al. (2020)

Only studies referring explicitly to the term "synbiotic" were considered. When available, the CFU numbers and mass, for the microorganisms and substrate counterparts, respectively, are presented.

confirmation of the substrate utilization (either by the coadministered or the host's microorganisms), making them a simple mixture of probiotics and prebiotics, not falling within the real definition of synbiotic.

9.3 Conclusion and future perspectives

Probiotic microorganisms, prebiotic substances, and synbiotic products have gained a high interest among the scientific community, clinicians, and food and pharmaceutical industries. Several scientific reports, including some clinical trials, have supported the use of probiotics, prebiotics, and synbiotics as an efficient health-promoting strategy. Despite these advances in knowledge, the molecular mechanisms underlying the beneficial effects of these products have not yet been fully elucidated. In this scope, future studies aiming at a comprehensive and in-depth analysis regarding the mechanisms of action underlying their health benefits should be carried out. Furthermore, additional clinical trials concerning the "best" probiotic, prebiotic, and synbiotic products, would be of extreme importance, in terms of efficacious dose, duration of treatment, efficiency, tolerance, and safety, for a given health condition and considering the consumer's unique gut environment. Indeed, such data would facilitate the selection process of these products for both the industry and the consumers.

Note: Several *Lactobacillus* species mentioned throughout this chapter have been recently classified. To facilitate the reading, the species were referred to as in the original papers. The correspondent nomenclature is as follows (the former nomenclature appears first followed by the new name):

Lactobacillus casei = *Lacticaseibacillus casei*
Lactobacillus paracasei = *Lacticaseibacillus paracasei*
Lactobacillus rhamnosus = *Lacticaseibacillus rhamnosus*
Lactobacillus plantarum = *Lactiplantibacillus plantarum*
Lactobacillus brevis = *Levilactobacillus brevis*
Lactobacillus salivarius = *Ligilactobacillus salivarius*
Lactobacillus acidophilus = *Lactobacillus acidophilus*
Lactobacillus delbrueckii subsp. bulgaricus = *Lactobacillus delbrueckii subsp. bulgaricus*

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CHAPTER

10

Evidence on the risks and safety of probiotics for humans

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10.1 Introduction

An assessment of the risks and safety of probiotics could rely on the authenticity and correspondence of the marketed product with the product studied (in vitro, ex vivo, and in vivo) in healthy and diseased subjects. Anything that cannot be shown to be false is authentic. The accepted benchmarks for the risks and safety of probiotics are: i) The conformity of the product according to current rules and regulations; ii) The manufacturers and distributors' declarations; and iii) The scientific evidence. These aspects will be discussed in the following sections of this chapter.

10.2 The conformity of the product according to current rules and regulations

For a long time, microbial strains were considered safe due to their historical use in food. This safety concept stems from the fact that strains that have been used as ingredients in food and nutraceuticals have a long history of use in large human populations and have not experienced particularly adverse effects. In terms of food and food supplement legislation, the target population has always been healthy. However, regardless of the historical use of the strain, if such a strain is the active ingredient in a product administered to a person for the dietary prevention or treatment of a disease, safety, and efficacy should be studied. Even strains with a very long history of safe use in healthy populations cannot automatically be assigned to the same level of safety in patients at risk or impaired. This concept is even more relevant when the probiotic product is a highly concentrated mixture of several strains.

In 2001, the FAO and WHO defined probiotics as "living microorganisms that, when used in sufficient quantities, are beneficial to health" ("Food and Agricultural Organization of the

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United Nations and World Health Organization. Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria," 2001). This definition is appropriate for food, dietary supplements, and pharmaceutical products, depending on the intended use and target audience. In general, it is a definition that guarantees the viability of the microorganism in the final product at the end of the stated shelf-life. However, this definition does not consider the number of dead cells present in the probiotic product. Dead bacterial cells are due to stress caused by industrial processes, such as biomass production and freeze-drying. Presently, dead cells are called "Parabiotics" (Nataraj, Ali, Behare, & Yadav, 2020). Their massive presence in low-quality probiotics can affect the immune and biological response of the receiver. Authorities do not request this information to be present on the labels of probiotic products. However, the presence of living microorganisms in products requires several control steps. The variability between batches at the same production facility is much less than the variability observed when production is transferred to another factory, even worse when production occurs on another continent where different supply sources exist. Generally, the quality of raw materials, production processes, and personnel training are considered essential factors. Depending on the production location, the environmental conditions, the equipment used, the medium used to cultivate the bacteria, and the quality control of the final product, even genetically identical stains may function differently (de Simone, 2019; Fenster et al., 2019).

The impact of such changes on efficacy and safety is usually not considered by the authorities. By understanding the mechanism of action (MOA) of a particular probiotic product, for example, its immunological or biochemical profile, the owner of the know-how can ensure that the limited variation occurring in the production of different batches does not affect the efficacy and safety of the product. Manufacturers who do not have information on the MOA will almost certainly release a product not comparable to the original product and not reproducible in terms of safety and efficacy. Presently, authorities do not request "bridging" experimental and clinical studies to ascertain that a new product is equivalent to the original one any time the production site or the producer is changed (de Simone, 2019).

A probiotic may be compliant with the rules and regulations of the authorities, but the current rules are inadequate to certify the safety and efficacy of the product. This "relaxed" attitude by the authorities is also reflected in the trademark policy for probiotic products. According to the European Union Intellectual Property Office (UEIPO), the holder of a trademark used to identify a probiotic preparation can change the strain, geographical origin, production method, concentration and keep the information hidden from the consumers, without the risk of having the trademark revoked or the need to inform about the occurring changes. American consumers are better protected. The U.S. Patent and Trademark Office (USPTO) considers the principle that because a brand can indicate continuity in the content and quality of a product, any significant change made to the goods sold under that trademark might lead to the brand's cancellation.

10.3 The manufacturers' and distributors' claims

The combination of a lack of control by the authorities on many aspects of probiotic quality and very lax regulation of branding offers an incredible opportunity for some manufacturers to market their products in a manner that deceives consumers. More often than not, probiotic

manufacturers, when falsely representing their products, are undetected. If the authorities catch them, they get away with a reprimand or a minimal fine. Either way, the details of the story are obfuscated or it becomes difficult to understand the nature and extent of the malfeasance. For this reason, having been one of the parties involved in a court proceeding in the US, we will examine and comment on the legal case of the probiotic VSL#3, hoping that it will shed light on how to reveal and prevent such misconduct.

In the US, the VSL#3 sellers were found liable for false advertising and ordered to disgorge USD 15 million. The Prof. De Simone Formulation established itself over the years as "the standard of excellence" in its therapeutic category. More than 70 human clinical trials were conducted, whose excellent results have been published in official medical and scientific journals. These trials confirmed that the De Simone Formulation is effective, among others, in the dietary treatment of ulcerative colitis and pouchitis, both severe chronic diseases (Bibiloni et al., 2005; Gionchetti et al., 2003, 2007; Huynh et al., 2009; Karimi, Peña, & Van Bodegraven, 2005; Kühbacher et al., 2006; Lee et al., 2012; Miele et al., 2009; Mimura et al., 2004; Ng et al., 2010; Pronio et al., 2008; Sood et al., 2009; Tursi et al., 2004, 2010; Ulisse et al., 2001). Regarding pouchitis, the De Simone Formulation is recognized by gastroenterology professional organizations as "the standard treatment," an achievement never earned by any other probiotic product. This formulation, produced by Danisco DuPont in the USA, had been marketed under the brand name VSL#3 (property of the Actial/VSL Group) from 2002 until May 2016. As detailed in the lawsuit, beginning in approximately mid-2013, Actial/VSL proposed to reduce the cost of manufacturing VSL#3 by changing the composition of the product to increase profits. They argued that since VSL#3 was not sold as a drug, a change in composition would go "unnoticed" as long as no one mentioned it. Prof. De Simone, the inventor of the product and sole owner of the know-how to produce the De Simone Formulation, rejected the request. It would have represented a violation of the trust that consumers had in VSL#3 and could have caused adverse consequences for their health. In light of Actial/VSL's mounting pressure, Prof De Simone terminated the "Know-How Agreement" under which he had given VSL Inc. the right to sell VSL#3 using the De Simone Formulation under the brand VSL#3. The above events were private affairs of which the public had no knowledge.

In May 2016, one of the U.S. distributors of VSL#3, Leadiant, publicly announced that the production of VSL#3 would be moved from the Danisco plant in the United States to a new facility in Italy (Centro Sperimentale del Latte; "CSL"). The distributor did not mention that with this change in manufacturer, it would change the VSL#3 formula from the De Simone Formulation to a new product never tested in humans. According to Leadiant's commercial statements regarding the transfer of VSL#3 production to a new facility located in Italy, the new product would be "the same quality product, containing the same genus and species of bacteria, in the same proportions that you have come to expect" and "How will this impact you and your patients? It won't. VSL#3, your first choice probiotic to manage Ulcerative Colitis, IBS and ileal pouch…" Consumers trusted the product called "VSL#3," a name that at the time denoted an effective and clinically proven product, namely the De Simone Formulation. By replacing the De Simone Formulation with a different, untested product and continuing to use the name "VSL#3," consumers believed that VSL#3 was the same product as before. There was a steady stream of communications directed at consumers and physicians designed to create the false impression that VSL#3 was the same product as before. At the end of August 2016, another distributor of VSL#3, Alfasigma, announced in a press release the following: "Legacy brand VSL#3 (www.vsl3.com), distributed in the United States

by Sigma-Tau Healthscience USA, Inc. under agreement with VSL Pharmaceuticals, has moved the manufacture of its brand, the most studied and recommended high-potency probiotic medical food, back to Italy, where it was originally developed and produced. The move includes the elimination of any traces of dairy in the manufacturing process, making it the only probiotic medical food available that is dairy-free. People who suffer from IBS, ulcerative colitis, or an ileal pouch, and who are also among the 30 to 50 million people in the United States who have allergies to milk or are lactose intolerant, can now take VSL#3 to manage their IBS, UC or ileal pouch." The August 2016 Press Release was an orchestrated campaign leading consumers and doctors to believe that this new Italy-made product was the same as the De Simone Formulation, concealing the fact that it was no longer available under the VSL#3 brand. It attempted to equate the effectiveness of the Italy-made VSL#3 with that of the De Simone Formulation. Alfasigma's deceptive advertising campaign included numerous false claims on Facebook as well. For example, on March 19, 2017, a Facebook user asked Alfasigma, "When did you reformulate VSL#3? Thanks!" on the VSL#3 Facebook page. In response, Alfasigma posted, publicly the following: VSL#3 Hi Timmy- VSL#3 contains the same 8 diverse strains and high potency that have effectively managed the symptoms of IBS, UC, and an ileal pouch for 15 years. By upgrading the manufacturing process, we are also happy to share that that VSL#3 is dairy-free, making it one of the few dairy-free probiotics available to patients. Now 30 to 50 million people who have allergies to milk or are lactose intolerant and who suffer from IBS, ulcerative colitis, or an ileal pouch will be able to take VSL#3 to help manage their symptoms. To further improve VSL#3, a small amount of cornstarch, an inactive ingredient that reduces moisture and preserves bacterial potency and stability, was added. VSL#3 unflavored packets have always contained cornstarch. Now we have added it to the capsules and DS. The inclusion of cornstarch does not affect the efficacy, potency, composition, and strain components of the product. Hope this info helps!

10.4 The scientific evidence

The Authorities do not consider all the issues related to the change of production and the know-how related to the safety and efficacy characteristics of the finished product. Authorities usually require a redeposit of the new label with the net weight of the product, the list of contained microbial strains, the number of CFU, the name of the producer, and the distributor. The consumer cannot rely on the correspondence between the label and the product sold under that label. Any decision by the USPTO regarding the trademark is contingent upon demonstrating that the new product that replaced the old one under the same brand name is different. The manufacturer generally claims that the new product is equal to or better than the old one since the strains have not been changed or just "modernized" from a taxonomic perspective. All the properties and virtues of the previous product are attributed to the new product using genetic testing as evidence. A plethora of molecular methods such as DNA-DNA hybridization technique, amplified ribosomal DNA restriction analysis, pulsefield gel electrophoresis (PFGE), and amplified fragment length polymorphism (AFLP) have been developed over time (Donelli, Vuotto, & Mastromarino, 2013). These methods permit to perform species and strain-specific typing of probiotic microorganisms. Still, they are not sufficient for assessing the stability of probiotic productions over time and the equivalence

of probiotic organisms and products. The assumption "If the strains are identical, then the products are identical" is just wrong and lends itself to misunderstandings and even cheating. Presently, the results of the genetic tests are profoundly influenced by the technique used and the bona fides of who materially performs the analysis. The uncertainty still affecting the use of genetic approaches in determining similarity among probiotic products is well represented by two recent publications providing the characterization of the VSL#3 formulation commercialized after 2016 (Douillard et al., 2018; Mora et al., 2019). Mora et al. in 2019 performed the genomic analysis on a sachet of VSL#3 and determined the presence of two different Bifidobacterium animalis subspecies lactis strains. They distinguished the two Bifidobacterium strains according to the presence or absence of a 54 bp deletion within the gene codifying for long-chain acyl-CoA synthetases. This enzyme is involved in cell membrane lipid production and renders B. animalis subsp. lactis more resistant towards oxidative stress (Oberg, Ward, Steele, & Broadbent, 2013). However, in 2018, the same authors examined eight strains provided individually by the manufacturer of the VSL#3. They reported the results in the publication "Comparative genomic analysis of the multi-species probiotic-marketed product VSL#3." This publication is the sole publicly available and most reliable source of the genomic sequences relative to the individual bacterial strains in the VSL#3 products. No one of the tested B. animalis subsp. lactis strains had the 54 bp deletion within the long-chain acyl-CoA synthetases gene (Douillard et al., 2018). The lack of discriminatory markers between these two B. animalis subsp. lactis strains is the unquestionable evidence that the VSL#3 products contain seven strains instead of eight as declared by the manufacturer. However, in 2019, the VSL#3 become an eight-strain product, according to the data generated by the same investigators. How is it possible that the genetic analysis of strains isolated from the bacterial mix (with all the related technical implications) is more accurate than that based on the same strains provided individually to the investigators by the manufacturer? A good workflow for the analysis of probiotics should rely on the phenotypic and genotypic characterization of contained strains followed by in vivo assays performed on animal models and clinical trials on adequate subjects. Phenotypic characterization of probiotic strains utilizes data supplied by all the typing methods, not based on DNA and RNA (Donelli et al., 2013). Although these methods are often laborious and time-consuming, they permit obtaining a broad spectrum of data such as a preliminary taxonomic description up to family and genus levels and enumeration of viable and dead cells. A significant corpus of data obtained by phenotypic characterization concerns the morphological and metabolic characteristics of microbial strains. This approach provides valuable information for discriminatory purposes and even physiological and biochemical features such as growth temperature, pH value, enzymatic activity, and compound metabolism (Nomura, Kimoto, Someya, & Suzuki, 1999; Yang, Cao, Cai, & Terada, 2010). The usefulness of the phenotypic characterization in determining the authenticity of probiotics and their variability played a pivotal role in underlining the modifications that the VSL#3 product underwent regarding the original De Simone formulation. Cinque et al. in 2016, through the use of specific stain and microscopy imaging, determined that the VSL#3 formulation produced in Italy presented significantly higher and lower proportions of the dead (postbiotic) and the live (prebiotic) bacteria, respectively than the original de Simone formulation. In the same paper, the authors carried out experiments assessing the two probiotic blends on the cell cycle and apoptosis. The original De Simone Formulation had a significantly higher capability to arrest the cancerous cells in G0/G1 and

induce the apoptotic death of cancer cells (Cinque et al., 2017). Biagioli et al. reported dissimilarities, significantly impacting patient safety between the VSL#3 products containing the original De Simone formulation and the VSL#3 manufactured after 2016 in Italy, using a murine model (Biagioli et al., 2017). Trinchieri et al. have provided further pieces of evidence about the difference between VSL#3 products manufactured at different sites in 2017 by comparing, in vitro, the production of metabolites and their effects on a rat intestinal epithelial cell line. They evidenced significant differences in the production and metabolism of 1,3 dihydroxyacetone (DHA) (Trinchieri et al., 2017). In this context, a comparative proteomic study performed by Razafindralambo et al. evidenced differences in protein abundances, identities, and origins of two series of VSL#3 samples manufactured in USA and in Italy (Razafindralambo, Correani, Fiorucci, & Mattei, 2020). Although genomics represents a powerful tool for microbial characterization, when taken alone, it does not represent an adequate method to properly assess functional equivalence between strains produced at different sites by different methods and can even be misleading. Information obtained by genomics should be validated and integrated with that provided by other functional platforms, for example, omics, to assess the products' real nature. Proteomics studies have succeeded in identifying proteins involved in the interaction of probiotics with the environment, such as their behavior in the nutrient matrix and ability to survive in the host gut, and detect changes in proteins that affect the efficacy of probiotics. For example, by combining functional proteomics and metabolomics analyses, Bianchi et al. distinguished not only the De Simone Formulation from the VSL#3 manufactured after 2016 but also the differences between various lots of VSL#3 produced by the same Italian manufacturer in the years (Bianchi et al., 2020).

10.5 Conclusions

The minimum requirements needed to claim the probiotic status of microorganisms include (i) assessment of identity down to the strain level, (ii) the evaluation of safety, (iii) the determination of probiotic potential through both in vitro and in vivo studies, (iv) its viability to guarantee efficacious dose of live bacteria throughout shelf life (Binda et al., 2020). Unlike the rigorous evaluation of safety and efficacy that drugs have to pass to assess their equivalence to previous original products, probiotics are subjected to less stringent regulatory rules. The rules are often not adequate to keep track of changes in probiotic formulations and to assure their effectiveness after the introduction of such changes. Scientifically, it should be mandatory that modifications of probiotic products will be followed by further evidence assessing the safety and efficacy of newly used formulations. This point assumes a particular relevance when the product is employed in managing illnesses in vulnerable subjects. In fact, despite the long history of safety reported for most probiotic microorganisms worldwide, their use may cause systemic infections, excessive immune stimulation in susceptible individuals, deleterious metabolic effects, or be responsible for the spreading of genes associated with harmful effects. Probiotics should be carefully evaluated for their infectivity, pathogenicity, and their ability to induce excessive immune stimulation in susceptible individuals (Zawistowska-Rojek & Tyski, 2018). One of the most confusing aspects of the probiotic market concerns the difficulties in monitoring the products over time to ensure the correspondence with the characteristics determined by scientific studies and clinical trials. Probiotics proven to be effective in treating

specific diseases or conditions are composed of ingredients, often not identified by a unique standard name but rather associated solely with a trademark recognized by physicians, experts, and patients as safe and effective. Although the brand should "confirm" the safety and efficacy for probiotics as long as the product remains unaltered, the lack of regulations, together with the strong legal protection of the rights associated with trademark ownership, make it possible to mislead consumers. Manufacturing processes may profoundly influence the properties showed by probiotics. Industrial production is characterized by several parameters, such as growth conditions, culture media, the use of cryoprotectants, and storage conditions, each potentially affecting the properties of probiotic microorganisms (Jankovic, Sybesma, Phothirath, Ananta, & Mercenier, 2010; Zacarías et al., 2017). The microbial strains may acquire different characteristics during the industrial fermentation process, mainly linked to differences in the substrates used for final commercial production and genetic changes accumulating within their genomes during extensive cultivation. After several generations, a probiotic microorganism may undergo relevant genomic reshapings, seriously affecting its genomic stability, and its probiotics properties (Fusco, Fanelli, & Chieffi, 2021). The European Society has underlined the importance of considering the variability introduced by the producing processes in probiotic products for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) that recently published a position statement calling for better quality control of probiotics. They note that "procedures such as fermentation, matrix composition, cell harvesting, spray-drying, freeze-drying and storage conditions like temperature, humidity and pH, are just several out of a wider array of manufacturing determinants that can affect microbial survival, growth, viability and ultimately the study results and/or clinical outcomes." The FDA further notes: "Because, in many cases, there is limited ability to identify the identity of the clinically active component(s) of a complex biological product, such products are often defined by their manufacturing processes. Changes in the manufacturing process, equipment, or facilities could result in changes in the biological product itself and sometimes require additional clinical studies to demonstrate the product's safety, identity, purity, and potency …". If the probiotic products are manufactured differently, they will behave differently, and studies conducted with one product are not transferable to other products, regardless of genetics. All scientific claims must refer to a specific strain/strain combination, a specific dose in a relevant product, and a specific population. Different strains produced using different methods and reagents will have other effects. Any reformulation will require a thorough reassessment to ensure that the documented properties of the original formulation are retained. We have examined a complicated judicial affair (De Simone v. VSL Pharmaceuticals, Inc. et al., Case Number:8:15-cv-01356, Federal Court of Maryland, US) to confirm that rules and regulations governing the safety of probiotics are circumventable. According to an ancient proverb, "made the law, found the trick". Sometimes, however, there are exceptions. On November 20, 2018, a jury unanimously found the US distributors Leadiant and Alfasigma liable for committing false advertising in violation of the Lanham Act. It awarded to the company owned by Prof. De Simone, ExeGi Pharma LLC, damages of $15 million in consideration for such false advertising. On November 21, 2018, the Court entered a final judgment based on this verdict, reconfirmed in the Court of Appeals on February 17, 2020. On July 23, 2019, some consumers who purchased the VSL#3 beginning in June 2016 brought a Class Action against the VSL#3 distributors (Alfasigma USA Inc., VSL Pharmaceuticals Inc., and Leadiant Biosciences Inc). The lawsuit argues that the new formulation was "fundamentally different" from the previous version, and that "not even a single

clinical study" had been performed to support the company's claims of the new product's efficacy and safety. The Class Action was filed with the aggravating circumstance of the civil "RICO Act" (Racketeer Influenced and Corrupt Organizations Act), a federal law of the United States designed to combat organized crime. On December 20, 2020, the Maryland Federal Court rejected the attempt to dismiss the RICO claims filed by Alfasigma USA Inc., VSL Pharmaceuticals Inc., and Leadiant Biosciences Inc. (https://www.classaction.org/news/class-action-accuses-pharma-companies-of-secretly-reformulating-vsl3-probiotics-product).

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CHAPTER

11

Probiotics in pregnancy and lactation

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11.1 Introduction

Maternal health is of paramount importance for the well-being of the growing fetus (Dhillon, Singh, & Kaur, 2020). The first 1000 days of life represent a critical window for health interventions. Different maternal intervention strategies such as healthy nutrition, exercise, dietary supplements, or probiotic consumption can be used in order to prevent the negative effects of programming during the first 1000 days, which is from conception to 2 years after birth. Effects of maternal probiotic consumption can be observed from the period prior to pregnancy to the end of lactation and can influence a wide range of important maternal and infant outcomes (Rodriguez-Gonzalez, Castro-Rodriguez, & Zambrano, 2018). The effects can be observed in many physiological functions of the mother and the infant, such as vaginal, gut and breast health due to microbial balance, metabolic status, immunological balance, and mental health (Fig. 11.1).

Usually, the key steps in probiotic supplement development are placebo-controlled clinical studies. Several recent meta-analyses were published using the data from clinical studies of probiotics during pregnancy and lactation (Amalia, Orchard, Francis, & King, 2020; Jarde et al., 2018; Kuang & Jiang, 2020; Li et al., 2019a; Li et al., 2019b; McFarland, Evans, & Goldstein, 2018; Pérez-Castillo et al., 2021; Sun, Chang, & Zhang, 2021). However, the efficacy of probiotics is both strain-specific and disease-specific (McFarland et al., 2018), and it is reasonable to expect that such studies will not observe any relevant statistical significance. The power of meta-analysis of probiotics on strain level is still low since there are not enough comparable clinical studies using the same probiotic strain (Amalia et al., 2020; Pérez-Castillo et al., 2021). Moreover, many clinical studies do not report the probiotic strains administered (Pérez-Castillo et al., 2021).

Many lactobacilli have been proven to have probiotic features and it is necessary to mention at this point that several representatives of lactobacilli belonging traditionally to the genus *Lactobacillus* (*L.*), has been recently assigned to 23 new genera (Zheng et al., 2020). For

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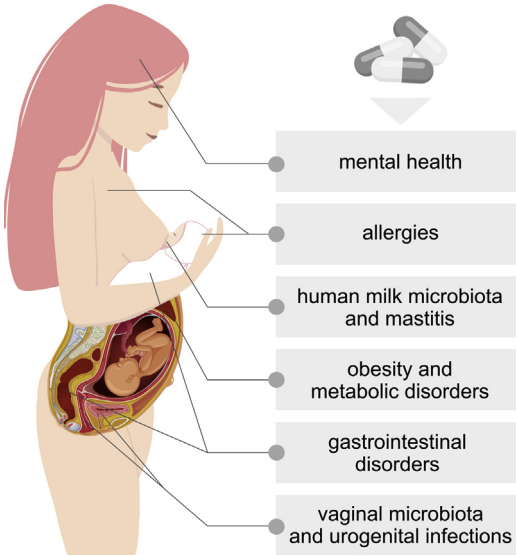


FIG. 11.1 Effects of probiotics during pregnancy and lactation on maternal and infant health. Effects of maternal probiotic consumption can be observed from the period prior pregnancy to the end of lactation and can influence a wide range of important maternal and infant outcomes such as gastrointestinal disorders, mastitis, urogenital infections, metabolic disorders, infantile allergies, and maternal mental health.

example, in accordance with the new taxonomic classification, *Lactobacillus rhamnosus* has been renamed into *Lacticaseibacillus rhamnosus (L. rhamnosus)*, *Lactobacillus vaginalis* into *Limosilactobacillus vaginalis (L. vaginalis)*, and *Lactobacillus reuteri* into *Limosilactobacillus reuteri (L. reuteri)*.

The focus of this chapter is probiotic interventions for mothers during pregnancy and lactation. It is known that maternal probiotic supplementation can be beneficial also for infants, although a limited number of studies monitored also potential beneficial effects on infants. Since Chapter 12 deals with probiotics in pediatrics, we discussed only studies where solely mothers were supplemented with probiotics, although in several studies along with mothers, infants were supplemented as well.

11.2 Vaginal microbiota and vaginal infections

The idea of using lactic acid bacteria to treat and prevent vaginal infections is not new; it appeared in the early 1980s, as a consequence of discovering the composition of the vaginal microbiota (Reid, 2017). Prophylactic and therapeutic potential of possible probiotics for vaginal health has been assessed in several preclinical and clinical studies, but by far the most commonly on healthy or on non-pregnant women affected with infections of vagina with bacteria or viruses (human papilloma virus [HPV], human immunodeficiency virus [HIV]). Although well-documented and reviewed by several authors, most of them are preliminar or pilot studies, not appropriately controlled, multicenter studies with solid research design and a large number of participants. It is well known that vaginal infections are associated with dysbiosis of microbiota in this specific ecological niche. In pregnancy, dysbiosis is considered an important risk factor for mother and a newborn as it can lead to several complications, such as infections, preterm birth, allergies, necrotizing enterocolitis, infantile colic,

cardiometabolic, and even autism spectrum disorders. Therefore, balanced vaginal microbiota and a healthy maternal vagina are crucial for the well-being of the infant, in the prenatal period and after delivery.

It has long been known that the human vaginal microbiota of healthy women, both non-pregnant and pregnant is dominated by lactobacilli. This observation has been confirmed in recent studies employing the next-generation sequencing (NGS) approach (Aagaard et al., 2012; Feehily et al., 2020; MacIntyre et al., 2015; Romero et al., 2014; Sroka-Oleksiak et al., 2020). Moreover, in pregnancy, the bacterial load and abundance of lactobacilli in relation to other representatives are increased. This was explained by increased estrogen levels, which promote glycogen deposition by vaginal epithelial cells and increased proliferation of vaginal microbiota, especially lactobacilli (Aldunate et al., 2015; Amabebe & Anumba, 2018; Donders, 2007; Griffin, Harding, & Sutton, 2015; Witkin, 2015). As a consequence, the vaginal microbiota of pregnant women is even more stable (MacIntyre et al., 2015; Romero et al., 2014). There is, however, less consistency among different studies in the abundance of different species of lactobacilli in the vaginal microbiome.

The vaginal microbiota was classified into five types (community state type [CST]) on the basis of similarities in bacterial composition (Gajer et al., 2012). Types CST I, CST II and CST III are characterized by domination of *L. crispatus, L. gasseri* and *L. iners*, respectively, while the remaining two types are not dominated by lactobacilli. In healthy pregnant women, CSTI and CST III types characterized by *L. crispatus* and *L. iners* were most common in the study of Romero et al. (Romero et al., 2014). Furthermore, four phylotypes (*L. vaginalis, L. jensenii, L. crispatus* and *L. gasseri*) had higher relative abundance in pregnant than in non-pregnant women. In the study of Dobrut et al. (2018) *L. crispatus* was dominant species in all three trimesters, followed by *L. gasseri*. The third best represented species was *L. rhamnosus* during first two trimesters, and *L. johnsonii* in the third trimester. Similarly, Aagaard et al. (2012) reported that overall diversity and richness were reduced in pregnancy, with dominance of lactobacilli species *L. iners, crispatus, jensenii*, and *johnsonii*. Feehily et al. (2020) used shotgun metagenomics to determine the vaginal communities in women at high risk of preterm labor (PTL). *L. crispatus, L. delbrueckii and L. gasseri* were the most abundant in no-risk group. Kumar et al. (2021) identified 19 species of lactobacilli, while *L. crispatus, L. iners, L. gasseri, L. fornicalis*, and *L. delbrueckii* were most represented.

Based on the differences among the samples of term and preterm delivering women, the abundance of *L. iners* was higher in the samples from PTL women in all three trimesters. On the contrary, only a small percentage of *L. crispatus* (from 0.53% to 0.58%) was identified in another study of the vaginal microbiota of healthy pregnant women (Sroka-Oleksiak et al., 2020), while *L. iners, L. gasseri*, and *L. plantarum* predominated. Representatives of other typical species (*L. acidophilus, L. jensenii* and *L. helveticus*) occurred in smaller amounts. In a systematic review, Van De Wijgert et al. (2014) observed that, in the majority of studies, *L. crispatus* (11 articles), or *L. iners* (15 articles) species were dominant, while other species are less common. These studies have shown that lactobacilli-dominated vaginal microbiota is associated with a healthy vaginal micro-environment and that bacterial vaginosis (BV) is associated with dysbiosis. The extent of dysbiosis correlates well with Nugent score and vaginal pH but not with the other Amsel criteria. Aagaard et al. (2012) confirmed the association of a vaginal microbiome dominated by *L. crispatus* with full-term pregnancies.

There are two ways of delivery of probiotic bacteria intended for maintenance or restoration of vaginal health, by oral consumption or by vaginal application. When probiotics are consumed in a form of capsules, tablets or powders, they are supposed to migrate from rectum to vagina, where they should at least temporarily colonize the vaginal epithelium (Nader-Macías, Tomás, & M., 2015). Another possibility is direct delivery of probiotics into vagina using dedicated formulations, such as tablets, creams, gels, suppositories, and rings (Tachedjian, Aldunate, Bradshaw, & Cone, 2017). There is increasing evidence that orally ingested lactobacilli reach and colonize vagina. Recent studies on pregnant women, however, are not very promising regarding colonization ability of probiotics since significant changes of vaginal microbiome composition or diversity most often have not been observed (Gille et al., 2016; Husain et al., 2020; Yang et al., 2020). This may be due to the choice of strains in these clinical studies, i.e. *L. rhamnosus* GR-1 and *L. reuteri* RC-14, which belong to two species not typically found among the prevailing species in the microbiota of healthy pregnant women. Indeed, Yefet et al. (Yefet, Colodner, Strauss, Zeletova, & Nachum, 2020) reported that the colonization ability of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 during two months of oral consumption was much lower than it was observed in non-pregnant woman in previous studies, after vaginal (Gardiner, Heinemann, Bruce, Beuerman, & Reid, 2002) or oral application (Reid et al., 2003). There have been several strains tested *in vitro*, and new strains with better *in vitro* performance from currently used probiotics for vaginal health, are still being identified (Happel et al., 2020). However, only few clinical studies on pregnant or non-pregnant women have been performed, predominantly with a combination of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 (Table 11.1). Combination of these two strains is often chosen because most probiotic product for vaginal health available on the market contains them (Anukam et al., 2006; Gille et al., 2016; Martinez et al., 2009). The treatment of pregnant women with particular probiotic strains, applied orally or directly into vagina, showed certain beneficial role on the prevention and/or treatment of bacterial vaginosis and related outcomes, such as PTL, Group B *Streptococcus* (GBS) colonization and premature rupture of membrane (PPROM).

11.3 Bacterial vaginosis in pregnancy

Bacterial vaginosis (BV), or vaginal dysbiosis, is a common vaginal condition associated with changes in the vaginal microbiome composition. BV is characterized by a decrease of *Lactobacillus* load and increase of the load and diversity of other facultative anaerobic and anaerobic bacteria (Van De Wijgert et al., 2014). BV is often associated with vaginal inflammation. The main negative effects of BV comprise the increased risk of acquiring sexually transmitted infections like HIV and, in pregnant women, increased risk for urogenital infections and abortion chorioamnionitis and adverse pregnancy outcomes, such as PROM (Redelinghuys, Geldenhuys, Jung, & Kock, 2020).

The traditional methods for the diagnosis of BV are the assessment of clinical Amsel's criteria (Amsel et al., 1983) and the Nugent Score for Gram stain of vaginal swabs (Nugent, Krohn, & Hillier, 1991). Based on Nugent's score, the vaginal microbiota can be described as normal, intermediate (4–6), or indicating BV-indicating (7–10) microbiota. While the normal microbiota is represented by high proportion of lactobacilli morphotypes, the microbiota

TABLE 11.1 Selected randomized, placebo-controlled studies of effects of probiotics administration during pregnancy on the vaginal microbiota and bacterial vaginosis (BV) and on the adverse pregnancy outcomes (preterm labor) and early-onset Group B *Streptococcus* (GBS) disease[a].

References	Country	Probiotic strains	No. of Participants (randomized) - population characteristics	Treatment regime during pregnancy and lactation; main outcome measure	Main outcomes and results
Sharpe et al. (2021)	Canada	*L. rhamnosus* GR-1 and *L. reuteri* RC-14	139 pregnant women, at 23-25 wk of gestation	Two capsules daily for 12 wk; Group B *Streptococcus* (GBS) vaginal/rectal colonization (swab for GBS at the end of intervention only)	The probiotic consumption did not reduce the rate of GBS colonization.
Ho et al. (2016)	Taiwan	*L. rhamnosus* GR-1 and *L. reuteri* RC-14	99 GBS positive pregnant women, at 35-37 wk of gestation	Two capsules daily, until delivery, GBS colonization rate before and after intervention	Oral probiotic reduced the vaginal and rectal GBS colonization rate in pregnant GBS positive women
Farr et al. (2020)	Austria	*L. jensenii* Lbv116, *L. crispatus* Lbv88, *L. rhamnosus* Lbv96 *L. gasseri* Lbv150	82 GBS positive pregnant women, at 32-36 wk of gestation	Twice daily, for 14 days, colonization with GBS after treatment, neonatal sepsis, gestational age at delivery, neonatal birth weight, and mode of delivery	Oral probiotics did not significantly reduce GBS during pregnancy, positive trend was observed
Gille et al. (2016)	Germany	*L. rhamnosus* GR-1 and *L. reuteri* RC-14	320 pregnant women, >18 yr, less than 12 completed wk of pregnancy	1 capsule daily for 8 wk; vaginal swabs pre-intervention and shortly after intervention	Proportion of normal vaginal microbiota (Nugent score) not increased in probiotic or placebo group
Yang et al. (2020)	Canada	*L. rhamnosus* GR-1 and *L. reuteri* RC-14	86 asymptomatic pregnant women, Nugent score ≥4 (BV), 14-16 wk of gestation	1 capsule twice daily for 12 wk, pregnancy outcomes, vaginal microbiota, concentrations of cytokines and chemokines	The Nugent score returned to normal (<4) in 30% of the women in both groups; no differences in Interleukin-4 and Interleukin-10 between placebo and probiotic groups; no difference in the microbiota between the placebo and probiotic groups

(*Continued*)

TABLE 11.1 Cont'd

References	Country	Probiotic strains	No. of Participants (randomized) - population characteristics	Treatment regime during pregnancy and lactation; main outcome measure	Main outcomes and results
Husain et al. (2020)	UK	*L. rhamnosus* GR-1 and *L. reuteri* RC-14	304 apparently healthy women aged 16 yr or older; 9-14 week of pregnancy	1 capsule daily from recruitment until the end of pregnancy; vaginal swabs at 18-20 wk and at 34-36 wk	The rates of BV (Nugent score ≥7) or prevalence of pathogens did not differ between groups at 18–20 wk' gestation; no differences in the alpha diversity or composition of the bacterial communities
Daskalakis and Karambelas (2017)	Greece	*L. rhamnosus* (DSM 14870) and *L. gasseri* (DSM 14869)	115 women with PPROM between 24 and 34 wk of gestation	Vaginal probiotic formula in combination with standard antibiotic or only antibiotic, for 10 days; gestational age at birth, duration of the latency period, and birth weight	Vaginal probiotics as an adjunct to antibiotic prophylaxis in women with PROM prolonged the latency period and improved the perinatal outcome
Stojanović, Plećaš, & Plešinac (2012)	Serbia	*L. rhamnosus* BMX 54	60 pregnant women, treated or untreated, between 16 and 22 wk of gestation	vaginal; once a week for 12 wk, vaginal and cervical swabs for analysis of pH, presence of pathogenic microorganisms, quality of vaginal discharge	Vaginal administration of *L. rhamnosus* BMX 54 is effective in preventing the development of abnormal vaginal microbiota

[a] No permission required.

No. of Participants – number of randomized participants; *L.*, *Lactobacillus* (in accordance with the new taxonomic classification. Zheng et al. (2020) also Lacticaseibacillus (L. rhamnosus) and Limosilactobacillus (L. reuteri).

typical for BV contains less lactobacilli-like morphotypes and much more pathogenic morphotypes. More advanced approaches for the assessment of changes in microbiota are available nowadays, such as high-throughput sequencing and qPCR, which enable to monitor slight changes in terms of abundance and composition on the species level. Vaginal icrobiota dominated by *L. iners* most often indicates vaginal dysbiosis (Lennard et al., 2018) and the decreased abundance of *L. reuteri*, *L. crispatus* and *L. jensenii* is often associated with persistent BV and genital inflammation. Nevertheless, the presence and abundance of *L. iners* cannot be considered as a marker for BV, since this species is present also in a healthy vagina (Redelinghuys et al., 2020). Also in pregnant women (68 subjects), lactobacilli were less frequently prevalent in women with BV (Aman, Zaheen, & Tariq, 2019). *L. iners* was commonly present in all groups of women, that is, those with BV, without BV or intermediate. Women harboring *L. gasseri* and *L. iners* were more susceptible to BV from those colonized by *L. crispatus* leading to the conclusion that *L. crispatus* individually or in combination with *L. jensenii*, *L. helveticus* and *L. acidophilus* may have good probiotic potential to combat BV (Aman et al., 2019).

Symptomatic BV is usually treated with antibiotics, such as metronidazole, clindamycin or tinidazole, applied orally or intravaginally. It is well known that antibiotic treatment may lead to recurrence of BV, may lead to the development of antibiotic resistance and may negative influence vaginal lactobacilli population when vaginal formulations are used for delivery of antibiotics, therefore alternative therapies are needed (Gottschick et al., 2017). Probiotics may have a positive effect on the treatment of BV, but there is a lack of strong evidence, not only in pregnant women but also in non-pregnant. Two comprehensive meta-analysis were performed on non-pregnant women, showing significant short-term effect of probiotics in the treatment of vaginal infections (Jeng, Yan, & Chen, 2020) and that probiotics may improve the cure rate of BV but may not be a substitute for traditional antibiotic therapy (Li et al., 2019a). Nevertheless, these studies were performed on non-pregnant women only, the probiotics used were diverse, the application routes were diverse (oral formulations, fermented milk, vaginal formulation), and they do not provide enough evidence for the efficiency of probiotic therapy either on its own or following antibiotic therapy. Moreover, the studies on pregnant women are still too few (Table 11.1).

Gille et al. (2016) evaluated in a randomized, placebo-controlled, triple-blind, parallel group trial the possible positive effects of oral consumption of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 on the maintenance or restoration of a normal vaginal microbiota during pregnancy. The microbiota was evaluated on the basis of Nugent score of vaginal swabs taken before and after intervention, i.e. 8 weeks of probiotic consumption. Contrary to their hypothesis, the proportion of normal microbiota slightly decreased in both groups, but with no significant differences across groups. An important observation is that the rate of BV was low already before intervention started. The authors discussed that the in the future experiments it would be reasonable to prolong the treatment period or to choose the vaginal application instead of oral. Another limitation may be also the way of evaluation, using Gram staining and Nugent score.

Another study on healthy pregnant women, using the same probiotic combination and application route (oral) as in a study of Gille et al. (2016), was carried out in 2019 by Husain et al. (2020). Unlike in the previous study, the probiotics were consumed from early pregnancy until delivery, and in addition to determination of Nugent score, the vaginal

microbiota was assessed also by vaginal community profiling by 16S rDNA NGS. Although the daily dose was 2-fold higher, the duration of intervention longer and the trial was multicenter, the outcomes were similar as in the study of Gille et al. (2016). The oral administration of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 daily from early pregnancy did not affect the prevalence of BV or common neonatal pathogens, or alter the composition of the vaginal microbiota at 18–20 weeks' gestation. No significant differences in alpha diversity or composition of vaginal microbiota between the groups were observed at either 9 to 14 or 18 to 20 weeks' gestation. In addition, the rate of BV was lower than anticipated, which reduces the power of the study.

Yang et al. (2020) recruited only pregnant women with Nugent score ≥4, indicating BV. The choice of the probiotic strains was the same as in the above-mentioned studies of Gille et al. (2016) and Husain et al., (2020) and the duration of oral intake 12 weeks. The microbiota of pregnant women with an intermediate Nugent score (n = 42) and those with a BV Nugent score (n = 24) at the time of study entry were not different between the groups ($p > 0.5$) before the intervention. After the intervention (at 35 weeks), no differences in the microbiota of women consuming probiotics were observed. The authors explained that this observation may have been a result of the subjects ingesting fermented foods during the study. The high prevalence of *L. rhamnosus* in both groups was unexpected as this species is not one of the five lactobacilli (*L. crispatus*, *L. iners*, *L. gasseri*, *L. jensenii* and *L. reuteri* or *L. vaginalis*, depending on the country) detected in most microbiota studies of healthy women.

11.4 Premature rupture of membrane and preterm labor

There is increasing evidence that vaginal microbiome alterations may be associated with premature rupture of membrane (PROM), preterm labor (PTL), and various adverse maternal health outcomes and neonatal outcomes. Preterm infants commonly experience increased adverse newborn outcome risks. Since the transition from normal vaginal microbiome to dysbiosis is associated with the decrease of the abundance and species diversity of lactobacilli, it is believed that probiotics have great potential in restoring the vaginal microbiome and therefore in prevention of these negative outcomes.

The PROM is a common cause for almost one third of preterm deliveries. Infections seem to play a major role in the etiology of this complication. The risk of severe perinatal complications, such as chorioamnionitis, cord compression, abruptio placenta, neonatal sepsis, respiratory distress syndrome, intraventricular bleeding and even neonatal death, is also increased following PROM. Observational studies on pregnant women shows that vaginal infections and/or alteration of vaginal microbiota in the third trimester of pregnancy is often associated with PROM. The standard therapy in the increased risk of PPROM is antibiotic and corticosteroid treatment and planned delivery at 37 weeks of pregnancy (Genovese et al., 2016). Due to negative effects of antibiotic treatment, such as disruption of beneficial vaginal microorganisms and development of antibiotic resistance, the use of probiotics as adjuvant therapy was proposed and already examined in clinical studies.

Recent studies provided some evidence on the benefit of probiotic administration in women with a risk of PROM. In the study of Daskalakis & Karambelas (2017), vaginal probiotic capsules containing *L. rhamnosus* and *L. gasseri*, applied in addition to standard

intravenous/oral antibiotic treatment were given to women with a risk of PPROM diagnosis. The study showed a clear benefit of probiotic administration in women with PROM. Vaginal probiotics in addition to standard antibiotic treatment proved the beneficial effect of probiotics, since probiotic treatment resulted in prolonged latency period, higher gestational age at birth and birth weight, as well as improved perinatal and neonatal outcome. Kavak et al. (2014) investigated in women with PROM the efficacy of probiotic treatment with vaginal capsules containing *L. rhamnosus*, simultaneously with the antibiotic treatment (intravenous and oral) with ampicillin. Antibiotic therapy was given to both groups, and probiotic therapy was applied to test group until delivery. The treatment with *L. rhamnosus* resulted in prolonged latency period, prolonged pregnancy duration, improved APGAR scores and higher birth weights. The study, however, has some limitations, such as low number of participants (40) and retrospective nature, therefore it was not included in the list of studies in Table 11.1.

The effectiveness of the probiotic treatment in reducing the risk of PTL, that is, birth before 37 completed weeks of gestation, and other adverse pregnancy outcomes was addressed in various clinical studies. Jarde et al. (2018) analyzed 21 randomized controlled studies addressing these issues published up to September 2016. Among the participants there were healthy pregnant women (6 studies), Group B *Streptococcus* positive women (1 study), women with gestational diabetes mellitus (4 studies), obese women (1 study), women carrying a fetus at risk of atopic disease or allergies (8 studies), and women with bacterial vaginosis (1 study). The authors concluded that there is no evidence for the reduced risk of PTL before 34 weeks or before 37 weeks or other adverse pregnancy outcomes by probiotic treatment. Although some positive effects were observed by the authors, we should consider low prevalence of PTL and small sample sizes. Nordqvist et al. (2018) carried out a prospective observational cohort study on healthy pregnant women and found out that the consumption of probiotic milk during early pregnancy was significantly associated with lower risk of PTL, while similar was not observed when probiotics were consumed during late pregnancy. Pérez-Castillo et al. (2021) evaluated the studies published from 2001 to 2020 (46) which reported the results on the perinatal outcomes obtained in randomized controlled trials on pregnant woman treated with probiotics (Pérez-Castillo et al., 2021).

The analysis of 11 studies on women without previous pathologies revealed that probiotic consumption during pregnancy did not have significant impact on the incidence of PTL (<37 weeks) cases. Also, when trials in which the combination of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 were administered to participants, were analyzed separately, the effects of probiotics on the PTL were not significant. Similarly, the probiotic treatment during pregnancy was not demonstrated to be associated with any other perinatal outcome evaluated, such as birth weight, cesarean section rate, and weeks of gestation. The authors emphasized that reporting of perinatal outcomes was very heterogeneous across the studies, which makes comparisons among the studies more difficult.

11.5 Colonization with Group B *Streptococcus*

Colonization with Group B *Streptococcus* (GBS) is formed with bacteria that may colonize the vagina of pregnant women. When these bacteria are transferred during the labor to the infant, they have the potential to cause pneumonia, septicemia, and meningitis. GBS

vaginal/rectal colonization in pregnancy has been associated with early-onset GBS disease (EOGBSD), a leading cause of neonatal morbidity and mortality of infants in the first days of life. Intrapartum prophylactic intravenous antibiotics application is a common strategy to combat vaginal GBS colonization and prevent newborn infection in Europe, USA and other counties (Renzo et al., 2015). It is based on a universal intrapartum GBS screening strategy using a rapid real time testing. Due to the drawbacks of intrapartum antibiotic administration, such as promotion of development of antibiotic resistance in microbiota and negative effects on the development of early microbiota in infant, alternative strategies are needed. Jois et al. (Jois, Tan, & Silva, 2020) analyzed four clinical studies dealing with the use of probiotics to reduce the risk of GBS colonization prior to delivery, which were published between 2014 and 2018 (Aziz et al., 2018; Hanson, Vandevusse, Duster, Warrack, & Safdar, 2014; Ho et al., 2016; Jois et al., 2020; Olsen, Williamson, Traynor, & Georgiou, 2018). The authors concluded that there is still limited evidence on the efficiency of probiotics to minimize the risk of GBS colonization, therefore this approach may not be recommended for the regular use yet.

The study of Ho et al. (2016) is one of the first placebo-controlled *in vivo* studies of the effects of probiotics in GBS positive pregnant women. *L. rhamnosus* GR-1 and *L. reuteri* RC-14 were orally applied to pregnant women who were found positive by screening for GBS colonization at 35 to 37 weeks and till the delivery. All the GBS positive participants at the time of delivery received also intrapartum antibiotic prophylaxis. On the basis of significant difference between the probiotic and placebo group, the authors concluded that oral probiotics reduced GBS colonization in pregnant women. In a study of Sharpe et al., 2021, the same probiotic strains or placebo were consumed orally by pregnant women from 23 to 25 weeks until 35 to 37 weeks of gestation, when the rate of GBS vaginal/rectal colonization was assessed according to the common screening for GBS colonization. The rates of vaginal/rectal GBS colonization did not differ significantly between groups. The authors commented that the study should have been more efficient if only GBS-positive participants had been involved. In the study of Farr et al. (2020), only GBS-positive women were randomized and assigned to probiotic or placebo group. Probiotic supplement consumed orally for two weeks, contained four lactobacilli strains belonging to species *L. jensenii*, *L. crispatus*, *L. rhamnosus* and *L. gasseri*. The analysis of the vaginal swabs taken 3 to 4 weeks after the initial visit, did not reveal any significant difference among probiotic and placebo group in the proportion of participants who were still GBS-positive. Some other studies demonstrated potential of probiotics to impact vaginal GBS in pregnancy (Hanson et al., 2014; Martin et al., 2019; Olsen et al., 2018) are not included in the Table 11.1 and discussed here due to limitations such as low number of subjects, too short length of intervention or absence of placebo control.

Several authors of original papers and reviews discussed the limitation of the studies published such as small sample size, only one hospital or other institution involved, too short term of observation, no data on neonatal outcomes available. Another common weakness, especially in the analyses of microbiota, is too few samplings since the microbiota is subjected to changes over time. Moreover, cohort studies lack appropriate control therefore confounding factors may have significant influence on the results. In conclusion, bigger, standardized, controlled and multi center studies are needed to obtain conclusive evidence on the efficiency of probiotics in pregnant women.

11.6 Allergies

Allergy is a very heterogeneous disease that may be manifested over a wide spectrum of disorders, such as asthma, atopic dermatitis, atopic rhinitis, food allergy and eosinophilic esophagitis. These anomalous immune responses may be initiated or influenced by an array of factors, such as exposure to air pollution, inflammation, infection, alterations in the specific microbiome, and inherent genetic predisposition (Krempski, Dant, & Nadeau, 2020). The hallmark of allergy is the imbalance in T cells type (T helper-1: Th1 and T helper-2: Th2). In healthy newborns, the immune response is skewed toward Th2, and gradually shifts to Th1 biasness in the early years. In the case of atopic children (IgE mediated allergy), the immune response stays biased toward Th2 while Th1 type immune response is insufficient (Royer, Rudolph, & Barrett, 2013).

The development of the immune system starts already in pregnancy where various factors influence the development of neonatal immunity. Probably, the most important drivers of appropriate immune development are proper epithelial barrier activity, microbial exposure and microbiome establishment. Several theories tried to provide explanations for the increased incidence of allergies in recent years, such as the "germless theory" and the "dual allergen exposure hypothesis". The "germless theory" suggests that limited exposure to viral or bacterial pathogens in childhood leads to Th2-skewed immune response (Amalia et al., 2020; Li et al., 2019b; Sun et al., 2021), which may result in the development of allergic diseases (Saliganti, Kapila, Kapila, & Bhat, 2017). The "dual allergen exposure hypothesis" suggests that the decision whether the immune system is primed to develop allergy or tolerance depends on whether the first exposure to food allergens takes place through the skin or gut during the first year of life (Pierau, Arra, & Brunner-Weinzierl, 2021). It should be pointed out that microbiome seems to play an important role in both theories, since the development of infants´ microbiota is influenced heavily by environmental factors (Jungles, Jungles, Greenfield, & Mahdavinia, 2021) and plays a major role in the adequate development of the newborn (Mesa et al., 2020). The microbial colonization of the new-born is in close relation with the bacteria it has come into contact, and at the same time the improper functioning of the epithelial barrier is often the result or cause of microbial dysbiosis on particular epithelia surface (Zhu, Zhu, Tran, Sivamani, & Shi, 2018).

No effective treatment for allergies has been found and the allergen avoidance seems to be the only solution which may not always be possible. However, many primary prevention strategies are being studied such as emollients, early allergen exposure, breastfeeding, microbial exposures, vitamin D and UV light, water hardness, immunotherapy, and probiotics (Bawany, Beck, & Järvinen, 2020; Royal & Gray, 2020). Probiotic interventions in prenatal and lactation period may be a possible preventive approach. Indeed, large-scale observational, retrospective cohort study from Norway assessed the protective role of probiotic milk products against allergic diseases in 40,614 children. Consumption of probiotic milk in pregnancy was associated with a slightly reduced relative risk of atopic eczema at 6 months and of rhinoconjunctivitis between 18 and 36 months. Notably, the consumption of probiotic milk products by both pregnant women (during the gestation period) and infants after 6 months of age led to a significant decrease in the incidence of rhino-conjunctivitis (Bertelsen et al., 2014).

In addition, several controlled clinical studies were performed in order to evaluate probiotics for prevention of infantile allergies (Table 11.2). Amalia et al. (2020) performed

TABLE 11.2 Selected placebo-controlled studies of possible benefits of administration of probiotics to the mother during the pregnancy and lactation for prevention of allergies

References	Country	Probiotic strains	No. of participants (randomized) - population characteristics	Treatment regime during pregnancy and lactation; main outcome measure	Main outcomes and results
Kalliomäki et al. (2001)	Finland	*L. rhamnosus* GG	159 - family history of atopic disease	From 36 to 38 wk and 6 mo during breastfeeding or if no breastfeeding, to children; atopic disease during 2 yrs	*Lactobacillus GG* was effective in prevention of early atopic disease in children at high risk
Rautava, Kalliomäki, & Isolauri (2002)	Finland	*L. rhamnosus* GG	62 - from atopic families	From 36 wk and during breastfeeding; immunoprotective effect (serum IgE in blood, TGF-β1 and TGF-β2 in milk) and occurrence of allergies during 2 yrs	Increased the immunoprotective potential of breast milk (increased TGF-β2); significantly reduced risk of developing atopic eczema during the first 2 yrs
Huurre, Laitinen, Rautava, Korkeamäki, & Isolauri (2008)	Finland	*L. rhamnosus* strain GG and *B. lactis* Bb12	171 - from allergic families who received dietary counseling	From 14 wk to the end of exclusive breastfeeding; occurrence of allergies (atopic sensitization) during 1 yr	Protective effect against sensitization in infants with a high hereditary risk due to maternal sensitization, partly by resulting in a beneficial composition of the breast milk
Prescott et al. (2008)	New Zealand	*L. rhamnosus* HN001 or *B. lactis* HN019	69 - participant or partner with allergic disease	From 35 to 38 wk to 6 mo; cord blood plasma and breast milk were assayed for cytokines (IL-13, IFN-γ, IL-6, TNF-α, IL-10, TGF-β1, total IgA in breast milk) and sCD14	Neonates of mothers in probiotic group had higher CB IFN-γ levels, plasma CD14 and a higher proportion had detectable blood IFN-γ levels, breast milk IgA, which was more readily detected, TGF-β1 levels were higher in early breast milk (week 1) from the probiotic groups
Kim et al. (2010)	Korea	*B. bifidum* BGN4, *B. lactis* AD011 and *L. acidophilus* AD031	112 - a family history of allergic diseases	From 32 wk to 3 mo after delivery; the occurrence of eczema during 1 yr	The probiotic group had significantly lower prevalence of eczema and the cumulative incidence of eczema was reduced; no difference in serum total IgE or the sensitization against food allergens
Boyle et al. (2011)	Australia	*L. rhamnosus* GG	250 - participant, partner or child with allergic disease	From 36 wk to delivery; cumulative incidence of eczema during the first yr	Prenatal probiotic treatment was not associated with reduced risk of eczema

References	Country	Probiotic strains	No. of participants (randomized) - population characteristics	Treatment regime during pregnancy and lactation; main outcome measure	Main outcomes and results
Rautava, Kainonen, Salminen, & Isolauri (2012)	Finland	*L. rhamnosus* LPR and *B. longum* BL999 or *L. paracasei* ST11 and *B. longum* BL999	241 - allergic disease and atopic sensitization	From 32 wk to 2 mo after delivery; incidence of eczema during 2 yrs	Both probiotics reduced incidence of eczema and no adverse effects were observed
Ou et al. (2012)	Taiwan	*L. rhamnosus* GG	191 - with any allergic diseases	From 24 wk gestation and after delivery also to non-breastfeeding neonates for 6 mo; sensitization and developing of allergic diseases during 3 yrs	Reduced severity of maternal allergic disease through increment of Th1 response, but not the incidence of childhood allergic sensitization or allergic diseases in probiotic group
Benor et al. (2014)	Israel	*L. acidophilus* NCFM and *B. lactis* Bi-07	58 - mothers of infants with birthweight of 1,500 g or less	From 72 h after birth; the occurrence of NEC, sepsis, and death in infants	No statistical significance, results tend toward beneficial impact
Dotterud, Storrø, Johnsen, & Oien (2010); Simpson, Dotterud, Storrø, Johnsen, & Øien (2015)	Norway	*L. rhamnosus* GG, *B. animalis* subsp. *lactis* Bb-12 and *L. acidophilus* La-5	415 - healthy	From 36 wk to 3 mo after delivery; atopic sensitization, atopic dermatitis, asthma and allergic rhinoconjunctivitis during 6 yr	Lower cumulative incidence of atopic dermatitis at 2 yrs and trend toward a lower cumulative incidence at 6 yr in the probiotic group
Wickens et al. (2018)	New Zealand	*L. rhamnosus* HN001	423 - father had a history of treated asthma, eczema or hay fever, women healthy	From 14 to 16 wk till 6 mo postpartum; prevalence of eczema, wheeze or atopic sensitization during 1 yr	Maternal probiotic supplementation without infant supplementation may not be effective for preventing infant eczema
Takahashi et al. (2019)	Japan	*L. casei* LC5, *B. longum* BG7, and *Bacillus coagulans* SANK70258	60 - lactating women who exhibited allergies for 2 mo (1–3 mo postpartum)	Open label pilot study, from 2 mo (1–3 mo post-partum); level of TGF-β and IgA in human milk	TGF-beta 1, TGF-beta 1, and IgA levels were significantly correlated at baseline. However, the correlation between TGF-beta 1 and IgA became non-significant by the end of the intervention ($p = 0.063$)
Forsberg et al. (2020)	Sweden	*L. reuteri* DSM 17938	58 - at least one parent or sibling with clinical symptoms or history of allergic disease	From 20 wk to 1 yr after delivery; immune cell populations during pregnancy and immune phenotyping to 4 dy after delivery	Immunomodulatory effects among activated and resting Treg cells during the second half of pregnancy

No permission required.

meta-analysis on the use of probiotic supplementation in pregnant mother, breastfeeding mother, and infant for the prevention of atopic dermatitis in children. When combining results of all studies supplementing probiotics to the pregnant mother and breastfeeding mother, the overall RR and CI indicate that children whose mothers were given probiotic supplementation during pregnancy and breastfeeding had a 28% less risk of developing AD at follow-up compared to those in the control group. Probiotic supplementation was effective in both, high-risk and normal population, but it was more effective in the high-risk population. In addition, it seems that a mixture of probiotics is more effective that single strain (Amalia et al., 2020; Li et al., 2019a; Sun et al., 2021). However, very recent update of guidelines from the European Academy of Allergy and Clinical Immunology (EAACI) does not recommend for or against prebiotics, probiotics or symbiotics for pregnant and/or breastfeeding women and/or infants alone or in combination with other approaches to prevent food allergy in infants and young children (Halken et al., 2021). On the contrary, World Allergy Organization (WAO) guideline determined that there is a likely net benefit from using probiotics resulting primarily from prevention of eczema. Both of the organizations state that these recommendations are conditional and supported by very low-quality evidence (Ricci et al., 2016).

Mechanisms of beneficial effect of probiotics administered to pregnant and lactating mothers remains elusive. Many *in vitro*, *ex vivo*, animal and human clinical studies tried also to elucidate the possible mechanisms of beneficial probiotic action for prevention and mitigation of allergy. In general, beneficial probiotic effect on the immune system could be mediated via pattern recognition receptors (PRR) such as TLR-2, harmonization of Th1/Th2 cytokine response, Th17 suppression, induction of tolerogenic DC, induction of secretory IgA, and via probiotic metabolites, such as short-chain fatty acids (SCFA) and polyamines. In addition, the immunologic composition of breast milk could also be altered by probiotic supplementation(Kuitunen, Kukkonen, & Savilahti, 2012). Although mechanisms of probiotic actions are unclear for many probiotic strains, especially for probiotic mixtures, the recent meta-analysis by Sun et al. (2021) predicts that a probiotic supplement is expected to become a novel potential strategy for infant eczema and atopic eczema prevention.

11.7 Gastrointestinal disorders

Probiotics are effective for treatment of microbial-dysbiosis-related disorders, such as antibiotic-associated diarrhea (Liao, Chen, Wen, & Zhao, 2021). In addition, probiotics intervention could also represent a safe strategy for regulation of maternal microbiome in order to restore the community of symbiotic microbiomes and provide beneficial effects on maternal, fetal and infant health (Sanz, 2000; Yao et al., 2020). Common gastrointestinal disorders in pregnancy are nausea, esophageal reflux, vomiting, constipation, and constipation-related symptoms, such as hemorrhoids (Yao et al., 2020).

11.7.1 Constipation in pregnancy

Up to 40% of women suffer from constipation in pregnancy and lactation (Cullen & O'Donoghue, 2007). According to Rome III criteria, constipation is diagnosed when 25% of

bowel movements are associated with at least two symptoms and last for 3 months, such as straining, sensation of anorectal obstruction during defecation, sensation of incomplete evacuation, need for manipulation to facilitate defecation, and less than three defecations per week (Mearin et al., 2016). Several probable causes for constipation in pregnancy were described in literature (Fan, Kang, Xiao, Li, & Yang, 2020; Zahoor, Babar, Javed, & Hussain, 2018) and the condition is accompanied with specific changes of maternal intestinal microbiota. Whether dysbiosis is merely a consequence or causes constipation it remains unclear (Dimidi, Christodoulides, Scott, & Whelan, 2017; Jin et al., 2020; Yao et al., 2020).

Treatment of constipation during pregnancy may include increased fluid intake, increased use of dietary fiber and use of bulk-forming agents, stool softeners and laxatives (Zahoor et al., 2018). Their adverse effects, such as abdominal pain and diarrhea, limit their use and such treatments during pregnancy need special attention due to concerns for the safety of the mother and her child. Scientific evidence suggests that some probiotic strains are promising for alleviating constipation. However, there are only three trials that evaluated probiotics for improvement of constipation and constipation related symptoms in pregnancy and lactation, of which one was a pilot study (De Milliano, Tabbers, Van Der Post, & Benninga, 2012) and two were randomized, controlled trials (Mirghafourvand, Homayouni Rad, Mohammad Alizadeh Charandabi, Fardiazar, & Shokri, 2016; Sakai et al., 2015). In the pilot study of De Milliano et al. (2012), multi-strain probiotic preparation was tested for 4 weeks on 20 pregnant women with constipation between 12 and 34 weeks of gestation. To evaluate the outcomes participants completed non-validated questionnaires. Compared to baseline, a significant decrease in sensation of anorectal obstruction, a sensation of incomplete evacuation, straining during defecation, episodes of abdominal pain and presence of reflux episodes were found. In the randomized, double-blind, placebo-controlled single-center study, 40 women who had natural childbirth were randomized to the group consuming either one bottle/day of fermented milk containing *Lactobacillus casei* Shirota, or placebo, for 6 weeks after childbirth (Sakai et al., 2015). Subjects filled in a diary on their bowel habits and answered questionnaires on constipation-related symptoms and quality of life during the study period. The probiotic group showed better scores on several parameters and decreased incidence of hemorrhoids while no statistically significant effect was observed on stool consistency and frequency. In another randomized, controlled trial, 60 constipated pregnant women were randomly put into two groups, probiotic group received 300 g of yogurt enriched with *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* Bb-12 while the control group received conventional yogurt for 4 weeks (Mirghafourvand et al., 2016). Participants completed a socio-demographic questionnaire, constipation checklist, and quality of life questionnaire. The study found no statistical significance between the groups in the mean scores of physical and mental aspects of quality of life after the intervention with the adjusting of baseline scores. However, several parameters improved compared to baseline suggesting that consumption of probiotics and conventional yogurt can play a role in improving the symptoms of constipation during pregnancy (Mirghafourvand et al., 2016).

Since constipation is a multifactorial problem, which is directly correlated with enteric and central nervous system, gut luminal environment and immune system, probiotics probably have several modes of action in improving constipation (Dimidi et al., 2017). Limited number

of studies suggest that probiotics may: i) help to regulate the nervous system to normalize gut motility (Bar et al., 2009; Kunze et al., 2009; Wang et al., 2010); ii) affect the composition of intestinal microbiota and SCFA production (Ishizuka et al., 2012; Matsumoto et al., 2006); iii) stimulate mucin production (Caballero-Franco, Keller, Simone, & Chadee, 2007); and iv) modulate the mucosal immune barrier and normalize inflammation-related dysmotility albeit not specifically in constipation (Dimidi et al., 2017).

11.7.2 Infantile colic

Infantile colic is another dysbiosis-related condition that is a potential target for maternal probiotic intervention (Ouald Chaib, Levy, Ouald Chaib, & Vandenplas, 2020). Colic is often reported gastrointestinal disorder in infants and is according to Rome IV criteria, diagnosed in infants younger than 5 months when recurrent and prolonged periods of infant crying, fussing, or irritability occur without evidence of infant failure to thrive, fever, or illness (Zeevenhooven, Koppen, & Benninga, 2017). An extensive number of possible factors have been proposed and many therapies are used such as dietary, pharmacological, and behavioral interventions (Baldassarre et al., 2018). As mentioned, dysbiosis of infant's gut microbiota may play an important role in the development of infantile colic and intervention with probiotics seems to be a promising strategy for treatment and prevention (Skonieczna-Zydecka et al., 2020). However, only two controlled clinical trials evaluated the effect of solely maternal supplementation of probiotics during pregnancy and lactation on the incidence and severity of infantile colic. Baldassarre et al. (2016) evaluated the effect of a mixture of probiotics (n = 33) or placebo (n = 33) administered to pregnant mothers 4 weeks before the expected delivery until 4 weeks after delivery, on levels of several immunological factors in milk and newborn stool samples as well as on incidence of gastrointestinal events in newborns. Probiotic maternal consumption had a significant impact on mean values of IL6 in colostrum, on IL10 and TGF-1 in mature breast milk and had higher values of fecal sIgA. A mixture of probiotics may also have consequences on inconsolable crying and regurgitation in the first month of life, however this data cannot be considered conclusive because the sample size calculation power was not adequate (Baldassarre et al., 2016).

In the study by Pourmirzaiee et al. (Pourmirzaiee, Famouri, Moazeni, Hassanzadeh, & Hajihashemi, 2020), the case group consisted of 87 pregnant women, who received daily doses *L. reuteri* LR92 DSM26866, and the control group with 88 pregnant women received the placebo for the last 4 weeks of pregnancy. Infants were followed for 5 months on signs and symptoms of colic with the repetitive examination by a blinded pediatrics assistant to record the occurrence of colic and it's grading. Infants of mothers from probiotic group were less likely to have colic and cases in the probiotic group were less severe, suggesting that maternal prenatal supplementation with probiotic *L. reuteri* LR92 DSM 26866 during the last 4 weeks of pregnancy can prevent the occurrence and reduce the severity of infantile colic (Pourmirzaiee et al., 2020). The mechanism of beneficial effect of maternal probiotic supplementation on incidence and severity of infantile colic remains elusive. The positive effects could be mediated through changes in immunological parameters in breast milk (Baldassarre et al., 2016) or through the transfer of the administered probiotic strain to infant gut and direct impact on the infant's intestinal microbiota or infant's immune system (Nocerino et al., 2020).

11.8 Obesity and metabolic disorders

Obesity in women of reproductive age is increasing worldwide and its negative impact on various pregnancy outcomes, including the risk of gestational diabetes mellitus (GDM), is now realized (Poston et al., 2016). GDM is defined as any degree of glucose intolerance with onset or first recognition during pregnancy (American Diabetes Association, 2011). Maternal obesity and GDM are independently associated with adverse maternal and neonatal outcomes. Women with GDM are more susceptible to other pregnancy complications including pre-eclampsia, PTL, and metabolic syndrome. In addition, GDM increases the risk of fetal macrosomia, neonatal hypoglycemia, and hypocalcemia during the perinatal period, and also the risk of developing childhood obesity and metabolic syndrome later in life (Buchanan, Xiang, & Page, 2012). In the Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study Catalano et al. (2012) demonstrated that maternal obesity and diabetes are the most potent predictors of childhood obesity, as women with obesity and GDM have a 1.73- and 2.19-fold higher risk, respectively, of having newborns with high birth weights compared with that of pregnant women with a healthy weight. Thus, optimal management of GDM, obesity and pregestational diabetes in pregnancy has the potential to lessen the trans-generational impact of metabolic disease.

Systematic review and meta-analysis performed by Oteng-Ntim et al. (Oteng-Ntim, Varma, Croker, Poston, & Doyle, 2012) revealed that lifestyle interventions for obese and overweight women during pregnancy restrict gestational weight gain and a trend was evident toward reducing the prevalence of GDM. However, many research gaps still exist regarding the effectiveness of lifestyle intervention in pregnancy. In the UK Pregnancies Better Eating and Activity Trial (UPBEAT) study, for example, Poston and colleagues (Poston et al., 2015) assessed a complex behavioral intervention based on a healthy diet and increased physical activity and compared it with standard antenatal care in the UK. In women who received the intervention, a significant improvement was reported in dietary pattern, physical activity was increased, and total gestational weight gain was lower than in controls. However, the intervention in obese pregnant women did not reduce the incidence of gestational diabetes or large-for-gestational-age babies. Similarly, in overweight and obese pregnant women, no evidence was found that maternal dietary and/or lifestyle intervention during pregnancy modifies the risk of early childhood obesity (Louise et al., 2021). Moreover, the Cochrane Review evaluating the combination of diet and exercise interventions for the prevention of GDM concluded that there is moderate-quality evidence that this combination of lifestyle interventions can reduce the risk of GDM (Shepherd et al., 2017). Obviously, a behavioral intervention addressing diet and physical activity in women with obesity during pregnancy is not adequate to prevent metabolic disorders including gestational diabetes or to reduce the incidence of large-for-gestational-age infants.

We already know that gut microbiota and its genome (microbiome) play an important role in individual health via affecting not only the host's intestinal homeostasis including digestion and immune system but also endocrine function, metabolism, as well as neurological system. Recent evidence highlighted the role of the gut microbiome in weight management. Obesity-associated gut microbiota alters host energy uptake, insulin sensitivity, inflammation and fat storage. Moreover, the gut microbiota-derived metabolites could control appetite directly by affecting the central nervous system or indirectly through modifying the gut

hormones secretion (Ejtahed & Hasani-Ranjbar, 2019; Martin, Sun, Rogers, & Keating, 2019; Vernocchi, Del Chierico, & Putignani, 2020). Several hypotheses have been put forward to link gut microbiota dysbiosis with glucose intolerance. Researchers have identified that a high-fat diet (HFD) is associated with modulation of gut microbiota composition and elevation of Gram-negative/Gram-positive bacteria ratio leading to the accumulation of lipopolysaccharides (LPS) in plasma. Elevation of Gram-negative bacteria and LPS is associated with increased gut epithelial permeability, low-grade inflammatory pathways, attenuation of insulin signaling, and glucose intolerance (Hasain et al., 2020).

Considering gut microbiota and pregnancy, Koren et al. (2012) were among the first researchers to report a direct link between gut dysbiosis and inflammation, adiposity, as well as insulin resistance in late pregnancy. After that, the decreased richness of the enteric microbiota has been associated with elevated insulin resistance, proinflammatory markers, and adiposity. In particular, a rise in the Firmicutes/Bacteroidetes ratio is related to low-grade inflammation and to an increased capability of harvesting energy from food (Cortez et al., 2019; Le Chatelier et al., 2013; Pascale, Marchesi, Govoni, Coppola, & Gazzaruso, 2019). Based on the current evidence, gut dysbiosis in GDM women is characterized by decreased microbial richness and diversity (Crusell et al., 2018; Koren et al., 2012), an increase in Firmicutes/Bacteroidetes (F/B) ratio and Gram-negative bacteria such as *Parabacteroides*, *Prevotella*, *Haemophilus* and *Desulfovibrio*, a reduction in the relative counts of the health-promoting bacteria, and decreased SCFA-producing bacteria of the genera *Faecalibacterium*, *Ruminococcus*, *Roseburia*, *Coprococcus* and *Akkermansia* (Cortez et al., 2019; Huang, Thonusin, Chattipakorn, & Chattipakorn, 2021). Most of the changes are associated with elevated blood glucose. Although there was evidence suggesting the alterations of gut microbiota composition in GDM when compared to the non-GDM group, the trend of the alterations in some bacteria was inconsistent. For example, the abundance of the genus *Faecalibacterium* and genus *Blautia* can be either increased or decreased in GDM, depending on their subgenus (Crusell et al., 2018).

11.8.1 Probiotics as preventive or curative treatments in metabolic disorders

Considering that the maternal gut microbiome is altered in diabetes and obesity, both of which are linked to adverse pregnancy outcomes, modulation of the gut microbiome by probiotics before and during pregnancy could become a new strategy for the prevention of adverse pregnancy outcomes. The results of meta-analyses that determine the efficacy of probiotics in various preventive or curative treatments are rarely convincing, and studies of the effect of probiotics on metabolic outcomes in pregnant women with metabolic disorders are no exception. Assessment of four randomized controlled trials involving 288 pregnant women with GDM found that a 6 to 8 week probiotic intervention did not improve fasting blood glucose (FBG) or LDL-cholesterol levels (Taylor et al., 2017). Although insignificant, the meta-analysis showed a reduction of 0.13 mmol/L in FBG compared to the placebo group. Moreover, probiotic supplementation resulted in a significant reduction in insulin resistance, suggesting the potential role of probiotics on glucose regulation in women with GDM. In total, 12 randomized control trials involving 1196 pregnant women were included in a meta-analysis on the effect of probiotics on the glucose levels of pregnant women (Peng, Wu, & Chao, 2018).

Results have shown that the use of probiotics significantly reduced the FBG level, insulin concentration, and Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) but only in pregnant women without GDM. A similar meta-analysis performed by Łagowska et al. (Łagowska, Malinowska, Zawieja, & Zawieja, 2020) showed the opposite results to the previous ones, namely that the supplementation of probiotics had a positive effect on glycemic control, thus lowers serum glucose levels, insulin levels, and HOMA-IR index but only in pregnant women with GDM. Zhang et al. (2019) who included for analysis eleven randomized trials involving 719 participants have observed positive effects of probiotic supplementation in pregnant women with GDM as well. Probiotic supplementation improved FBG, fasting serum insulin, HOMA-IR, triglycerides and HDL-cholesterol. While Ramanathan et al. (2021), who included 9 studies with a total of 1053 participants, found a significant decrease in fasting plasma glucose (FPG) and a significant difference between probiotics (intervention) and control groups, Masulli et al. (2020) observed no effect of probiotics supplementation on the incidence of GDM, with a small but significant reduction of FPG. A Cochrane Review evaluating the use of probiotics to treat GDM published in 2020 found evidence of a reduction in markers for insulin resistance (HOMA-IR) and insulin secretion in probiotics compared with placebo (Okesene-Gafa et al., 2019). However, a recent Cochrane Review that systematically assessed the effects of probiotic supplementation during pregnancy on the prevention of GDM has not clearly identified the effect of probiotics on the risk of GDM while according to results suggested that probiotics probably increase the risk of pre-eclampsia. There were no other clear differences between probiotics and placebo among the other primary outcomes. Probiotics make little to no difference in the risk of cesarean section, maternal weight gain during pregnancy, large-for-gestational-age infants and neonatal adiposity (Davidson, Barrett, Price, Callaway, & Dekker Nitert, 2021).

As might be expected, it was difficult to identify uniform or consistent findings across this heterogeneous group of studies in the meta-analysis. The discrepancy is mainly the result of the different studies included in the meta-analyses, and the different methods of statistical analysis. Most of the studies use different doses and different strains of probiotic bacteria, alone or in combinations, which makes it impossible to compare such studies in the meta-analysis as probiotic effects are strain-specific. The reporting of strains is heterogeneous across the studies, with authors reporting culture collection numbers (i.e., *L. rhamnosus* ATCC 53103), commercial designations (i.e., *L. rhamnosus* GG), or even strain is not reported. Furthermore, the doses of individual probiotic microorganisms are diverse; treatment duration is ranging from few weeks to several months with different starting points during pregnancy. Most included samples consist of relatively small groups of women, with or without metabolic disturbances. The results of the individual studies are also affected by other factors that are not considered, for example, the mother's diet that can profoundly influence the mother's gut microbiota. In addition, the efficacy of any dietary intervention, including probiotics, may depend on the characteristics of the individual, such as genotype, presence or absence of various diseases (which may alter metabolism), intake of supplements and drugs and other factors. Moreover, gut microbiota composition may affect the bioavailability of nutrients, and consequently the nutritional status and metabolism of the host. These are a number of factors that contribute to the inconsistency of the results of clinical studies (Table 11.3).

In summary, pregnant women are receptive to nutritional counseling, however the poor compliance associated with lifestyle interventions in general calls for new solutions.

TABLE 11.3 Selected, placebo-controlled studies of effects of probiotics administration during pregnancy on the glycemic control, incidence of gestational diabetes mellitus (GDM) and on the weight control including gestational weight gain (GWG).

References	Country	Probiotic strains	No. of participants (randomized) - population characteristics	Treatment regime during pregnancy and lactation; main outcome measure	Main outcomes and results
Asemi et al. (2013)	Iran	Probiotic yoghurt *L. acidophilus* LA5 and *B. lactis* Bb12	70 primigravida pregnant women at their third trimester of gestation	200 g/day probiotic yoghurt or conventional yoghurt (placebo) for 9 wk; fasting plasma glucose and serum insulin levels before and after treatment	No effect
Asgharian, Homayouni-Rad, Mirghafourvand, & Mohammad-Alizadeh-Charandabi (2020)	Iran	Probiotic yoghurt *L. acidophilus* LA5 and *B. lactis* Bb12	128 women with pre- or early-pregnancy BMI ≥ 25	100 g/day probiotic yoghurt or conventional yoghurt (placebo) for 4 wk; fasting plasma glucose, the incidence of gestational diabetes	Significant lower plasma glucose level in the probiotic than in the placebo group, no differences between the groups in frequency of GDM
Dolatkhah et al. (2015)	Iran	*L. acidophilus* LA-5, *B. lactis* BB-12, *Str. thermophilus* STY-31, *L. bulgaricus* LBY-27	64 pregnant women with GDM screened during 24–28 wk of gestation	1 capsule daily of probiotic for 8 wk; fasting plasma glucose and serum insulin levels before and after treatment, HOMA-IR, weight gain	Significantly higher decrease of FBG in probiotic group, significantly lower weight gain in the probiotic group in the last 2 wk of the study
Lindsay et al. (2014)	Ireland	*L. salivarius* UCC118	175 obese and overweight pregnant women	1 capsule daily of probiotic from 24 to 28 week of gestation; fasting glucose before and after intervention, the incidence of gestational diabetes	No effect
Ilmonen, Isolauri, Poussa, & Laitinen (2011)	Finland	*L. rhamnosus* GG and *B. lactis* Bb12	256 pregnant women without metabolic diseases, at <17 wk of gestation	Parallel-group nutrition counseling and probiotics; 85 diet/probiotics; 86 diet/placebo; 85 control/placebo (25–35 yr); BMI and adiposity defined as a waist circumference of 80 cm or more	The risk of central adiposity was lowered in women in the diet/probiotics group.

References	Country	Probiotic strains	No. of participants (randomized) - population characteristics	Treatment regime during pregnancy and lactation; main outcome measure	Main outcomes and results
Luoto, Laitinen, Nermes, & Isolauri (2010)	Finland	*L. rhamnosus* GG and *B. lactis* Bb12	256 pregnant women, at the first trimester of gestation	Parallel-group nutrition counseling and probiotics; 85 diet/probiotics; 86 diet/placebo; 85 control/placebo, pregnancy outcome and infant growth	Reduced frequency of GDM; 13% (diet/probiotics) v. 36% (diet/placebo), no significant differences in prenatal or postnatal growth rates among the study groups
Callaway et al. (2019)	Australia	*L. rhamnosus* GG and *B. lactis* Bb12	411 obese and overweight pregnant women, at 20 wk of gestation,	1 capsule daily of probiotic from enrollment until birth; the frequency of GDM at 28 wk of gestation	No effect
Wickens et al. (2017)	New Zealand	*L. rhamnosus* HN001	423 women at 14–16 wk of gestation, >16 yr (212 probiotic, 211 placebo)	1 capsule daily of probiotic from enrolment until birth; GDM at 24–30 wk	GDM prevalence was significantly lower in the HN001 group
Okesene-Gafa et al. (2019)	New Zealand	*L. rhamnosus* GG and *B. lactis* BB12	230 women with a singleton pregnancy at 12 to 17 wk of gestation and BMI >30 kg/m^2	Randomization to a dietary intervention or to routine dietary advice; and to daily probiotic or placebo, from enrollment until birth; proportion of women with excessive GWG, adjusted for baseline BMI	Dietary education and or probiotics did not alter rates of excessive gestational weight gain

According to Isolauri and colleagues (Isolauri, Rautava, Collado, & Salminen, 2015) the modest results obtained in the lifestyle intervention studies can be partly explained by failure to reshape the unfavorable intestinal microbiota composition often associated with obesity and metabolic disorders. Probiotics can be a complimentary treatment to dietary interventions. Probiotics can support the host in the prevention or improvement of metabolic disorders by balancing the aberrant indigenous microbiota, by supporting the repair of increased intestinal permeability, regulation of inflammation and energy uptake. The strain-specific nature of probiotic effects highlights the need for rigorous *in vitro* and preclinical tests prior to human clinical trials. The selection of probiotic bacteria and their combinations should be based on identified mechanisms and targets.

11.9 Human milk microbiota and lactational mastitis

Historically, bacteria in human milk were viewed as a cause of milk spoilage, mastitis, and the route for transfer of diseases (Fernández, Pannaraj, Rautava, & Rodríguez, 2020). Several discoveries at the beginning of 21st century led to the proposal that human milk contains its own site-specific microbiota (Martín et al., 2004), which is the source of the majority of bacteria found in breastfed infants' gut, including gut-associated obligate anaerobes (Jost, Lacroix, Braegger, Rochat, & Chassard, 2014; Pannaraj et al., 2017). In addition, an increasing number of studies support the hypothesis that the milk microbiome influences health outcomes in infants (Groer, Morgan, Louis-Jacques, & Miller, 2020). The origin of bacteria that can be found in human milk is still unknown and to date, no experimental evidence has conclusively demonstrated their origin. Thus, human milk microbes might originate from multiple sources. A pre-lactational mammary gland microbiome may act as the initial seed for the human milk microbiome; however, the maternal skin, infant oral cavity, and maternal gastrointestinal tract are also very likely sources (Stinson et al., 2021).

Human milk microbiota is composed of a plethora of organisms from four domains of life – bacteria, viruses, fungi, and archaea (Stinson et al., 2021). Research on the topic can be divided regarding the methods used, that is, culture-dependent and culture-independent techniques. Both methodologies have their strengths and limitations, while culture techniques cover only viable and culturable organisms and DNA-based techniques cover also uncultivable organisms and provide a more comprehensive picture of microbial diversity, although we get no information about their viability. Research using both techniques agreed that the most common bacteria found in human milk are members of the genera *Staphylococcus*, *Streptococcus*, *Propionibacterium*, and *Corynebacterium*. In addition, culture-independent studies have shown a wide diversity of bacterial signatures belonging to more than 800 different bacterial species, including some gut-associated strict anaerobes (Fernández et al., 2020). It is worth mentioning that human milk contains also bifidobacteria, lactobacilli and other commensals, which are important for the establishment of infant gut microbiota and human health (Groer et al., 2020).

The knowledge about the factors that shape human milk microbiota is still limited, however, several studies pointed out sample collection, human milk oligosaccharides and other milk metabolites, maternal diet, maternal body mass index, gestational age, mode of delivery, infections such as mastitis, postpartum period, geographical location, and maternal

treatments such as antibiotics, chemotherapy, prebiotics, and probiotics (Demmelmair, Jiménez, Collado, Salminen, & McGuire, 2020; Fernández et al., 2020; Treven et al., 2019).

11.9.1 Lactational mastitis and probiotics

One of the factors that profoundly affect human milk microbiota is lactational mastitis. Mastitis is defined as the inflammation of a mammary gland, due to milk stasis and infection. The occurrence of mastitis can be explained as dysbiosis of human milk microbiota where the overgrowth of one species leads to decreased bacterial diversity, accompanied by dramatic enrichment of aerotolerant bacteria and depletion of obligate anaerobes (Rodríguez & Fernández, 2017). The characteristic changes of mammary gland microbiota due to mastitis are loss of bacterial diversity, enrichment of aerotolerant and depletion of obligate anaerobes, increased abundance of opportunistic pathogens and presence of biofilm-related genes. Thus, most often biofilm-forming bacteria overgrowth in narrow milk ducts causes the obstruction of milk flow and milk retention finally leading to inflammation (Angelopoulou et al., 2018). Mastitis is classified from two perspectives: clinical manifestation (clinical or subclinical) or course of disease (acute, subacute granulomatous, recurrent and chronic). In each of these courses, some characteristic bacterial species are dominated, for example, *Staphylococcus aureus* in acute mastitis or *Staphylococcus epidermidis* and coagulase-negative staphylococci in subacute mastitis (Rodríguez & Fernández, 2017).

Antibiotic therapy is the most common treatment of mastitis although treatment is often not successful due to antibiotic resistance of biofilm-forming bacteria. In addition, antibiotic therapy is one of the main drivers of dysbiosis of mucosal surfaces, such as lactating mammary gland, leading to decreased bacterial diversity and a lower abundance of beneficial bacteria, such as lactobacilli and bifidobacteria (Fernández et al., 2020; Soto et al., 2014). Indeed, antibiotic therapy in the perinatal period is also a risk factor for the (re)occurrence of mastitis (Betzold, 2007; Rodríguez & Fernández, 2017). Consequently, more efficient treatments which could replace antibiotics are needed. Although, still in the experimental stage, probiotics and bacteriocins are the most promising non-pharmacological strategies for the treatment of mastitis (Angelopoulou et al., 2018). There are also several intervention possibilities for preventing mastitis, which may include specialist breastfeeding advice, holistic approaches and probiotics (Crepinsek, Taylor, Michener, & Stewart, 2020).

Only five randomized control studies evaluated the effect of probiotic supplementation for prevention or treatment of mastitis, using four probiotic strains. All studies were conducted in Spain. The strains used were *L. salivarius* CECT5713 (Arroyo et al., 2010; Jimenez et al., 2008), *L. gasseri* CECT5714 (Jimenez et al., 2008), *L. fermentum* CECT5716 (Arroyo et al., 2010; Hurtado et al., 2017; Maldonado-Lobón et al., 2015), and *L. salivarius* PS2 (Fernandez et al., 2016). All studies showed the beneficial effects of tested probiotics for the treatment and/or prevention of mastitis, although all studies had some methodological flaws such as problems with randomization, blinding, sample size or drop-outs (Barker, Adelson, Peters, & Steen, 2020).

In addition, there are several *in vitro* and animal studies, pointing out the probiotics as a promising strategy for the treatment or prevention of mastitis and shedding the light on possible mechanisms of probiotic action. Some research was conducted on bovine mammary epithelial cells since bovine mastitis causes substantial economic loss in the dairy industry.

It should be emphasized that the interpretation of these results for treatment of human lactational mastitis should be taken with care since the biology of the ruminant mammary gland differs from the human mammary gland by the lack of entero-mammary pathway of immune cells (Rainard, 2017). Mechanisms of probiotic action for prevention of mastitis may include direct influence through microbe-microbe interaction which might be possible via modulation of human milk microbiota or via direct interaction with colonizing pathogen. Several studies reported characterization of potential probiotics which inhibited adhesion and/or growth of mastitis-causing pathogens (Armas, Camperio, & Marianelli, 2017; Assis et al., 2015; Bousmaha-Marroki, Boutillier, Marroki, & Grangette, 2021; Diepers et al., 2017; Pellegrino et al., 2019; Wallis, Krömker, & Paduch, 2019). It is important to note that the presence of probiotics in the mammary gland is obligatory in order to prevent adhesion and inhibit the growth of potential bacterial pathogens. Indeed, probiotics supplemented to mother during pregnancy and lactation might be transferred to breast milk and influence breast milk microbiota (Abrahamsson, Sinkiewicz, Jakobsson, Fredrikson, & Björkstén, 2009; Andres et al., 2017; Mastromarino et al., 2015; Treven et al., 2015, 2019; Zaidi, Moore, & Okala, 2021). The enhancement of the epithelial barrier is another possibility for the beneficial act of probiotics in the mammary glands; however, there is a substantial lack of studies proving such mechanism (Rainard & Foucras, 2018). The study of Zheng et al. (2021) fills this research gap by showing that *L. casei* Zhang increased the expression of the tight junction proteins in bovine mammary epithelial cells as well as in mice mammary tissue after *E. coli*-induced mastitis. The modulation of the immune system through interactions with the immune cells might also be an important way of probiotic beneficial action for the prevention and treatment of mastitis. Different probiotic strains modulated cellular or systemic immune response by restriction of inflammasome activity and induction of autophagy (Zou et al., 2020), regulation of LPS-induced inflammatory response (Fukuyama et al., 2020), increase the expression of several pro-inflammatory cytokines (Souza et al., 2018), increase of TGF-1 in milk (Messias et al., 2019) and limiting detrimental inflammatory responses, partly via promoting TLR2 and NOD1 synergism (Wu, Liu, Yang, Wang, & Zhu, 2016). Nevertheless, more controlled clinical studies are needed to support the rationale of probiotic intervention for the treatment or prevention of mastitis.

11.10 Mental health

Perinatal depression can be divided in relation to its onset. Depression onset during pregnancy can be referred to as antepartum depression, whereas depression onset during postpartum can be referred to as a postpartum depression. However, many studies do not distinguish between participants with a previous history of depression from those with de novo postpartum depression although the timing of depression onset likely has different implications for disease etiology (Qiu, Hodges, Clark, Blankers, & Galea, 2020). The incidence rate for depression during prepartum and postpartum are 1.6% and 5.7% (Banti et al., 2011), respectively and global prevalence for the first-time onset of depression during postpartum has been reported to be as high as 18% (Hahn-Holbrook, Cornwell-Hinrichs, & Anaya, 2018). The exact causes of perinatal depression are still unknown and different biological and psychological models try to explain the cause of depression (Yu et al., 2021). One of the important

factors in this aspect is gut microbiota and the gut-brain axis. Gut dysbiosis is a usual characteristic of depressive disorders and there is general consensus is that the gut bacterial composition of depressive patients is notably different from their healthy counterparts (Tyagi, Tasleem, Prakash, & Chouhan, 2020). The prospect of using certain probiotics as an alternative treatment for depression was so far proven by many studies and the term psychobiotic was coined in order to describe probiotic bacteria for that particular application (Poluektova, Yunes, & Danilenko, 2021). Psychobiotics may be characterized as probiotic strains that, when consumed in appropriate quantities, impart positive psychiatric effects in psychopathological conditions (Tyagi et al., 2020).

Several dozen studies showed the positive effects of probiotics on the symptoms of depression, anxiety in randomized, double-blind, placebo-controlled studies on different populations (Poluektova et al., 2021), however, controlled clinical studies regarding the mental health of pregnant and lactating women are scarce. Only one clinical study evaluated the effect of probiotics on anxiety and depression in pregnant and lactating women as the primary outcome (Dawe, McCowan, Wilson, Okesene-Gafa, & Serlachius, 2020) while three evaluated quality of life and maternal depressive symptoms as a secondary outcome (Browne, Bolte, Besseling-van der Vaart, Claassen, & de Weerth, 2021; Mirghafourvand et al., 2016; Slykerman et al., 2017). Interestingly, in all four studies the study population, number of participants probiotic strains or mixtures and time frame of the intervention/follow were different. In the study of Mirghafourvand et al. (Mirghafourvand et al., 2016) the mixture of *L. acidophilus* La-5 and *B. animalis* subsp. *lactis* Bb-12 was tested on constipated women (n = 60) for 4 weeks during pregnancy (24–28 week) with 2 weeks follow-up period. Slykerman et al. (2017) tested *L. rhamnosus* HN001 on women where her or the unborn child's biological father had with a history of asthma, hay fever or eczema requiring medication (n = 423); from 14 to 16 weeks of gestation up till 6 months post-birth and mothers competed for a questionnaire about their psychological wellbeing at 6 and 12 moths postpartum. Dawe et al. (2020) tested the mixture of *L. rhamnosus* GG and *B. animalis* subsp. *lactis* Bb-12 on pregnant women with obesity (n = 230) from 12 to 36 weeks of pregnancy. Browne et al. (2021) tested the probiotic multispecies mixture consisting of 9 probiotic strains of different *Bifidobacterium*, *Lactobacillus* and *Lactococcus* species on pregnant women with pre-existing symptoms of depression (n = 40), from 26 to 30 weeks gestation until delivery. The participants filled in questionnaires at baseline, at 8 weeks after starting the treatment and 4 weeks post-partum. The results of these studies are also diverse. Only in the study of Slykerman et al. (2017), women who received HN001 had significantly lower depression and anxiety scores in the postpartum period, while the other three studies revealed no significant changes in measured outcomes. Taking together, these studies once more emphasize the importance of specific probiotic strain, study population, time of treatment and follow-up period, on the outcomes of the probiotic intervention.

Many *in vitro* and *in-vivo* studies on mainly on rats and mice explained the role of intestinal microbiota and the mechanism of antidepressant activity of probiotic bacteria (Poluektova et al., 2021; Tyagi et al., 2020). It should be noted that extrapolation of this knowledge onto pregnant and lactating women should be taken with care since many physiological changes during pregnancy and lactation may cause different physiological response to probiotic treatment. Anyway, probiotics may exert antidepressant activity by several mechanism such as: increasing contents of SCFA in caecum (Hao, Wang, Guo, & Liu, 2019), stimulation of vagus

nerve (Bercik et al., 2011; Bravo et al., 2011; Malick et al., 2015; Takada et al., 2016); altering the activity of hypothalamic-pituitary-adrenal axis and normalizing corticosterone levels (Ait-Belgnaoui et al., 2014; Allen et al., 2016; Desbonnet et al., 2010; Gareau, Jury, MacQueen, Sherman, & Perdue, 2007); influence on tryptophan metabolism and serotonin production (Desbonnet, Garrett, Clarke, Bienenstock, & Dinan, 2008; Luo et al., 2014; Valladares et al., 2013), anti-oxidant activity which has been shown to alleviate mood disorders (Asemi, Zare, Shakeri, Sabihi, & Esmaillzadeh, 2013; Divyashri, Krishna, Muralidhara, & Prapulla, 2015; Pandey, Singh, Chaudhari, Nampoothiri, & Kumar, 2015); regulation of the activity of gamma-aminobutyric acid γ-aminobutyric acid (GABA) (Ko, Lin, & Tsai, 2013); and synthesis of a wide range of neuroactive compounds such as GABA, serotonin, dopamine, adrenaline, norepinephrine, and histamine (Poluektova et al., 2021).

In addition, a plethora of bioactive components which might be produced by probiotic bacterial cells or are part of probiotic cell components might take part in antidepressant mechanism of probiotics. For example, extracellular vesicles derived from *Lactobacillus plantarum* were shown to increase the brain-derived neurotrophic factor (BDNF) expression in cultured hippocampal neurons and produce antidepressant-like effects in mice (Choi, Kim, & Han, 2019). With so many possible mechanisms there is also an option that mode of action of probiotics is determined not just by one molecule but, rather, by the totality of metabolites secreted by the specific strain (Poluektova et al., 2021).

11.11 Conclusions

In conclusion, probiotics might be effective for the prevention or treatment of several illnesses of the mother or her child during pregnancy and lactation, although, larger controlled clinical trials are needed to prove that a specific probiotic strain is effective for a specific application. Many novel potential probiotic strains are described in literature almost every day, however, clinical trials are performed only on several strains for many different applications. For example, the strain *L. acidophillus* La-5 was tested in clinical trials for the prevention of allergies, metabolic disorders and constipation. On the contrary, for some applications, such as treatment or maintaining vaginal health, only a few different strains were tested in clinical trials. These shortcomings open up new opportunities for the development of new probiotics for treatment or prevention of health problems of pregnant women, nursing mothers and their infants.

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CHAPTER

12

Probiotics in Pediatrics

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12.1 Introduction

Although it has been known for more than a century that occupational microbiota has beneficial effects on the organism, interest in the human microbiome and its relationship to health has increased markedly in recent years. Research by the US Human Microbiome Project (Human Microbiome Project Consortium) and the European MetaHIT project (Arumugam et al., 2011) have revolutionized the world of science and confirmed to us that humans are superorganisms whose functioning represents an amalgam of microbial and human properties and many diseases are the result of the loss of harmony between us and our bacteria. Although there is still a long way to go, the knowledge and modulation of the microbiota open the door to a great deal of research to treat and prevent many diseases, making it one of the most important scientific revolutions in medicine.

Human beings have the same number of bacteria as cells in our organism (Sender, Fuchs, & Milo, 2016), being present in all parts of the body although most of them are located in the skin and those cavities of the organism that communicate with the exterior, which are fundamentally the vagina and the digestive tract, especially in the large intestine. This is what we call autochthonous microbiota (Suarez, 2013). The relationship we maintain with it is usually mutualistic since its microorganisms provide us with a series of advantages ranging from protection against invasion by pathogens and the development of the immune system to collaboration in the digestion of dietary components and the provision of vitamins and other essential nutrients (Álvarez-Calatayud, Suárez, Requena, & Rodríguez, 2020).

Each individual has a peculiar microbial community that depends on their genotype and early exposure to microorganisms in their environment, but also on diet, lifestyle changes or

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therapy against infections. This implies that colonization from birth will be different depending on factors such as the type of delivery, the pattern of breastfeeding, the rural or urban environment in which we grow up, being born in a developing or developed country, the use of antibiotics, especially those used to combat infections during delivery and in early childhood, etc. (Matamoros, Gras-Leguen, Le Vacon, Potel, & de La Cochetiere, 2013) An inadequate development of our digestive microbiota during the first months of life due to an increase in the number of cesarean sections, premature abandonment of breastfeeding or, in adulthood, due to the abuse of antibiotics, an inadequate diet or the aging process, can lead to a state of dysbiosis with an alteration of the microbiota both qualitatively (species different from the usual ones) and quantitatively (lower concentration of beneficial bacteria). The consequence will be a decrease in their health effects and, frequently, the appearance of diseases such as, for example, diarrhea.

In 2001, a commission of international experts jointly convened by FAO and WHO defined probiotics as "live microorganisms that when administered in adequate amounts confer a health benefit to the host" (Food and Agricultural Organization of the United Nations and World Health Organization 2001). Since then, this definition has been the most widely accepted worldwide and has recently been confirmed by the ISAPP (International Scientific Association of Probiotics and Prebiotics) (Hill et al., 2014). Probiotic organisms would mimic the positive effects exerted by the indigenous microbiota, so in principle, any component of it could be a candidate for becoming a probiotic. However, most belong to two microbial groups: lactobacilli and bifidobacteria, although the yeast *Saccharomyces cerevisiae* and some species of *E. coli* and *Bacillus* are also used.

Prebiotics are food ingredients that produce a selective stimulation of the growth and activities of one or a limited number of genera/species of microorganisms in the intestinal microbiota conferring health benefits to the host (Gibson et al., 2017). The best-known prebiotics are fructooligosaccharides (FOS), galactooligosaccharides (GOS), inulin, lactulose, and, of course, breast milk oligosaccharides. A product that combines at least one probiotic and one prebiotic is called a synbiotic. If the prebiotic carbohydrate is utilized by the probiotic strain, its proliferation in the host intestine will be selectively promoted. It is interesting to note that the combination of probiotic and prebiotic in the same product can have a synergistic effect and confer greater benefits than those exerted by each of the components of the synbiotic separately (Swanson et al., 2020). Recently, the term postbiotic has been introduced to refer to substances produced by probiotics that exert metabolic and immunomodulatory effects on the host, but so far it has not had significant repercussions both from a research and clinical point of view (Salminen et al., 2021). Psychobiotics would be living organisms that, when consumed in adequate amounts, produce a health benefit in patients with psychiatric disorders (Dinan, Satanton, & Cryan, 2013).

Increasing importance is given to the modulation of the intestinal microbiota through the use of probiotics, prebiotics, and synbiotics to treat various diseases, mainly gastrointestinal problems, such as different types of diarrhea (community, antibiotic-associated, traveler's, lactose intolerance, etc.), functional disorders (infant colic or irritable bowel syndrome), or inflammatory processes (ulcerative colitis). They are also used successfully in various women's pathologies (vulvovaginitis and mastitis) and their effect has been evaluated in allergies such as atopic dermatitis and in the prevention of infections (from premature newborns to the elderly) (Alvarez-Calatayud, Leis, & Díaz, 2021).

In parallel to the numerous research projects that have appeared in recent years dedicated to expanding the knowledge of the native microbiota, research in experimental animals and in humans opens the door to future applications with the supplementation of these organisms and nutrients. There are studies that support their use in nutritional problems (obesity, malnutrition), neurological diseases and behavioral disorders (autism, depression, and anxiety), periodontal disease, and the eventual prevention of various types of cancer. Finally, progress is being made on the repercussions that alterations in the microbiota may have on the appearance of cardiovascular diseases, arteriosclerosis, diabetes, hypercholesterolemia, metabolic syndrome, etc. (Rodriguez, 2015).

Pediatrics is one of the disciplines in which meta-analyses and systematic reviews of contrasted clinical trials have demonstrated their efficacy in various pathologies, especially digestive ones. For this reason, their use is gradually becoming established in the routine clinical practice of pediatricians (Johnson & Versalovic, 2012). In addition, there are several lines of research open on probiotic supplementation in childhood. Below we will describe the main pediatric pathologies in which the main clinical practice guidelines recommend their use based on the scientific evidence available to date (Pérez Moreno, Maturana, Álvarez-Calatayud, & Bodas, 2016). The clinical applications of probiotics in pediatrics have been growing over the years. In addition to the classic and widely studied indications, such as acute diarrhea and antibiotic-associated diarrhea, this chapter reviews the scientific evidence on the use of probiotics in the rest of pediatric pathology.

12.2 Probiotics in newborns and infants

12.2.1 The usefulness of probiotics in perinatology

It had been postulated that fetuses were totally free of germs since, on the one hand, the placenta is a very effective barrier that blocks their passage by the hematogenous route, while the mucous plug of the cervix and the amniotic membranes would prevent colonization by the ascending route. However, microbial DNA has been detected in amniotic fluid, placenta, fetal membranes, umbilical cord blood, and meconium (Aagaard et al., 2014; Jimenez et al., 2008). However, the presence or absence of microbiota in embryonic tissue by maternal-fetal transmission remains is still a controversial issue, as there are insufficient studies to ensure direct colonization of the fetus by viable microorganisms under normal conditions (De Goffau et al., 2019).

The mother's gut microbiota affects the health of the fetus, whether this interaction is solely through metabolic products, through colonization of the fetus by bacteria or both. However, there is an involvement of the intestinal microbiota in the development of the embryo through various metabolites, such as short-chain fatty acids (SCFA) and aromatic compounds, which trigger changes in the expression of genes related to the immune response in order to prepare the fetus for contact with the massive load of foreign microorganisms and promote immunotolerance mechanisms. This suggests that during pregnancy some bacteria are transferred from the mother to the fetus, providing it with an initial microbiota that would produce initial intestinal colonization and would constitute a first stimulus for the development of the child's immune system (Lahtinen et al., 2009). The incidence of such

diseases, such as asthma, rhinoconjunctivitis, and eczema, has greatly increased in the pediatric age group during the second half of the 20th century, to the point that these diseases currently constitute one of the main public health problems in the world. For this reason, in recent years numerous studies have been carried out on the possible beneficial effects on offspring of the administration of probiotics to pregnant women (Stojanovic, Plecas, & Plesinac, Stojanovic et al.).

12.2.2 Probiotics and prevention of preterm birth

Preterm deliveries are responsible for 60 to 80% of neonatal mortality and carry significant morbidity for survivors. Between 30% and 50% of preterm deliveries are associated with maternal infection. It has been postulated that certain probiotics could exert beneficial effects in the prevention of preterm labor due to their ability to displace and inhibit the growth of pathogenic germs in the vagina, in addition to interfering with the inflammatory cascade that precedes preterm labor. This is why in recent years several clinical trials have been conducted on the administration of probiotics to pregnant women. These are clinical trials with a small number of participants where a significant decrease in the risk of suffering a genital infection after the use of probiotics has been observed, although there is still little evidence of a significant decrease in the risk of presenting preterm labor (Madan et al., 2012), so more clinical trials with larger samples of pregnant women are needed before we can systematically recommend the use of probiotics during pregnancy to prevent preterm labor.

12.2.3 Prevention of necrotizing enterocolitis

There are significant differences in the pattern of colonization between term and preterm infants. The gut of preterm infants is generally colonized by *Bacteroides*, *E. coli*, and *Klebsiella* while beneficial commensal organisms, such as bifidobacteria and lactobacilli, tend to appear only from the third week of life onwards. They are therefore more likely to be colonized by fewer and more pathogenic microorganisms, with preterm infants, especially those of very low birth weight, being at a disadvantage compared to term neonates in relation to the development of the intestinal microbiota. Among the factors that contribute to this, in addition to intestinal immaturity, are premature rupture of membranes, maternal infection, increased incidence of cesarean section, exposure to broad-spectrum antibiotic therapy in the perinatal and postnatal period or to other gut-modifying medications (H_2 blockers), alterations in intestinal motility, fasting periods, microbial resistance due to admission to intensive care and lower consumption of breast milk (Grishin, Papillon, Bell, Wang, & Ford, 2013).

Necrotizing enterocolitis (NEC) is the most severe acquired disease of the gastrointestinal tract in preterm infants, with an incidence of around 5 to 10% in very low birth weight infants and even somewhat higher in extremely low birth weight infants less than 28 weeks. This pathology is associated with higher morbidity and mortality in this population. Bacterial colonization is considered a prerequisite for the development of NEC and, combined with prematurity and enteral formula feeding, predisposes the newborn to this severe inflammatory condition (AlFaleh & Anabrees, 2014).

Most meta-analyses that have studied the use of probiotics in neonates have as their primary objective the evaluation of their use in the prevention of NEC. According to these, enteral supplementation with some strains significantly reduces the incidence of severe NEC (stage II or higher) compared to placebo, and some of them even point to a decrease in mortality. However, no significant decrease in nosocomial sepsis or parenteral nutrition days has been observed. The beneficial effect of probiotics on gastrointestinal maturity and function could improve enteral tolerance as well as reduce the risk of necrotizing enterocolitis in preterm infants. Some trials have also examined this point, observing that infants on probiotics achieved complete enteral feedings significantly earlier than in control groups. Further trials are needed to evaluate this potential benefit of probiotics, in view of the important role of enteral intolerance and consequent nutritional deprivation in the postnatal growth restriction of extremely low birth weight infants (Deshpande, Rao, Keil, & Patole, 2011).

Recently, the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) (van den Akker et al., 2020) and American Gastroenterological Association (AGA) (Su et al., 2020) published their recommendations on the use of probiotics for preventing NEC. Although most of the studies use *lactobacilli* and *bifidobacteria*, they do so with different doses, varying also in the interval and duration of treatment. The time to initiate probiotic treatment has not been established either, depending on the policy of each Neonatal Intensive Care Unit (NICU). This heterogeneity makes it difficult to extrapolate the results of the studies and to establish specific treatment guidelines. However, it should be taken into account that the published results of probiotic supplementation may vary from unit to unit depending on the different enteral feeding policies (early vs. delayed, minimal vs. nutritional, breastfeeding vs. formula) and the use of antibiotics in suspected or confirmed sepsis, as this affects colonization in preterm infants.

The safety profile of probiotics is important, especially in the preterm neonatal population. No systemic infections caused by the microorganisms used as probiotics have been documented in the various trials analyzed in meta-analyses. However, caution should be exercised before adopting probiotics as prophylaxis, since some cases of sepsis due to lactobacilli have been reported in premature infants with short bowel and in immunocompromised infants and children (Tolín, Butragueño, Sánchez, & Salcedo, 2016).

In view of the multiple clinical trials showing beneficial effects of probiotics and the paucity of data linking them to serious adverse effects, it appears that the updated evidence strongly supports a change in clinical practice. At the present time, more than 25 randomized trials indicate that adverse effects of probiotic use are infrequent. Despite this strong evidence, there is still a reluctance to incorporate probiotic prophylaxis among neonatologists and scientific societies. Some authors, however, argue that current evidence from randomized clinical trials indicates that there is safety and efficacy in the use of probiotics in extremely preterm infants (those at highest risk for NEC, death, and feeding intolerance) and that the practice of evidence-based medicine in these infants should not be taken to such an extreme, considering the potential benefit that would be lost while probiotic prophylaxis is not implemented. Many level III NICUs (in Japan, Italy, Finland, and Colombia) have been using them routinely for more than a decade and have not documented significant adverse effects (Alonso, Oikonomopoulos, Suarez, & Notario, 2016).

12.2.4 Probiotics and prebiotics in infant milk formulas

Breast milk remains the ideal and exclusive food for the healthy infant in the first months of life because its composition is specifically adapted to the digestive characteristics and nutritional and growth needs of the infant. In addition to nutrients, human milk contains bioactive compounds responsible for a wide range of beneficial effects such as promoting the maturation of the immune system, protection against infections, and cognitive development, among other functions. Among these bioactive compounds, we have known for some years that human milk is not sterile and contains bacteria (more than 250 species have been isolated in healthy women) and oligosaccharides in large quantities, called human milk oligosaccharides (recognized by the acronym HMO) whose function is to promote the growth of bacteria in the infant's intestine, especially bifidobacteria, and are therefore considered the first prebiotics with the beneficial implications this has for the health of the infant (Martín et al., 2003).

Milk formulas emerge as an alternative in infant nutrition when breastfeeding is not possible. The ultimate goal of infant milk formulas is to approach the "gold standard", breast milk, not only by copying its components but also by trying to achieve similar functionality, measurable by its effects on the infant. In fact, the microbial profile of term, vaginally delivered, exclusively breastfed infants is the standard of beneficial microbiota and should serve as a reference for the development of infant formulas. The composition of formulas has changed as the nutritional needs of infants have become more widely known. It has been known for many years that breast milk evolves and changes continuously, adapting to the needs of each infant throughout breastfeeding and that it maintains some basic differences with respect to cow's milk, which is the starting point for the development of milk formulas. The four components (probiotics, prebiotics, synbiotics, and postbiotics) can be added to infant milk formulas (Maldonado Lozano, 2014; Salminen, Stahl, Vinderola, & Szajewska, 2020).

These milk formulas are safe and have been shown to be well tolerated by infants, produce no adverse effects, and do not alter growth. In addition, they have a number of beneficial clinical effects such as lower incidence of gastrointestinal and upper respiratory tract infections, less use of antibiotics, lower incidence of allergic processes, mainly atopic dermatitis, in at-risk infants, and lower incidence of constipation. In addition, there are numerous studies linking the composition of the intestinal microbiota and the development of the immune system and how it may modulate the response and the development of long-term allergy and tolerance mechanisms. The use of *L. rhamnosus* GG strain in hydrolyzed milk formulas increases the percentage of infants acquiring immunological tolerance in those allergic to cow's milk proteins (Canani et al., 2012).

The review of the Nutrition Committee of the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) establishes, regarding the possible benefits in the administration of formulas supplemented with probiotics and prebiotics to infants under 4 to 6 months of age, and according to the available evidence, that no consistent clinical effects have been found, although it admits that their addition (both in starter and follow-on formulas) may have beneficial effects on stool frequency and consistency, gastrointestinal infections, reduction in the use of antibiotics and colic irritability. In conclusion, this

Committee does not recommend the use of formulas supplemented with probiotics and/or prebiotics on a routine basis, although it is possible that the addition of certain strains may be advisable. The poor quality of the methodological studies (type of probiotic, duration of intervention) leads the Committee to believe that there is still too much uncertainty. Even so, because more studies with greater methodological rigor are needed, it does not recommend the systematic use of a formula supplemented with these components in infants (ESPGHAN Committee on Nutrition 2011).

Many experts consider that breast milk is an excellent source of commensal and probiotic bacteria for the infant' intestine, as well as containing a large number of oligosaccharides with a prebiotic effect, so it is not surprising that there are profound differences between the intestinal microbiota of breastfed infants and those fed with infant formula. For this reason, many of us think that a rational supplementation of infant milk formulas, supported by controlled clinical trials, with specific probiotic strains, with adequate dosage, and with prebiotics could be justified in the future (Maldonado, Cañabate, & Maldonado, 2016; Skorka, Piescik-Lech, Kolodziej, & Szajewska, 2018).

12.3 Digestive pathology

12.3.1 Acute infectious diarrhea

Most evidence on the efficacy of probiotics has been described for the treatment of diarrhea, estimating that 40% of their use is in this pathology. It is well known that the effect of probiotics is strain specific. Therefore, analyzing different strains and species as a whole does not seem to be the most appropriate approach when evaluating the efficacy of probiotics in the treatment of acute diarrhea. Thus, in recent years, numerous systematic reviews and meta-analyses have been published in which each strain is studied separately and are included in the different clinical practical guidelines (CPG) (Guarino et al., 2014; Salazar-Lindo, Polanco Allué, & Gutiérrez-Castrellón, 2014; Szajewska et al., 2014).

The World Gastroenterology Organization (WGO), in its latest update in 2017 has significantly expanded, in view of the clinical trials performed, the number of probiotic preparations for childhood diarrhea although it continues to emphasize that the strains with the highest level of scientific evidence are *Lactobacillus rhamnosus* GG and *Saccharomyces boulardii* CNCM I-745 (Guarner et al., 2017). The latest revision of the Probiotics and Prebiotics Working Group of the ESPGHAN has decreased the level of evidence and the degree of recommendation for the use of probiotics in infant diarrhea although the most recommended strains continue to be *S. boulardii*, *L. rhamnosus* GG, and, to a lesser extent, *Lactobacillus reuteri* DSM 17938 (Szajewska et al., 2020).

In general, the efficacy of probiotics in the treatment of acute diarrhea in children is produced by shortening the duration of the episode by one day, reducing the number of stools on the second day of treatment, and the percentage of diarrhea that lasts more than 4 days. It seems that the beneficial effects are more noticeable the earlier probiotics are administered in the course of the disease, and no adverse effects are evidenced with their administration. The beneficial effect is more significant in diarrhea caused by rotavirus (Espín & Díaz, 2016).

12.3.2 Antibiotic-associated diarrhea

The consumption of antibiotics is very frequent in developed countries, being three times higher in children than in the adult population. Antibiotic therapy can alter the resistance to colonization of the intestinal microbiota, giving rise to a variety of symptoms, among which diarrhea stands out. We refer to antibiotic-associated diarrhea (AAD) as that which appears from the beginning of antibiotic treatment until 3 to 8 weeks later, being unexplained by any other cause. Its incidence is 11 to 40% in children, according to different statistics, and practically all antibiotics can trigger it, although it is more frequent with broad-spectrum antibiotics (Sanders, Lenoir-Wijnkoop, & Salminen, 2014).

Trials have been able to demonstrate that probiotics in combination with antibiotics reduce the risk of antibiotic-associated diarrhea, although there are, for the moment, no conclusive studies to routinely recommend the joint use of probiotics and antibiotics. However, some strains, mainly *S. boulardii* and *L. rhamnosus* GG, have demonstrated their efficacy in reducing the incidence of AAD. Patients treated with probiotics at high doses (≥5 × 109 CFU/day) have a lower prevalence of diarrhea than the placebo-treated group, with 7 patients needing to be treated with probiotics to avoid one case of antibiotic-associated diarrhea (NNT = 7). Their administration should be carried out at the beginning of treatment and not when the symptoms have been triggered and should be continued throughout the antibiotic treatment (Johnston, Goldenberg, Vandvik, Sun, & Guyatt, 2011). Recent WGO (Guarner et al., 2017) and ESPGHAN (Szajewska et al., 2016) reviews agree that the strains with the most scientific evidence for the prevention of diarrhea in children receiving antibiotic therapy are *L. rhamnosus* GG and *S. boulardii* CNCM I-745, and therefore recommend their use.

12.3.3 Pediatric functional gastrointestinal disorders

Pediatric Functional Gastrointestinal Disorders (PFGD) are defined as a combination of chronic and recurrent digestive symptoms not explained by structural or biochemical abnormalities. In infants, gastroesophageal reflux (GER), colic, and constipation are the main reasons for consultation (23%, 21%, and 18% of all consultations in children under 6 months of age, respectively), while in children over 4 years of age, abdominal pain and constipation are the most common, with prevalences between 5 and 38%.

Probiotics could act, on the one hand, on microbiota alteration and, on the other hand, improving intestinal motility, modifying the perception of intestinal pain by inhibiting intestinal contractility and decreasing intestinal inflammation, as well as inhibiting the growth of gas-producing coliform bacteria in children with colic. Numerous clinical trials have been published on different probiotics in infant colic, especially *L. reuteri* DSM17398, with good results, which is why this strain is recommended in the latest revision of the WGO guidelines for both treatment and prevention (Guarner et al., 2017; Ong, Gordon, Banks, Thomas, & Akobeng, 2019; Szajewska, Gyrczuk, & Horvath, 2013).

In relation to GER and constipation, there is very limited information, and the studies show that although those who receive the probiotic have a higher stool frequency/day at 2, 4, and 8 weeks compared to placebo, no differences are found in stool consistency although a recent systematic review does not support the use of probiotics for the treatment of functional constipation in children (Wojtyniak & Szajewska, 2017). However, the use of prebiotics

increases the water retention capacity of the stool and stimulates the growth of *bifidobacteria*, increasing the average number of stools and decreasing their consistency. Inulin and fructooligosaccharides (FOG) have dose-dependent laxative effects that are attributed to increased microbial biomass as a result of their fermentation in the colon. In a placebo-controlled study in infants evaluating the use of fructose oligosaccharide, the use of prebiotics was shown to be effective with a significant increase in the number of stools and a decrease in stool consistency (Moore, Chao, & Yang, 2003).

The efficacy of probiotics in functional abdominal pain and irritable bowel syndrome (IBS) would be based on their properties on intestinal homeostasis, with the maintenance of the intestinal barrier and modulation of innate and acquired intestinal and systemic immune responses. The most recent meta-analyses corroborate that probiotics decrease IBS symptoms with certain single or combined strains. Some probiotic strains have been shown to be more effective than placebo in the treatment of pediatric patients with functional disorders associated with abdominal pain especially in IBS. Proof of this is reflected in the recommendations of the latest and recent revision of the World Gastroenterology Organization (WGO) guideline on Probiotics and Prebiotics (Guarner et al., 2017). In adults, it has been shown that prebiotics is a healthy, feasible, and effective alternative to the long-term low-FODMAP diet that can negatively impact the microbiota (Huaman et al., 2018).

12.3.4 Probiotics in the treatment of inflammatory bowel disease

In inflammatory bowel disease (IBD), the results of clinical trials with probiotics are contradictory. None have demonstrated efficacy in Crohn's disease, whereas in ulcerative colitis, it appears that the use of VSL#3 contributed to inducing and maintaining remission (Miele et al., 2009). The European Guidelines of the ECCO (European Crohn's and Colitis Organization) reserve a possible therapeutic role for probiotics in certain indications in IBD. Regarding the use of prebiotics such as Plantago ovata, inulin, and barley-derived fiber, the ECCO guidelines do not offer clear indications, given the scarce scientific evidence (Turner et al., 2018). In line with the 2018 guidelines of the ESPGHAN Porto IBD Group, there is limited evidence in favor of using the mixture of 8 strains VSL#3 or *L reuteri* ATCC 55730 as an adjuvant to standard therapy for induction of remission in mild-to-moderate pediatric ulcerative colitis (Miele et al., 2018). It seems evident that this is a promising field to be developed. Further studies are needed to assess which species and strains and what doses should be used.

12.3.5 Short bowel syndrome

In recent years, several studies have been published on the importance of the intestinal microbiota in the etiopathogenesis and symptomatology of short bowel syndrome. However, we should not forget that in many cases we are dealing with immunosuppressed patients, so caution in its administration should be maximized. At present, controlled studies in children with a larger sample size are needed to be able to draw practical conclusions, although probiotics could be used in clinical practice in the short and medium-term (Sentongo & Cohran, 2008).

12.3.6 Bacterial overgrowth syndrome

This is an alteration in the distribution of the intestinal microbiome in children, characterized by abnormal bacterial proliferation in the small intestine, with the presence of bacterial microbiota typical of the colon with a predominance of strict anaerobes and coliforms. The lack of response to the usual medical treatment with antibiotics has made it necessary to investigate other therapeutic approaches, such as the use of probiotics. These would replace the altered microbiota with another with the capacity to inhibit abnormal bacterial growth. The use of certain strains (*L. rhamnosus* GG and *Lactobacillus plantarum* 299V) has been shown to be effective in the treatment of children diagnosed with short bowel syndrome when antibiotics were not effective, inducing an anaerobic microbiota in the colon, increasing short-chain fatty acids in the stool, reducing inflammation, and improving nutritional status (Quigley & Quera, 2006).

12.3.7 Celiac disease

Children with celiac disease have a higher number of Gram-negative bacteria and a lower number of Gram-positive bacteria in their microbiota. In addition, in post-treatment, there are fewer lactobacilli, enterococci, and bifidobacteria, more diversity of eubacteria, and more bacteroides, staphylococci, *Salmonella*, *Shigella*, and *Klebsiella* than in healthy children. It seems that a gluten-free diet for at least two years would be necessary to restore the microbiota and metabolome of these children. However, alterations in the microbiota have been observed not only in untreated celiac patients but also in some who follow a gluten-free diet. Also, some celiac children continue with symptoms, despite receiving a gluten-free diet and normalizing their intestinal mucosa. These findings suggest that dysbiosis could be associated with the persistence of gastrointestinal symptoms in treated celiac patients, thus opening new perspectives for the treatment of this type of patient (Olivares et al., 2018).

12.3.8 Lactose malabsorption

The intestinal microbiota also plays an important role in the tolerance and intolerance of those who maldigest lactose, by conditioning colonic compensation, one of the determining factors of symptomatology. Probiotics in fermented milk derivatives could improve lactose tolerance by modulating the intestinal microbiota, favoring colonic compensation, as well as its degradation by bacterial enzymes. Thus, some strains of *Lactobacillus acidophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, improve its digestibility. The European Food Safety Authority (EFSA) expert panel has found evidence in 15 of the 18 scientific studies presented to affirm that live yogurt bacteria (*L. bulgaricus* and *Streptococcus thermophilus*) improve lactose digestion in individuals with lactose intolerance (Levri, 2005). In fact, the use of yogurt microorganisms to improve lactose intolerance is, at the moment, the only health claim accepted by the CE regulatory framework (Oak & Jha, 2019). A recent systematic review supports the beneficial effects of probiotic supplementation on breath H2 test results and lactose intolerance symptoms as evidenced by decreases in hydrogen exhalation concentration and reduced symptomatology (Leis, de Castro, de Lamas, Picáns, & Couce, 2020).

12.3.9 *Helicobacter pylori* infection

As an adjuvant treatment to *H. pylori* eradication regimens, probiotics could decrease treatment side effects and improve eradication rates. Furthermore, by different immunological and non-immunological mechanisms, such as the production of antimicrobial substances (short-chain fatty acids and bacteriocins), probiotics would compete for *H. pylori* adhesion receptor binding sites on epithelial cells, strengthening the gastric mucosal barrier (stimulating mucin production) and modulating the immune response against pathogens.

Different meta-analyses and studies on probiotics and *H. pylori* have shown that administration of *Lactobacillus casei* DN-114 001 and *S. boulardii* CNCM I-745 strains, according to the 2017 revision of the WGO guideline (Guarner et al., 2017), can improve, as adjuvant therapy, eradication rates and decrease the adverse effects of antibiotic therapy. They may be especially useful in patients with *H. pylori* infection refractory to previous treatment or a history of poor gastrointestinal tolerance of antibiotics. According to 2017 ESPGHAN/NASPGHAN *H. pylori* guidelines, the addition of probiotics to eradication therapy to reduce side effects or improve eradication rates is currently not recommended (Jones et al., 2017). In contrast in adults, given the available evidence, the fourth edition of the Maastricht consensus on the treatment of *H. pylori* infection stated that "certain probiotics show promising results as adjuvant treatment in reducing side effects" (Malfertheiner, Megraud, O'Morain, Gisbert, & Kuipers, 2016).

12.3.10 Non-alcoholic fatty liver disease

In non-alcoholic hepatic steatosis, among other agents, intestinal bacteria, bacterial endotoxins, and TNF-α appear to modulate liver injury. Experimental studies with VSL#3 in rats with non-alcoholic fatty liver disease demonstrate a decrease in liver injury, total hepatocyte fat, and alanine aminotransferase (ALT) levels (Soriano, Sánchez, Poca, Román, & Guarner, 2016).

12.3.11 Hepatic encephalopathy

In experimental studies in cirrhotic rats, a strain of *Lactobacillus johnsonii* has been used for the treatment of hepatic encephalopathy. This antioxidant-containing treatment resulted in a suppression of bacterial translocations, a reduction in ileal and cecal contents of *enterobacteria* and *enterococci*, and a decrease in malondialdehyde levels, compared to the antioxidant-only group. However, in a similar study with *L. rhamnosus* GG, the same results were not found (Miloh, 2015).

12.3.12 Liver transplantation

Administration of *L. casei* DN-114001 (10^{10} CFU) for 2 months to children aged 3 to 17 years at least 2 years post-transplantation, immunosuppressed with tacrolimus, without biliary complications and not requiring antibiotic treatment, was safe and resulted after 2 months in a decrease in β-glucuronidase and β-glucosidase activity, enzymes known to contribute to mucosal tissue damage and mutagen production, reaching basal levels after 4 months (Infante et al., 2008).

12.3.13 Cystic fibrosis

The microbiota of children with cystic fibrosis (CF) is significantly different from that of healthy controls. *Eubacterium rectale, Bacteroides uniformis, Bacteroides vulgatus, Bifidobacterium adolescentis, Bifidobacterium catenulatum,* and *Faecalibacterium prausnitzii* are less prevalent in children with CF than in those with CF who took probiotics (Leis & Peña, 2016).

12.3.14 Pancreatitis

The use of a strain of *L. plantarum* has been effectively tested in the prevention of systemic complications due to bacterial translocations in adult patients with severe acute pancreatitis, with a high decrease in mortality. All these studies open a new therapeutic strategy for the prevention of septic complications in critically ill patients. The use of probiotics in the prevention of sepsis associated with severe acute pancreatitis and post-surgical sepsis has a grade of recommendation B (level of evidence 2B) (Lifschitz & Szajewska, 2015).

12.4 Non-digestive pathology

12.4.1 Cow's milk protein allergy

Cow's milk protein allergy (CMPA) is the most common food allergy in the pediatric age group. It usually appears in the first months of life after the cessation of breastfeeding and manifests with a wide variety of clinical symptoms (from banal digestive processes to anaphylaxis) and may be the result of immune mechanisms (immediate IgE-mediated reactions) or a non-immunological mechanism. Treatment is based on the elimination of the suspected allergen (cow's milk proteins) with the use of substitute milk formulas, generally formulas with a high degree of hydrolysis (Muraro et al., 2014).

It is precisely in this type of special milk, where probiotics and prebiotics are supplemented to avoid the symptoms of CMPA and to acquire immunological tolerance. Although the role of their use in this pathology has yet to be determined and more studies are needed to evaluate their efficacy, numerous controlled clinical trials with different strains are beginning to appear with encouraging results and their use is gaining acceptance in the CPG (Sidbury et al., 2014).

12.4.2 Atopic dermatitis

Atopic dermatitis (AD) is a chronic, relapsing, and pruritic inflammatory skin disease that affects 10 to 20% of children in our environment. It can appear alone or associated with allergic rhinitis, asthma, and food allergy. In 60% of cases, it appears during the first year of life. In moderate and severe cases, it has a significant impact on the quality of life of the patient and his or her parents, due to its psychological, economic, and occupational repercussions. The main symptom of AD is pruritus. The pediatrician should try to unmask triggering factors that are perpetuating eczema lesions: excessive hygiene, lack of use of moisturizing creams, textiles with fiber, and the practice of swimming. Topical corticosteroids are the first-line drugs in the treatment of outbreaks.

Although little studied until now, the microbiota of the skin probably protects this organ and its appendages from many of its diseases and probably contributes to the maintenance of health beyond the dermal area. It is possible that some of the microbes of our skin may be involved in the etiology of many dermatological processes such as atopic dermatitis. This is precisely why probiotics are beginning to be used for the treatment of various dermatological conditions, both in oral and topical preparations. Certainly the studies with the most scientific evidence point to their administration in the prevention of early atopic dermatitis in children at high risk of suffering it, being *Lactobacillus rhamnosus* GG the most studied strain. Even so, most CPG and systematic reviews are of the opinion that more well-designed clinical trials are needed before their routine use can be recommended (Cuello-Garcia et al., 2015; Garcia-Larsen et al., 2018; Szajewska & Horvath, 2018). Specifically, the WAO suggested: "a) *using probiotics in pregnant women at high risk for having an allergic child; b) using probiotics in women who breastfeed infants at high risk of developing allergy, and c) using probiotics in infants at high risk of developing allergy*." All recommendations were conditional and supported by a very low quality of evidence (Fiocchi et al., 2015).

Although the majority of clinical trials are concerned with prevention, increasingly there are studies in relation to the treatment of AD which is the skin disease on which most studies have focused to evaluate the effect of probiotics on the evolution of the disease. The benefit appears greatest if treatment is prolonged, in patients over three years old and in those with a family history of the disease. In the study published by Navarro-López et al. (2018) a probiotic mixture composed of *Bifidobacterium lactis*, *B. longum* and *Lactobacillus casei* was tested and administered for 12 weeks. At the end of the intervention period, clinically and statistically significant differences were observed, reaching a reduction in the SCORAD index of 80% in the probiotic group (Navarro-López et al., 2018).

12.4.3 Prevention of respiratory and otorhinolaryngologic infections

With regard to the treatment of respiratory infections with probiotics, the studies carried out to date show great heterogeneity, some of them finding a positive effect of probiotics in terms of both reduction of the symptoms derived from the infection and the duration of the episodes, although it seems that more studies with a higher level of evidence are needed as the results are not conclusive. The Cochrane review published by Hao in 2015 includes 13 clinical trials with a total of 3720 participants of all ages. The authors concluded that people who ingest probiotics compared to placebo had fewer upper respiratory infections, with shorter duration of infections, reduced use of antibiotic therapy, and lower absenteeism from work. (Hao, Dong, & Wu, 2015).

A recent systematic review of the use of probiotics in the prevention of upper respiratory tract infections shows that most studies show at least a beneficial effect. Even a minimal reduction of 5 to 10% in the incidence of these infections would have an important clinical and economic impact on society. Furthermore, long-term administration of probiotics appears to be safe in children and none of the studies have reported serious adverse effects related to the probiotic strain. However, more studies are needed to recommend probiotics as prevention of extraintestinal infections (Ozen, Sandal, & Dinleyici, 2015).

12.4.4 Prevention of urogenital infections

Lactobacilli are important components of the normal urovaginal microbiota, where they are found attached to the cells of the urinary and vaginal epithelium. Laboratory and clinical studies show that *lactobacilli* are protective against urogenital infections, both through their competition for the ecological niche and through immunomodulation and production of antimicrobial substances and H_2O_2, which inhibit the growth of pathogenic microorganisms.

Urinary tract infection (UTI) in pediatrics is estimated to be 2 to 5%, being the most prevalent serious bacterial infection in febrile infants without focus. Initially, the empirical use of prebiotics is based on the fact that a decrease in the colonization of uropathogens in their reservoirs (intestine, vagina) would be associated with a lower risk of urinary tract infections. Probiotics used for the prevention or treatment of UTI should be supported by clinical trials demonstrating their efficacy for this specific target (McTaggart et al., 2015).

12.4.5 Human immunodeficiency viruses (*HIV*) infection

In the context of HIV infection, studies have shown how the microbiome can affect the risk of transmission of infectious diseases and therefore play a role in prevention at that level. Both the vaginal and penile microbiota modify the risk of sexual acquisition of HIV due to their influence on inflammatory pathways on the one hand and the metabolization of antiretroviral drugs on the other (Klatt et al., 2017; Liu et al., 2013). Recent studies have shown how an altered vaginal microbiome also increases the risk of vertical transmission of HIV (Frank et al., 2012). These studies illustrate the influence of the microbiome on the risk of transmission of infections, its potential in prophylaxis, as well as its implications in pharmacokinetics through bacterial drug metabolization. One preventive strategy that has been validated for its results and whose success is probably due in part to modifications in the microbiome is circumcision, which reduces the risk of HIV transmission and is among the measures recommended by the WHO in high-endemic countries.

12.4.6 Coronavirus infection

Currently, there is no evidence that probiotics or prebiotics can help prevent or alleviate the symptoms of COVID-19, but, as we have discussed, there is evidence that some strains reduce the incidence of common respiratory infections and, especially, prevent, and shorten the duration of viral diarrhea (d'Ettorre et al., 2020). Therefore, although without proof of scientific evidence, for the time being, the possible benefits of probiotics in specific situations can be considered and discussed (ISAPP Newsbib35). All patients hospitalized by COVID-19 are treated with antibiotics and concomitant treatment with probiotics may be beneficial as demonstrated by several meta-analyses.

12.4.7 Dental caries

L. rhamnosus GG inhibits the adherence of *Streptococcus mutans*. Moreover, it competes with cariogenic microorganisms and reduces the risk of caries. In a study of 594 children aged 1

to 6 years, in which milk with *L. rhamnosus* GG was used 5 days a week for 7 months or milk without supplementation, significantly fewer caries and *S. mutans* were found in relation to the unsupplemented group (Ashwin et al., 2015). Therefore, further randomized research is needed to confirm its role in caries prevention.

12.4.8 Probiotics and vaccines

We have known for decades that there is an individual predisposition in the immune response to vaccines. The intestinal microbiota plays an essential role in the development and regulation of the immune system and therefore its structure and composition can condition the way individuals can respond to vaccines (Zimmermann & Curtis, 2018). Although there are promising data on the effects of probiotics as immunization adjuvants, more studies with sufficient statistical power are needed to understand their immunomodulatory properties. The disparity of the observed results may be due to multiple factors such as small sample size, age, the strain used, the duration of treatment, and its temporal relationship with the administration of the vaccine (Zimmermann & Curtis, 2018).

12.4.9 Obesity

Recent studies relate cesarean delivery with dysbiosis in children, due to delayed and reduced exposure to microbial colonization and, therefore, increased risk of developing obesity later in life. The use and abuse of antibiotics at various stages of life (pregnancy, birth, lactation, and early childhood) cause a dysbiosis of the intestinal microbiota with an increase in *proteobacteria* and other anaerobic populations (enterobacteria, clostidia, and bacteroides) and a decrease in beneficial microbiota (bifidobacteria and lactobacilli) which, together with other factors (genetics, diet, lifestyle, etc.), can become a potential risk for the later development of overweight and obesity. *Bifidobacterium* and *Staphylococcus aureus* populations in children are possible direct and inverse predictors of the development of obesity in adulthood (Rodriguez et al., 2013).

Dietary habits, and especially the decrease in dietary fiber intake, have been linked to certain patterns of microbiota composition. The progressive loss of microbial diversity over generations in industrialized societies has been associated with the emerging increase in chronic non-communicable diseases. Diet has strong implications for the development of diseases such as obesity, metabolic syndrome, malnutrition, eating disorders, inflammatory bowel disease, and colorectal cancer, among others. For this reason, studies are needed to identify microbiota-related prognostic biomarkers for these disorders. In turn, healthy diets and specific nutritional interventions, including increased dietary fiber and the consumption of probiotics and prebiotics, could be valuable for the restoration of a more diverse microbiota with the capacity to prevent these diseases. Examples include, on the one hand, a recent meta-analysis showing that the administration of certain probiotic strains significantly reduces body weight, body mass index, and fat percentage in obese individuals (Borgeraas, Johnson, Skattebu, Hertel, & Hjelmesæth, 2018) and, on the other hand, a proposed dietary intervention as a model of the new Precision Medicine and Nutrition (De Filippis, Vitaglione, Cuomo, Canani, & Ercolini, 2018).

12.4.10 Severe malnutrition

Probiotics help to balance the intestinal microbiota, having a useful role in malnutrition where there is a significant risk of bacterial overgrowth and secondary immunodeficiency. Their usefulness is also reflected by their antimicrobial activity (production of acetic acid, lactic acid, and bacteriocins) improving the barrier function. At present, there are no multicenter studies that clarify the benefits and recommend the use of probiotics in severe malnutrition, but it is clear that these therapeutic agents play an important role in immunity and in the balance of the intestinal microbiota, having an important impact on malnutrition (Kerac, 2009).

12.4.11 Hypercholesterolemia

There are intestinal bacteria that are capable of enzymatically modifying cholesterol and converting it into coprostanol, which is its main microbial metabolite in mammals, and which is excreted in the feces. This function is acquired in humans from 6 months of age. Probiotics have the ability to hydrolyze conjugated bile acids, precipitating with cholesterol at a pH below 5.5, and eliminating it in the feces. This loss by the liver is compensated by the production of new bile acids from cholesterol, which could have positive effects on hypercholesterolemia. Studies with probiotic fermented milk are still inconclusive, although some show a decrease in serum cholesterol and low-density lipoprotein (Guardamagna et al., 2014).

12.4.12 Diabetes mellitus type 1

Several investigations highlight the role of the intestinal microbiota in the pathogenesis of type I diabetes, associated with dysbiosis, especially of *Lactobacillus* and *Bifidobacterium*, and alteration of the development of immunity. Recent studies in children at high genetic risk of type I diabetes demonstrated significant differences in the microbiota between children who develop the disease and those who do not. However, the results are not consistent, due to confounding factors such as environmental factors, especially the geographic location and diet (Davis-Richardson & Triplett, 2015, Kelly, Minuto, Cryan, Clarke and Dinan, 2017).

12.4.13 Autism spectrum disorders

There are several studies that address the possible important role that the gut microbiota could play in social behavior, as described in several recent papers, although mostly conducted with experimental animals. The origin of autism spectrum disorders (ASD) seems to occur in the prenatal stage, which is a window of time in which important neurological connections are formed. Maternal infections during gestation and elevated cytokine levels in both mothers and children with ASD appear to play a role. Few studies have been done describing the gut microbiota of individuals with ASD, but the results seem to indicate a higher content of toxin-producing clostridial groups in ASD. It is possible that a lower proportion of beneficial bacteria, such as bifidobacteria and eubacteria, is related to frequent gastrointestinal problems in patients with ASD, such as diarrhea, constipation, meteorism, abdominal distension, and various food intolerances (Li, Han, Dy, & Hagerman, 2017). In

summary, alterations in the composition of the intestinal microbiome in children with ASD seem to be confirmed. However, the available data do not allow us to define a characteristic profile of these patients, as the results are contradictory, perhaps due to the heterogeneity of the patients recruited. These intestinal dysbioses are generally associated with gastrointestinal symptomatology and can sometimes be correlated with the severity of autism although they are not present in all cases (Ho et al., 2020).

The use of special diets, the use of probiotics and prebiotics, and the use of fecal transplantation open a door to hope for the benefit that modification of the intestinal microbiota in neurological and behavioral disorders, such as ASD. The probiotics and prebiotics could improve not only gastrointestinal symptoms in these patients but also behavioral disturbances. In fact, there are several meta-analyses that have assessed the efficacy of probiotics and prebiotics in these patients with improvements in their digestive symptoms as well as in the behavioral attributes of the ATEC autism severity assessment scale, such as language, sociability, learning, or behavior (Xu, Xu, Li, & Li, 2019). Trials have been developed in these patients using a fecal transplant with good safety and excellent results (Kang et al., 2017). Although, in general, promising results have been suggested more well-designed, randomized, placebo-controlled clinical trials are needed to validate the efficacy of probiotics, prebiotics, or fecal transplantation in the treatment of ASD to identify appropriate strains, doses, and duration of treatment. For this reason, caution is necessary, as one of the main goals in these children and adolescents is to improve their quality of life and we should not make the mistake of and we must not fall into the error of creating false hopes for patients and families.

12.4.14 Attention deficit hyperactivity disorder

Attention deficit hyperactivity disorder (ADHD) is today the most prevalent neurodevelopmental disorder. Despite many years of research, its etiology is still unknown. Among the different factors that have been implicated in the risk of developing ADHD, changes in the composition of the intestinal microbiota have also been related, suggesting a link between the microbiome and this pathology. Specifically, it is known that children born by cesarean section are 5 times more likely to develop the disorder than those born vaginally. Preliminary studies in humans also suggest that dietary components that modulate the gut microbiota may influence the development of ADHD or its symptoms, although further studies are needed to confirm this hypothesis and to determine what role the gut microbiota may play in ADHD, as this could open new avenues for early intervention and improve the management of the disease (Cenit, Campillo, Codoñer-Franch, Dinan, & Sanz, 2017).

12.5 Conclusions

The intestinal microbiota is conditioned by genetic and environmental factors and dysbiosis can be a cause of digestive and extra digestive pathology in the short, medium, and long term. Many studies have shown the positive effect of the use of probiotics and prebiotics on the health of children, especially in relation to immunity and inflammation. Pediatrics is one of the medical specialties where controlled clinical trials with probiotics have demonstrated their efficacy and safety in various pathologies, especially digestive.

The effect of the probiotic should be evaluated according to the strain used, the dose, the time of administration, and the duration of administration. In the treatment of acute infectious diarrhea and in the prevention of antibiotic-associated diarrhea, the use of probiotics has been shown to have the highest level of evidence. The inclusion of probiotics and prebiotics in the Clinical Practice Guidelines for pathologies in children and adolescents requires more randomized and follow-up studies, with adequate sample sizes, to demonstrate their long-term efficacy and safety.

Abbreviations

AAD	Antibiotic-associated diarrhea
AD	Atopic dermatitis
ADHD	Attention Deficit Hyperactivity Disorder
AGA	American Gastroenterological Association
ALT	Alanine aminotransferase
ASD	Autism Spectrum Disorders
CE	Conformité Européenne
CF	Cystic fibrosis
CFU	Colony forming units
CMPA	Cow's milk protein allergy
COVID-19	Coronavirus disease 2019
CPG	Clinical practical guidelines
DNA	Deoxyribonucleic acid
ECCO	European Crohn's and Colitis Organization
EFSA	European Food Safety Authority
ESPGHAN	European Society for Paediatric Gastroenterology Hepatology and Nutrition
FAO	Food and Agriculture Organization
FODMAP	Acronym for fermentable oligosaccharides, disaccharides, monosaccharides and polyols
FMT	Fecal microbiota transplantation
FOS	Fructo-oligosaccharides
GER	Gastroesophageal reflux
GOS	Galacto-oligosaccharides
HE	Hepatic encephalopathy
HIV	Human immunodeficiency viruses
HMO	Human milk oligosaccharides
Hp	*Helicobacter pylori*
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
ISAPP	International Scientific Association of Probiotics and Prebiotics
LGG	*Lactobacillus rhamnosus* GG
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEC	Necrotizing enterocolitis

NICU	Neonatal Intensive Care Unit
PFGD	Pediatric Functional Gastrointestinal Disorders
SCFA	Short chain fatty acids
SCORAD	SCORing Atopic Dermatitis
US	United States
UTI	Urinary tract infections
VSL#3	Mixture of 8 probiotic strains (*Lactobacillus plantarum, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus paracasei, Lactobacillus acidophilus, Bifidobacterium breve, Bifidobacterium longum, Bifidobacterium infantis* and *Streptococcus salivarius* subsp. *thermophilus*)
WHO	World Health Organization

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CHAPTER

13

Probiotics in old age

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13.1 Introduction

Aging is the process that refers to the effects of time on the human body and each individual has a different aging rate. This process reflects the biological effects of the gradual accumulation of wear and tear on the various tissues, organs, and systems of the human body. Biological aging is affected by the genetics, environment, and lifestyle of the individuals (Khan, Singer, & Vaughan, 2017).

This process can also be associated with a different composition of the intestinal microbiota or its reconfiguration, and an increased presence of certain pathogens when compared to younger adults (Odamaki et al., 2016). The term inflammaging has been used to describe chronic low-grade inflammatory processes related to aging, while immunosenescence refers to the dysregulation of the immune function in older adults. Both processes are caused, among other factors, by changes in the composition of the microbiota as the organism ages. A pioneering study on specific types of intestinal microbiota and the health of older adults identified two types of metabolism of intestinal bacteria: i) proteolytic and putrefying bacteria that produce harmful metabolites; and ii) saccharolytic fermentative bacteria that produce final metabolites such as the lactic acid (Bischoff, 2016).

Several studies have been carried out to evaluate the effects of commercial probiotic formulations on health conditions related to aging, including gastrointestinal disorders, reduced immunity and infectious diseases, cognitive impairment, among others. In this sense, it is crucial to learn which were the variables, endpoints, approaches, and results from these studies seeking to build evidence for the prescription of probiotics in old age.

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13.2 Age-related changes in microbiota

The gut microbiota is relatively stable in adulthood. Nonetheless, aging causes significant changes in its composition, function, and biochemical activity. Structural and functional alterations in the intestines involve changes in its regenerative capacity, formation, and integrity of the epithelial barrier, composition of the mucus layer, and peristalsis (Bosco & Noti, 2021). In association, changes in dietary patterns, polypharmacy (especially the use of antibiotics and nonsteroidal anti-inflammatory drugs), the reduction or absence of physical activity, and inadequate diets work in synergy and contribute to age-related dysbiosis (Rondanelli, 2015; Salazar, Valdés-Varela, González, Gueimonde, & de los Reyes-Gavilán, 2017).

The processes of immunosenescence and inflammaging have been associated with changes in the microbiota and result in increased pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin 6, and interleukin 8 (Peterson, Sharma, Elmén, & Peterson, 2015). These changes have significant impacts on health, longevity, nutritional status, and disease progression, especially of sarcopenia and frailty (Biagi et al., 2010; Bischoff, 2016; Claesson et al., 2012; Kang et al., 2021).

The gut microbiota of older adults is characterized by reduced diversity of bacteria, modified predominant species, decrease of beneficial microorganisms, increase in facultative anaerobic bacteria, and reduced production of bacterial metabolites (especially short-chain fatty acids). The bacterial metabolism in the colon is altered from a predominantly saccharolytic metabolism (present in younger people) to a predominantly putrefactive metabolism (Salazar et al., 2017) (Table 13.1).

Intervention studies that evaluated the use of probiotics in older adults have shown positive impacts on the microbial profile of the gut microbiota, such as the increase of bifidobacteria, lactobacilli, and enterococci (Ahmed, Prasad, Gill, Stevenson, & Gopal, 2007; Lahtinen et al., 2009), and the reduction of opportunistic pathogens such as *Clostridium difficile*, *Enterococcus faecium*, *Clostridium perfringens*, *Campylobacter*, and enterobacteria (Ahmed et al., 2007; Rampelli et al., 2013). Thus, the modulation of the gut microbiota has been recognized as a possible health-promoting strategy in this age group (Hamilton-Miller, 2004).

TABLE 13.1 Main microbiota changes related to aging.

↓ bacterial diversity
↓ availability of total short chain fatty acids
↓ beneficial microorganisms
↑ levels of lactate, methane, and branched chain fatty acids (valeric, isovaleric, isobutyric, and caproic acids)
↑ facultative anaerobic bacteria
↓ Firmicutes, mainly *Clostridium* cluster XIVa and *Faecalibacterium prausnitzii*, and Actinobacteria (mainly bifidobacteria)
↑ Proteobacteria
↓ Firmicutes, Bifidobacteria, *Clostridium* cluster XIV, *Faecalibacterium prausnitzii*, *Blautia coccoides-Eubacterium rectale*
↑ Enterobacteriaceae, *Streptococcus* spp., *Staphylococcus* spp., *Enterococcus* spp. e *Enterobacter* spp.
↑ or ↓ Bacteroidetes and Lactobacilli
Predominantly putrefactive bacterial metabolism

Source: Peterson et al. (2015), Rondanelli (2015), Salazar et al. (2017).

13.3 The role of probiotics and their indication in old age: What is being prescribed?

The World Health Organization defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Food and Agriculture Organization & World Health Organization, 2001). Probiotics can be found in fermented foods, pills, powders, and drops (Liu, Tran, & Rhoads, 2018). The most widely used strains are the lactic acid bacteria, especially the genera *Bifidobacterium* and *Lactobacillus*, which are, respectively, the dominant and subdominant groups of the gut microbiota (Bermudez-Brito, Plaza-Díaz, Muñoz-Quezada, Gómez-Llorente, & Gil, 2012; Butel, 2014). These genera also include the strains *Lactobacillus rhamnosus GG*, *Lactobacillus casei*, *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Bacillus coagulans*, *Bacillus clausii*, *Bifidobacterium longum*, *Bifidobacterium infantile*, *Streptococcus bacteriums thermophilus*, and *Escherichia coli* (Liu, Alookaran, & Rhoads, 2018; Wilkins & Sequoia, 2017).

Probiotics have received increased attention as part of a healthy diet. Studies are being carried out to determine their potential beneficial effects on the prevention and treatment of a wide variety of systemic conditions, including inflammatory and autoimmune diseases such as rheumatoid arthritis, ulcerative colitis, multiple sclerosis, hepatic encephalopathy (Liu et al., 2018), depression, anxiety (Liu, Walsh, & Sheehan, 2019), infectious diarrhea, allergies, obesity (Butel, 2014), constipation (Liu et al., 2018), among others. However, in an immunologically vulnerable population, probiotics must be used with caution (Wilkins & Sequoia, 2017).

Evidence suggests that the use of probiotics in older adults may have promising effects on the potential to improve cognitive function or to reduce cognitive decline (Kim et al., 2021; Ruiz-Gonzalez, Roman, Rueda-Ruzafa, Rodriguez-Arrastia, & Cardona, 2021; Zhu, Zhao, Zhang, Chen, & Wang, 2021), to improve the gastrointestinal clinical condition (Ghafar, Yaakup, Ali, & Shah, 2020; Rondanelli, 2015), and to modulate the immune response (Hutchinson et al., 2021). Thus, probiotics should be considered as a low-risk adjuvant treatment for specific indications (Setbo, Campbell, O'Cuiv, & Hubbard, 2019). Nonetheless, data analysis is made difficult by the limited number of studies and their methodological inconsistencies (Butel, 2014; Eastwood, Walton, Van Hemert, Williams, & Lamport, 2021; Wachholz, Dos Santos Nunes, Do Valle, Jacinto, & Villas-Boas, 2018).

The World Organization of Gastroenterology is responsible for the most recent guideline about the use of probiotics. However, there is no specific information on their use in the older groups (World Gastroenterology Organisation, 2017). This work aims to contribute to this discussion by approaching the effects of probiotics on the main clinical conditions assessed in older adults.

13.4 Constipation

There is an increased prevalence of constipation with age. This condition is intensified due to several factors such as dietary inadequacies (low intake of fiber and water and high consumption of processed foods), absence or decrease in physical activity, and dehydration. These are enhanced by anatomical and functional changes related to aging, such as mucosal atrophy, reduced intestinal peristalsis, dysmotility, and changes in pH. This

condition is further intensified by the side effects of medication, especially antibiotics and anti-inflammatory drugs, and also by other common situations in this age group, such as diverticular disease and hypochlorhydria (BRASPEN, 2019).

The consequences of constipation include fecal incontinence, fecal impaction, urinary retention, hemorrhoids, anal fissures, rectal prolapse, and cardiovascular alterations, which have significant impacts on the health and life quality of the older adults (Martínez-Martínez, Calabuig-Tolsá, & Cauli, 2017). In this sense, probiotics may be an important alternative for the treatment of constipation and a replacement for traditional drug treatments such as laxatives, which have side effects and a high cost to the health of the patient when chronic use is made (Huang, Zhu, Qu, & Qin, 2018).

In 2017, a systematic review evaluated the efficiency of consuming probiotics in the treatment of constipation in older adults. Nine studies were included, being four placebo-controlled randomized clinical trials and five observational studies. The clinical trials showed that the consumption of probiotics significantly ameliorated constipation by 10 to 40% when compared to placebo. The effects were considered small but significant, especially for the bowel movement endpoint and the appearance of the feces. The strains and genera tested in those works included *Bifidobacterium longum*, *Bifidobacterium lactis*, *Lactobacillus*, and *Streptococcus*. Among the observational studies, there was only one report that indicated a significant effect of the administration of probiotic mixtures (*L. reuteri*, *L. rhamnosus*, and *P. freudenreichii*) on constipation. Due to the heterogeneity of the microorganisms used in the trial, it was not possible to establish a specific recommendation of strain and dosage. However, the authors suggest that the use of probiotics should be considered as an additional therapy to treat chronic constipation in older adults (Martínez-Martínez et al., 2017).

A randomized, double-blind controlled study assessed the effect of a probiotic mixture on older adults living in nursing homes. One group received a probiotic pill with a protective coating and the second group received the same bacterial strains without the protective coating. After 2 weeks there was a significant improvement in the effort to evacuate and the sensation of anorectal obstruction in the group that received the coated probiotic, besides a higher defecation frequency (Yeun & Lee, 2015).

Recently, a randomized clinical trial evaluated the effects of a mixture of probiotics containing *Lactobacillus* and *Bifidobacterium* in 72 hospitalized individuals (≥60 years) with constipation and multiple chronic diseases. A significant improvement in frequency and consistency of feces was observed after 7 days of intervention. The product was well tolerated and had no adverse effects (Ghafar et al., 2020).

Miller (2013) conducted a meta-analysis of 11 clinical trials to assess the efficacy of probiotic supplementation on intestinal transit time. Overall, the short-term treatment with probiotics (10–28 days) improved intestinal transit and reduced constipation in older individuals (≥40 years). The most significant effects were observed for *B. lactis* HN019 and *B. lactis* DN-173 010 (Miller & Ouwehand, 2013).

Most of the clinical trials, systematic reviews, and meta-analysis that indicate positive effects of the intervention with probiotics were carried out with pediatric, adult, or mixed-age populations (Dimidi, Mark Scott, & Whelan, 2020; Ford et al., 2014). However, although there is little evidence of this intervention in older adults, the set of results demonstrate that it is positive within this group. The benefits are observed mainly with the genera *Bifidobacterium* and *Lactobacillus* and the intervention is generally considered safe (Ghafar et al., 2020).

13.5 Diarrhea

The main cause of nosocomial diarrhea is infection by *C. difficile*, often affecting older adults receiving antibacterial treatments. It may have serious health impacts, increasing morbidity, mortality, and health care costs. Adjunctive therapy with probiotics has been tested in recent years, but results are still unclear, especially within older adults (Asempa & Nicolau, 2017; Mizusawa, Doron, & Gorbach, 2015).

Positive effects of this intervention have been found in two systematic reviews with meta-analysis, which assessed the efficacy of probiotics in the prevention of antibiotic-associated diarrhea and *C. difficile* infection using mainly the strains *Saccharomyces boulardii*, *L. rhamnosus* GG, and a mixture of probiotics (Chamberlain & Lau, 2016; McFarland, 2006). However, these studies were carried out with either a pediatric, adult or mixed population. In works carried out specifically with a group of older people, the results were variable and, in general, less significant when compared to the evidence produced in other age groups.

In the clinical trial by Hickson et al. (2007) with 135 patients (average age: 73; sd: 11 years), the administration of probiotic yogurt containing *L. casei* DN-114 001, *S. thermophilus*, and *Lactobacillus bulgaricus* reduced the incidence of diarrhea (frequency of diarrhea: 12% in the probiotic group vs. 34% in the placebo group; $p = 0.007$) and infection by *C. difficile* (frequency of infection: 0 in the probiotic group vs. 17% in the placebo group; $p = 0.001$) (Hickson et al., 2007).

The PLACIDE study (Allen et al., 2013) is the major multicenter clinical trial carried out on this topic, involving approximately 3000 hospitalized older adults (≥65 years). The study produced no evidence that the preparation containing lactobacilli and bifidobacteria was able to prevent diarrhea associated with the use of antibiotics and infection by *C. difficile*. Concerning secondary outcomes, such as the severity of diarrhea, frequency of the gastrointestinal symptoms, length of stay, and life quality, there were also no significant impacts of the intervention with probiotics. Therefore, the authors suggest that the benefits reported in the literature regarding the use of probiotics in the management of diarrhea are less effective in hospitalized older adults.

In another multicenter clinical trial with 1127 patients (>55 years), Rajkumar et al. (2020) verified no beneficial effects after the administration of *L. casei* DN114001 (Rajkumar et al., 2020). Accordingly, the case-control conducted by Alberda, Marcushamer, Hewer, Journault, and Kutsogiannis (2018) with 32 critically ill patients (average age in the probiotic group: 59.9; sd: 15.6) found no significant benefits after the consumption of *L. casei* (Alberda et al., 2018). In a group of older adults (>70 years) hospitalized due to femur fracture, the consumption of a mixture of probiotics *(L. casei*, *L. bulgaricus*, and *S. thermophilus*) had no effect on the reduction of the incidence of diarrhea (Mallina et al., 2018).

A systematic review with meta-analysis indicated that the effects of an intervention with probiotics vary according to the age of the subject. A comparison between outcomes in the adult age group (18–64 years) and the older adult age group (>65 years) evidenced that the beneficial effect of the intervention is age-dependent. Positive impacts of the use of probiotics were found in adults (RR, 0.47; 95% CI: 0.4–0.56) but not in older adults (RR, 0.94; 95% CI: 0.76–1.15) (Jafarnejad et al., 2016).

Thus, overall, the results suggest that the intervention with probiotics in the management of diarrhea in older adults has its singularities such as dose, duration of treatment, and

administered probiotic strain. Further studies are necessary to clarify such elements and target more specific and effective treatments.

13.6 Other gastrointestinal diseases

Studies have shown the beneficial effects of probiotics in gastric infection by *Helycobacter pylori* (Armuzzi et al., 2001; Homan, 2015), irritable bowel syndrome (Dale, Rasmussen, Asiller, & Lied, 2019; Didari, 2015; Ford et al., 2014), and inflammatory bowel disease (Abraham & Quigley, 2017; Derwa, Gracie, Hamlin, & Ford, 2017). However, these studies were carried out with an adult or mixed population, and it is not possible to isolate the effect of the intervention within the older adults age group. The potential of probiotics in the prevention and treatment of intestinal diseases is recognized (Bergonzelli, Blum, Brüssow, & Corthésy-Theulaz, 2005) but issues concerning strain, safe dose, and treatment duration indicate the necessity of more studies and standardization, specifically in older adults. It is also important to assess the integrity of the intestinal barrier due to the risk of bacterial translocation and sepsis. Caution is also recommended with immunocompromised patients with severe acute pancreatitis or those with central vein lines in situ (Abraham & Quigley, 2017).

13.7 Immunity markers

One of the most important consequences of aging is the decrease of the immune system or age-associated immune dysregulation. This condition also includes a decreased proliferative response to mitogens, low activity of natural killer cells (NK), progressive thymic involution, lower degree of diversity and proliferative response of T lymphocytes, and increased levels of pro-inflammatory cytokines. This imbalance is called immunosenescence and it can explain the high susceptibility of older adults to diseases (Salazar et al., 2017).

The gut microbiota plays a critical role in host health due to the interaction with the intestinal epithelium and the immune system. These consequences of the immunosenescence process may be mitigated by the modulation of the intestinal microbiota activity in older adults (Setbo et al., 2019).

Growing evidence suggests that probiotic supplementation may be effective in reducing age-related changes in the composition and diversity of gut microbiota composition. This can impact health outcomes and promote a healthy aging process with improved immunity. However, conflicting results were obtained from randomized clinical trials using a variety of probiotic strains in healthy older adults.

A recent systematic review assessed 17 placebo-controlled randomized clinical trials which analyzed different outcomes of probiotic treatments in healthy older adults, including 11 studies evaluating their effects on the immune system (Hutchinson et al., 2021). The three studies that examined the effects of probiotic supplementation on markers for humoral immunity found some kind of effect, for example, decreases in inflammatory markers such as interleukin 8 (IL-8), tumor necrosis factor (TNF-α), and C-reactive protein (CRP). Furthermore, other studies observed an increased production of anti-inflammatory cytokines such

as IL-10 and interferon-gamma (IFN-gamma), or a decreased production of pro-inflammatory cytokines such as TNF-α. However, the results reported in the different studies are inconclusive, showing changes in opposite directions. Regarding the IL-8, one study showed increases, while another study found decreases. These disparities may be in part attributed to the duration of the intervention since the longer study had more favorable results.

A systematic review with meta-analysis evaluated the association of pre-selected interventions on two inflammatory biomarkers, IL-6 and CRP, in middle-aged and older adults with chronic low-grade inflammation. Although the results were not specified for the older adult age group, they suggest a significant effect of probiotic supplementation on the reduction of these inflammatory biomarkers, besides greater reductions in IL-6 in longer treatments (Custodero et al., 2018).

In addition to humoral immunity markers, the review conducted by Hutchinson et al. (2021) showed that supplementation with probiotics can affect cellular immunity. The reviewed studies showed that this supplementation can neutralize the reduction of the production of T-naïve cells and the accumulation of memory-T cells, which are commonly associated with aging and can increase the number of regulatory T cells and B cells. One study found a decrease in the number of CD8 + CD28 null cells. This is a significant finding since the accumulation of these cells has been associated with a reduced immune response to infection and the immunization response in older adults. The impact of supplementation on the activity of natural killer (NK) cells was less conclusive. Some studies observed a positive impact on the activity and number of these cells, while other studies found no effect (Hutchinson et al., 2021).

These results indicate the beneficial use of probiotics in humoral and cellular immunity markers without establishing specific strains and doses. The findings regarding treatment length are promising and indicate that results are better with longer treatments.

13.8 Cold and other infections

The effect of probiotics on the incidence and duration of the common cold and other episodes of infection have already been studied in the older adults. In a pre-selected population, low and high doses of the heat-killed *L. pentosus* b240 were administered for 20 weeks and the incidence of the common cold was evaluated (Shinkai et al., 2013). Although the mean duration showed no differences between the groups, the incidence showed a trend of dose-dependent decline, in which the high-dose group had a significantly lower incidence rate.

The incidence and duration of common infectious diseases and their subcategories were investigated in a study on the effects of supplementation with a sweetened fermented dairy product containing *L. casei* DN-114001 combined with *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (Guillemard, Tondu, Lacoin, & Schrezenmeir, 2010). Although no significant changes were found in the incidence of common infectious diseases, episode duration and cumulative duration were significantly shorter in the treatment group compared to placebo.

In their systematic review, Setbo et al. (2019) showed the beneficial effect of probiotics on infections common in older adults. A case-control study showed a reduction in the duration of fever from 2.9 days in the controls to 1.5 days in the group treated with the *L. casei* Shirota (Yakult) strain. A randomized clinical trial also using Yakult in 72 older adults measured days

with fever and days with diarrhea for 6 months and found a reduction in febrile days and a decrease in diarrhea in 3 months, but the result has not been confirmed in 6 months. Another study found a significant decrease in respiratory tract infections and influenza-like illnesses in 98 residents of an elderly care facility. The residents were randomized to receive a placebo or treatment with *Lactobacillus coryniformis* 2 weeks before the influenza vaccine. The primary outcome was seroconversion, which improved significantly in the treatment group, corresponding to a statistically significant reduction in the incidence of respiratory symptoms and analgesic consumption. In contrast, a larger study using the *L. casei Shirota* (Yakult) strain in 737 residents of elderly care facilities showed no difference after 3 weeks of treatment before the seasonal influenza vaccine for days with respiratory symptoms or incidence of severe respiratory tract infection (Setbo et al., 2019).

On the other hand, a meta-analysis concluded that there is insufficient evidence to indicate the use of probiotics to reduce the incidence and duration of infections in older adults (Wacholz et al., 2018). Some studies found that supplementation with probiotics may help reduce the incidence of colds and infections or the duration of these conditions. However, when analyzed in a meta-analysis, these results are not confirmed. Therefore, more studies are needed to increase the evidence of the benefits of probiotics in common infections in older adults.

13.9 Bone health

Evidence from studies on the effect of probiotics on serum calcium levels and bone mineral density (BMD) shows that calcium levels were significantly higher in participants after intervention with probiotics compared to those in the control group. Two studies that analyzed BMD using dual-energy X-ray absorptiometry (Jafarnejad et al., 2016; Tu et al., 2015) showed no statistical difference in hip BMD in participants after intervention with probiotics, prebiotics, or synbiotics compared to the control group. Likewise, the spinal BMD also showed no statistical difference between the probiotic and control groups. Three studies examined the effects of supplementation with probiotics, prebiotics, or synbiotics on parathyroid hormone (PTH) and osteocalcin. PTH levels and osteocalcin secretion were not different between participants under the interventions and the control group (Holloway et al., 2007; Jafarnejad et al., 2016; Tu et al., 2015). Three studies assessed the effects of the same supplementation on alkaline phosphatase. The levels of this enzyme were not significantly different in the participants after the intervention as well as compared to the control group (Farhangi, Javid, & Dehghan, 2016; Jafarnejad et al., 2016; Tu et al., 2015).

The studies that evaluated the effect of probiotic supplementation on bone health found that increased serum calcium levels were the only positive result. There was no significant difference for the other outcomes, BMD, and hormone levels compared to the controls.

13.10 Alzheimer's disease

Alzheimer's disease (AD) is a neurodegenerative disease marked by the accumulation of beta-amyloid plaques, neurofibrillary tangles, and brain atrophy that cause cognitive and

behavioral damage (Paitel, Samii, & Nielson, 2021). According to the World Alzheimer Report 2019, more than 50 million people are living with the disease worldwide, and this prevalence is predicted to increase to 152 million people by 2050 (Alzheimer's Disease International, 2019). Although the high incidence of the disease, its pathogenesis is not fully understood (Borstel et al., 2021).

Recent experimental and clinical evidence has demonstrated the link between the gastrointestinal tract and the brain, the so-called gut-brain axis, and the activation of intestinal inflammation as a possible pathogenic cofactor in cognitive decline and the development of dementia. In this sense, the intake of probiotics has been indicated in the prevention and treatment of AD (Akbari et al., 2016). Recently, a systematic review with meta-analysis evaluated the effects of supplementing probiotics and prebiotics in people with mild cognitive impairment (MCI) and AD and indicated that probiotics supplemented in adequate amounts for 12 weeks or more can improve cognitive function in individuals with AD. However, because of the insufficient evidence from current randomized controlled trials and high heterogeneity, the authors recommend further large-scale randomized controlled trials to investigate the neuroprotective effects of probiotics at different stages of AD in adults and seniors. Seven out of the eight studies included in the review were assessed by the meta-analysis. The strains tested included *L. fermentum*, *L. plantarum*, *B. lactis*, *L. acidophilus*, *B. bifidum*, *B. longum*, *L. acidophilus*, *L. casei*, *B. breve*, *L. rhamnosus* GG, *Acetobacter aceti*, *Acetobacter spp.*, *L. delbrueckii*, *L. fermentum*, *L. fructivorans*, *Enterococcus faecium*, *Leuconostoc spp.*, *L. kefiranofaciens*, *Candida famata*, and *Candida krusei*. Strains belonging to *Bifidobacterium* and *Lactobacillus* genera are more often characterized as having great potential for psychobiotic candidates; however, the authors point out that not all probiotics have this property (Zhu et al., 2021).

Ji and Shen (2021) reviewed the advances in research on the effects of probiotic administration to control AD (Ji & Shen, 2021). The authors found that, in general, the currently available evidence shows that the administration of probiotics provides neuroprotective benefits by attenuating cognitive deficits and modulating dysbiosis in the intestinal microbiota, which may be related to oxidative and inflammatory pathways. The review included 35 studies, where 26 were based on animal models and nine on clinical trials. A total of 13 studies used single strain probiotics and the others used multi-strain probiotics (ranging from two to nine probiotic strains); four studies used milk fermented with probiotics or soy fermented with probiotics; two studies used designed probiotic strains, and four studies focused on the combined effect of probiotics with the AD drug memantine, selenium, or exercise. It is of note that species belonging to Bifidobacterium and Lactobacillus genera were the most frequently used in these studies.

In a recent systematic review and meta-analysis, Den, Dong, Chen, and Zou (2020) also found that supplementation with probiotics improves cognitive performance in patients with AD or mild cognitive impairment (MCI), possibly through decreased levels of inflammatory and oxidative biomarkers (Den et al., 2020). The review included studies published from 2016 to 2019, with an aggregated sample size of 297 individuals. All studies were randomized, double-blind, and controlled. Three of the studies selected individuals diagnosed with AD and two included adults with MCI, with a duration of intervention of 12 weeks. In addition, two studies used the analysis of a single strain of probiotics, while the other three studies used multiple strains. The review also showed that three studies found significant differences

in the improvement of cognition between the probiotic and control groups, one study found no significant difference and another study reported variable results. Strains tested included *B. bifidum, B. breve, B. lactis, Bifidobacterium longus, L. acidophilus, L. casei, and L. delbrueckii*. However, the authors also state that current evidence is insufficient, and large-scale, long-term randomized clinical trials are required to obtain more reliability.

The results suggest, in general, that the modulation of the gut-brain axis with probiotics has beneficial effects in the management of AD. Evidence to support the safe recommendations for intervention to manage this condition should be provided with further large-scale and well-designed clinical studies.

13.11 Parkinson's disease

Parkinson's disease (PD) is the second most common neurodegenerative disorder following Alzheimer's disease (AD) and is characterized by cardinal motor symptoms such as bradykinesia, rigidity, resting tremor, and cognitive impairment (Aarsland et al., 2021). It presents, in addition, a heterogeneous spectrum of non-motor symptoms that may precede the motor symptoms, for example, olfactory and gastrointestinal dysfunction (Sales et al., 2021). These features can severely affect the quality of life of people living with PD. Recently, the close bidirectional relationship between the central nervous system and the enteric nervous system has gained prominence in the literature, and the impact that gut-brain axis dysfunctions can cause. Restoration of the intestinal microbiota with probiotics has been identified in recent literature as a potential adjuvant in the management of PD (Tamtaji et al., 2019). A recent randomized, double-blind, placebo-controlled clinical trial examined the effects of a capsule-shaped probiotic product containing *L. acidophilus*, *B. bifidum*, *L. reuteri*, and *L. fermentum* on clinical and biochemical profiles in PD patients. A 12-week administration of probiotic was beneficial to improve MDS-UPDRS (Movement Disorders Society Unified Parkinson's Disease Rating Scale), high-sensitivity C-reactive protein, blood glutathione, malondialdehyde, and insulin metabolism, but did not affect other metabolic parameters (Tamtaji et al., 2019).

The effects of a new probiotic composed of lactic acid bacteria and bifidobacteria (SLAB51) (traded in Switzerland) on PD was tested in a PD model, first in vitro and subsequently in vivo, using dopaminergic neurons and behavioral tests (Castelli et al., 2020). The authors found that the administration of probiotics increases the protection of dopaminergic neurons, improves behavioral deficiencies, and neutralizes the neuroinflammation and oxidative stress characteristic of PD, both in vivo and in vitro. SLAB51 was then recognized as a candidate probiotic for prevention or treatment of PD or adjuvant therapy, confirming that the modulation of the intestinal microbiota affects different pro-survival pathways, leading to a possible delay in the progression of PD. However, they also claim that more studies are needed.

Gazerani (2019) conducted a review of the status of probiotics in the management of PD and found evidence from human and animal studies that the common benefit of using probiotics is the restoration of the intestinal microbiota and, consequently, the maintenance of immunological homeostasis (Gazerani, 2019). The hypotheses that predict the benefits of using probiotics in the management of PD include the ability to change the composition of the microbiota, improve gastrointestinal function and, consequently, reduce the risk of intestinal secretion, bacterial translocation, and the development of neuroinflammation associated

with the enteric nervous system. However, precise mechanisms underlying the effects of probiotics in PD are poorly understood. In addition, more consistent study designs are needed for probiotic strains, strain combinations, duration of intervention, and doses. There is a lack of information on whether persistent exposure of PD patients to probiotics could lead to long-term colonization of these individuals' microbiota, or whether it would return to its original state after discontinuing the intervention with probiotics. Therefore, although the beneficial potential of using probiotics in the management of PD has been demonstrated, the limited and nonuniform data strengthen the need for caution in interpreting and extrapolating the results.

13.12 Other psychiatric disorders

As stated before, the bidirectional link between the brain and the gut represents a symbiotic relationship that contributes to both the health of the host and disorders of the body and brain. It is believed that this communication pathway is underlain by neural, endocrine, and immune mediators. However, the mechanisms are still imprecise. In particular, there is growing evidence of an association of the gut microbiome with psychiatric and cognitive dysfunction (Soto et al., 2018; Zhu et al., 2020). In this sense, probiotics have been investigated continually for their potential to reduce cognitive deficits and improve cognition in the absence of clinical impairment (Eastwood et al., 2021).

A systematic review was conducted to assess the effects of probiotics on central nervous system function in animals and humans. Thirty-eight studies were included in the review, being 25 with animal models and 15 with clinical trials (2 studies were performed in both). The analysis of the studies indicated that *B. longum*, *B. breve*, *B. infantis*, *L. helveticus*, *L. rhamnosus*, *L. plantarum*, and *L. casei* were the species most effective in improving behaviors related to psychiatric disorders, including anxiety, depression, autism spectrum disorder (ASD), obsessive-compulsive disorder, and memory skills, including spatial and non-spatial memory. The authors called attention to the fact that, in most studies, the doses of probiotics that had an effect on behavior ranged from 10^9 to 10^{10} colony-forming units (CFU). They indicate that more experimental designs with behavioral and neuroimaging measures are needed (Wang, Lee, Braun, & Enck, 2016). The review could not assess the effect in the older adults because the studies included were carried out with adult, pediatric, or mixed-age populations. In a multicenter randomized clinical trial with 53 patients (≥65 years), Kim et al. (2021) found a significant reduction in intestinal bacteria causing inflammation in the older adults ($p < 0.05$) after administration of the probiotics *B. bifidum* BGN4 and *B. longum* BORI in a 12-week trial (Kim et al., 2021). Moreover, the same group demonstrated better results in the test of mental flexibility and stress score than the group of older adults who received the placebo ($p < 0.05$). Additionally, unlike a placebo, probiotics significantly increased the serum level of BDNF (neurotrophic factor vital for synaptic formation, plasticity, and neuroimmune responses) ($p < 0.05$). Other studies in adult populations found that the composition of the intestinal microbiome is associated with mood, knowledge, and behavior and that its manipulation through probiotic supplementation improves depressive and anxiety symptoms in healthy adults (Sanborn, Azcarate-Peril, Updegraff, Manderino, & Gunstad, 2018). However, it is not possible to assess the effect of this relationship in the older adults age group.

The results of several studies have supported the role of the gut microbiota and its modulation through the use of probiotics in the management of neurological disorders. However, the highly heterogeneous methodologies used in the studies make the critical interpretation of the available literature a challenge.

13.13 Conclusions

Different studies show the benefits of using probiotics in diarrhea, constipation, intestinal diseases, immunity markers, increased serum calcium levels, Alzheimer's disease, Parkinson's disease, and other neurological disorders. However, there are variations between studies, even for the same clinical condition, in the dose administered, duration of treatment, and strains used. These factors can interfere with the results and compromise the comparability between studies. Furthermore, there is a significant lack of data regarding the safety profile of these interventions. Considering its administration to older adults, individuals who often present multimorbidity associated with polypharmacy comprise typically the group with the growing use of probiotics in clinical practice. The body of evidence allows us to conclude that the interpretation and extrapolation of results of the effects of probiotic supplementation in older adults require caution because of the heterogeneity of the methodologies used in different studies. Thus, more clinical studies with standardization of methodologies regarding the dose, duration of intervention, strains, and sample representativeness in the age group are needed to support appropriate probiotic recommendations for this population.

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CHAPTER

14

Probiotics in sports and physical exercise

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14.1 Introduction

Gut microbiota is extensive and comprises several host microorganisms that directly influence nutritional processes, energy metabolism, nutrient absorption, immune system development, and barrier function, besides playing a regulatory role in inflammation and oxidative stress. Some situations, as exercise training, can alter the gut microbiome causing metabolic repercussions, especially in athletes (Mach & Botella, 2017).

Athletes are at risk of various GI disorders caused by inflammation, altered immune function, oxidative stress, insufficient nutrition, inadequate rest period, and psychological distress that directly influences the health of these individuals (Clark & Mach, 2016). During extensive and prolonged training, discomfort, abdominal cramps, reflux, nausea, vomiting, diarrhea, and splanchnic hypoperfusion may occur (de Oliveira, Burini, & Jeukendrup, 2014).

Sports athletes such as marathons, triathlon, and high endurance are the ones most likely to have health problems and are at risk of metabolic endotoxemia. There has been increasing scientific evidence regarding the nutritional supplementation with probiotics to avoid harmful conditions in trained individuals (Leite, Student, West, & Lancha, 2019). Probiotics are selective microorganisms that have beneficial characteristics like those that are part of the intestinal microbiota and can be considered as a strategy to prevent or treat several GI pathologies, mainly due to the role of homeostatic maintenance and control of the body immunological defenses (Wieërs et al., 2020; Williams, 2010).

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Supplementation of probiotics and their efficacy must be associated with the type of disease, pathophysiological characteristics, clinical indication, duration of treatment, and the specific case of each patient (Gupta & Garg, 2009). Probiotics can be ingested as food components or dietary supplements and maybe composed for a single or several types of microorganisms/strains (Kim et al., 2019).

In this context, the consequences acquired with the use of probiotics indicate positive effects on the GI tract and promote the better performance of professional athletes (Vitale & Getzin, 2019). The use of probiotics associated with physical exercise indicates favorable results in the immune system and improvement of inflammatory markers, being considered an important factor to reduce the incidence, duration, and/or severity of adverse events in these individuals (Rawson, Miles, & Larson-Meyer, 2018; Vitale & Getzin, 2019).

In this chapter, we discuss the basis of intestinal tract functions, exercise repercussions in the gastrointestinal (GI) tract, benefits, and repercussions of exercise in gut microbiota, as well as the impact of nutritional interventions, and supplementation of probiotics for athletes.

14.2 Morphofunctional considerations of the gastrointestinal tract

Digestion of nutrients is one of the main roles of the GI tract through various processes, which include secretion of enzymes and absorption of ingested food and liquids that move through the peristalsis of the intestinal smooth muscle (Liao, Zhao, & Gregersen, 2009). GI tract can be divided into an upper portion consisting of the mouth, pharynx, esophagus, and stomach, and a lower portion consisting of the small intestine, which is divided into the duodenum, jejunum, ileum, and colon (Greenwood-Van Meerveld, Johnson, & Grundy, 2017).

There are several events responsible for neural control within the GI tract and this coordination is mediated by a specific system known as the enteric nervous system (ENS), considered a third component of the autonomic nervous system (ANS) (Gibbons, 2019). Within the endocrine system, GI functions are performed by enteroendocrine cells (EEC) widely distributed throughout the GI regions that connect by afferent fibers to the CNS, sending intestinal reflexes and, at the same time, receive commands by efferent fibers that leave the nervous system and forms the characteristic intestine-brain axis (Gibbons, 2019). Information is related to motility, secretion, glucose metabolism, and control of food intake (Latorre, Sternini, De Giorgio, & Greenwood-Van Meerveld, 2016).

The enteric nervous system (ENS) is formed by the myenteric plexus and the submucosal plexus. The myenteric plexus is responsible for the contraction and relaxation of the GI smooth muscle, while the submucosal plexus regulates the secretory and absorptive functions, the local blood flow, and the neuroimmune response (Furness, 2000). The myenteric and submucosal plexus receive sympathetic and parasympathetic stimuli from sensory neurons that originate in the spinal ganglia, although the main mechanism regulated by the SEN occurs by the innervation performed by the neurons themselves (Galligan, 2002).

The GI motility is controlled in an extrinsically and intrinsically way (Spencer & Hu, 2020). Extrinsic or peripheral control is composed of sympathetic and parasympathetic fibers that act directly in brain-intestinal communication, whereas intrinsic control is released by

transmitters of enteric neurons that modulate the peristaltic reflex such as acetylcholine and substance P, representing main excitatory neurons, and vasoactive intestinal polypeptide (VIP) and nitric oxide, composing prevalent inhibitory neurotransmitters (Ameku, Beckwith, Blackie, & Miguel-Aliaga, 2020; Kellow et al., 1999).

Extrinsic gut enteric sensory neurons can be five types: vascular afferents neurons considered the most complex in both intramural and extramural axons in enteric ganglia; intraganglionic axons; mucosal afferents that innervate the subepithelial layer of the mucosa; muscular-mucosal afferents with deep mucosal endings; and intramuscular afferents with nerve endings within the longitudinal and/or circular layers (Brookes, Spencer, Costa, & Zagorodnyuk, 2013).

The GI tract receives extrinsic neural innervation from the parasympathetic and sympathetic pathways controlled by the caudal nucleus of the brainstem. While the sympathetic nervous system exerts an inhibitory effect on the GI muscle and mucosal secretion regulates the blood flow of the GI through vasoconstriction. The parasympathetic nervous system exerts both excitatory and inhibitory control over gastric and intestinal motility and tone (Travagli, Hermann, Browning, & Rogers, 2006).

In this sense, GI functions are highly regulated by a neuroendocrine communication system. This connection starts with the innervation of the GI tract by vagal neurocircuits that communicate with specific regions of the caudal brainstem, such as the nucleus of the solitary tract, dorsal motor nucleus of the vagus, and the ambiguous nucleus (Browning & Travagli, 2014).

While neurons in the nucleus of the solitary tract are responsible for receiving visceral sensory information, the dorsal motor nucleus of the vagus and the nucleus ambiguous is responsible for sending information to the vagal motor fibers. The efferent fibers of the dorsal motor nucleus of the vagus and the nucleus ambiguous make synaptic communication with postganglionic neurons located in the target organ to promote the modulation of motility and other visceral functions (Travagli & Anselmi, 2016).

The Vagus nerve innervates most organs, especially in the GI tract, and is a key component of the ANS (Bonaz, Sinniger, & Pellissier, 2021). It is a mixed (essentially sensitive) nerve containing 80% of afferent fibers that transmit somatic and taste sensations and 20% of efferent fibers representing the parasympathetic branch of the ANS that leads to the release of acetylcholine (ACh) at the neuromuscular junction, fibers intrinsic nerves or secretory cells, besides modulating GI motility as well as secretion at the level of the digestive tract (Yuan & Silberstein, 2016).

The presence of extrinsic autonomic innervation is also important for the maintenance of the GI tract functions, including the sympathetic (noradrenergic), which covers the splanchnic nerves, and the parasympathetic (cholinergic), with the vagal nerves. The vagus nerve originates from the central nervous system (CNS) and mainly innervates the parasympathetic nervous system connecting parasympathetic efferent fibers to the GI tract (Browning, Verheijden, & Boeckxstaens, 2017). In the GI tract, motility control is mediated by vagal function and any change in stimulation can cause its dysfunction (Bonaz, Sinniger, & Pellissier, 2017).

The regulation of GI functions can be via endocrine modulation, which comprises hormones excreted by enteroendocrine cells. These are known as incretins and are released and transported by the bloodstream through the portal circulation to the systemic circulation until reaching the target cells and their respective receptors (Parikh & Thevenin, 2021).

The intestine also performs immune functions, and the presence of inflammatory mediators can influence the recruitment of lymphocytes and other immune system cells to the intestinal wall including mast cells (Wouters, Vicario, & Santos, 2016). The interaction between the CNS and the intestinal immune system is of great importance in the maintenance of tissue homeostasis and the regulation of inflammatory responses in the mucosa. In the GI tract, the vagus nerve seems to play an important role as an anti-inflammatory through direct modulation of enteric neurons and indirect with splenic immune cells (Goverse, Stakenborg, & Matteoli, 2016). In this process, efferent vagal activation induces the release of acetylcholine and other immunomodulatory neurotransmitters by myenteric neurons located in the intestinal wall indicating the participation of the vagal cholinergic pathway in the decrease of cytokines release, restoring intestinal homeostasis (Mateolli & Boeckxstaens, 2013).

Regarding the intestinal microbiota, it is mainly composed of four types of bacteria in healthy humans: Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria. Moreover, another microorganism as fungi, viruses, and archaea also shares complex relationships with the human host, ranging from symbiosis to parasitism. The imbalance of the intestinal microbiota with a reduction in biodiversity and an increase in pathogenic bacteria is known as intestinal dysbiosis and is associated with the pathogenesis of several diseases, such as Crohn's disease, irritable bowel syndrome, ulcerative colitis, and colorectal cancer (Milani et al., 2017; Morgan et al., 2012).

It is important to mention that the organism has natural defenses against pathogenic microorganisms, the first defense being the mucous layer that covers the intestinal epithelium. This barrier is constituted by the polymerization of mucin-forming mucin-like proteins, which expand into the lumen due to their ability to bind with water. The second line of defense concerns the intestinal epithelium itself, consisting of enterocytes and specialized epithelial cells, such as goblet and Paneth cells, and the integrity of the epithelium is maintained through the action of adhesion proteins, tight junctions, and desmosomes that form complexes at the apical junctions of the epithelium (Geremia, Biancheri, Allan, Corazza, & Di Sabatino, 2014).

The altered intestinal microbiota contributes to the activation of the immune system, as the presence of pathogenic microorganisms is associated with the release of molecular patterns associated with pathogens and activation of Toll-like receptors (TRLs) through lipopolysaccharides (LPS) present in the membranes of Gram-negative bacteria. The activation of these receptors allows the translocation of nuclear transcription factor kappa B (NF-κB), a factor that induces the transcription of pro-inflammatory cytokines and increased oxidative stress, triggering a cascade of inflammatory reactions in the GI tract and activation of the immune system (Cătană et al., 2015).

14.3 Gastrointestinal tract and exercise

For a long time, studies involving exercise physiology were chiefly focused on adaptations related to the respiratory, cardiovascular, neuroendocrine, and neuromuscular systems (Adams & Linke, 2019; Ruegsegger & Booth, 2018). Still, the GI tract has also deserved great attention given the repercussions caused by physical exercise (Codella, Luzi, & Terruzzi, 2018; Dalton, Mermier, & Zuhl, 2019; Sohail, Yassine, Sohail, Al Thani, 2019).

During the 1800s, Beaumont made the first observations of how GI function was modified during exercise. Then, the physiologist Anton Julius Carlson became a pioneer by publishing on the subject (Carlson, Johnson, & Cavert, 1961). After Carlson, studies on exercise and the GI tract became less frequent. However, in the late 1960s, driven by the consumption of energy drinks and the practice of running, there was a greater interest in the study of GI function and physical exercise. From the 1980s onward, several researchers began to investigate the effects of exercise on the GI tract through clinical and epidemiological studies, in particular its harmful effects (Martin, 2011).

Among these repercussions, the appearance of GI symptoms in physical exercise practitioners and athletes is common, such as nausea, diarrhea, vomiting, and intestinal bleeding, particularly in high-intensity exercises. In addition, physical exercise also seems to act in the prevention of several diseases related to the GI tract, such as colon carcinoma, diverticulitis, cholelithiasis, and constipation. Thus, several studies have shown interest in the relationship between exercise and the GI tract, with an emphasis on disease prevention and treatment (Bilski, Mazur-Bialy, Wierdak, & Brzozowski, 2013; Silva et al., 2014).

The GI motility results from the activity of the digestive tract musculature. This intrinsic muscle activity is called motility. This varies according to the segment and circumstances, notably the dietary condition. Shortly after food ingestion, segmentation contractions predominate in the motor behavior, where neighboring GI segments contract simultaneously and continuously (Browning & Travagli, 2014). On the other hand, under fasting, the migratory motor complex occurs, a pattern of cyclic and consecutive motility, passing from the stomach until reaching, approximately 90 minutes later, the ileocecal valve. Given the similarity in morphology, such activity variation results from neurohumoral regulatory mechanisms (Hansen, 2003).

Motility of the GI tract is highly coordinated by neurons of the myenteric plexus present throughout the GI tract. The myenteric plexus regulates peristalsis, modulating the contraction of the muscle wall, both frequency, and intensity of contraction. The essential stimuli for the myenteric plexus come from the mechanical distension of the gastric wall through food, as well as from the irritation of the epithelium and the activity of the extrinsic nervous system (Spencer & Hu, 2020). Although the human stomach is anatomically a single viscera, its motor behavior is quite distinct in the proximal and distal portions. In the proximal portion, there is an accommodation of the ingested food without major changes in intraluminal pressure, thanks to receptive relaxation. After the gastric secretions are mixed, the food passes to the distal stomach from where it is emptied into the small intestine (Furness, 2012).

The practice of physical exercise per se promotes physiological adjustments whether in the neuromuscular, cardiovascular, respiratory, endocrine, or even in the GI tract (Gritti, Banfi, & Roi, 2000). However, such adjustments do not occur singly and linearly in the various systems, as they depend on the time, intensity, volume, and nature of the exercise, in addition to the energy sources that govern such activities.

Another very important point of exercise on the GI tract is related to the possible risks and benefits caused by this practice. In this sense, the impact of exercise and physical activity on the GI tract has been of emerging interest in the scientific community. For more than two decades, research has focused primarily on the risks of strenuous exercise, especially GI symptoms. However, over the past few years, interest has also turned to the potential benefits of exercise on the GI tract. Several studies indicate an inverse relationship between exercise

TABLE 14.1 Summary of the main benefits and risks of exercise on the gastrointestinal tract.

	Benefits	Risks
Esophagus	None	Acid reflux induction
Stomach	Low intensity GE	High-intensity GE and Acid production
Small intestine	None	High-intensity exercise interferes absorption and induces bleeding
Colon	Exercise risk of colon cancer and diverticulitis	High-intensity exercise bleeding
Liver	None	None

GE: gastric emptying.
Adapted from Bi and Triadafilopoulos (2003).

and the risk of GI diseases, such as colon cancer, diverticulitis, colitis, or constipation (Brown et al., 2018; de Oliveira Santos et al., 2021; Johannesson, Ringström, Abrahamsson, & Sadik, 2015; Klare et al., 2015; Onerup et al., 2017; Table 14.1).

Acute physical exercise seems to influence gastric motility, in a dose-response relationship with intensity, with low-intensity exercises seeming to induce an acceleration of the gastric emptying rate, while at high intensity there is a delay in gastric emptying. Different modalities, the volume of food intake, osmolality of energy drinks, and exercise duration also seem to have effects on gastric motility (Horner, Schubert, Desbrow, Byrne, & King, 2015).

The performance of aerobic exercise on a treadmill, of moderate-intensity (at 75% of the maximum heart rate) for 25 minutes, was able to increase gastric compliance in healthy men and women but did not change the perception of satiety in these individuals (Severo et al., 2019). Changes in gastric compliance can be attributed to neuroendocrine adaptations promoted by exercise (Severo et al., 2019). It is noteworthy that physical exercise can induce repercussions on the autonomic nervous system, thus influencing the GI tract. There is an increase in function mediated by the vagus nerve via the parasympathetic nervous system, which contributes to reducing sympathetic excitability and maintenance of sympathovagal balance (Besnier et al., 2017; Fu & Levine, 2013).

In addition, Carvalho et al. (2020) found that acute anaerobic exercise was able to increase gastric accommodation and reduce satiety in healthy men, with these effects being mediated by the secretion of lactate, CK, and some plasma cytokines, such as interleukins (IL)-6, -13 and tumor necrosis factor α (TNF-α). In addition, one of the possible explanations for this phenomenon concerns the activation of cholinergic pathways that increase gastric tone and increase the release of nitric oxide, promoting increased gastric accommodation mediated by physical exercise (Carvalho et al., 2020; Silva et al., 2021).

14.4 Microbiota, chronic diseases, and exercise

Exercise, especially moderate training, is associated with positive changes in the gut microbiome. This positive modulation has been studied in the context of several diseases, like atherosclerosis, obesity, diabetes, and immune-based diseases, as seen in Table 14.2

TABLE 14.2 Clinical trials showing a relationship between exercise and gut microbiome.

Author, year	Patients	Exercise	Outcomes
Kern et al. (2020)	88 obesity and overweight	habitual living (CON) active commuting by bike (BIKE) exercise of moderate-intensity (MOD, 50% of VO_2 peak-reserve) exercise of vigorous-intensity (VIG, 70% of VO_2 peak-reserve)	Shannon's diversity index increased by 5% in VIG (CI95 1–9%, p = 0.012) at 3 months compared with CON; Beta diversity changed in all exercise groups compared with CON, particularly the participants in VIG showed decreased heterogeneity. The inferred functional potential of the microbiota in the exercise groups was increased, primarily at 3 months and in MOD.
Motiani et al. (2020)	26 sedentary subjects with insulin resistance	sprint interval (SIT) moderate-intensity continuous training (MICT)	Both training modes reduced systematic and intestinal inflammatory markers (tumor necrosis factor-α, lipopolysaccharide-binding protein) (time P <0.05); Training modified microbiota profile by increasing *Bacteroidetes* phylum (time p = 0.03) and decreasing *Firmicutes/Bacteroidetes* ratio (time p = 0.04); There was a decrease in *Clostridium genus* (time p = 0.04) and *Blautia* (time p = 0.051); Only MICT decreased jejunal fatty acid uptake (p = 0.02).
Pasini et al. (2019)	30 patients with type 2 diabetes	program of endurance, resistance, and flexibility training	Chronic exercise reduced intestinal myocytes overgrowth, leaky gut, and systemic inflammation.
Liu et al. (2020)	39 medication-naive men with prediabetes	70-min high-intensity combined aerobic and resistance interval training sessions	Alteration in the gut microbiota promoted by exercise was significantly associated with the percentage reduction of HOMA-IR (p < 0.01); Among the 19 species significantly correlated with the improvements of glucose homeostasis and insulin sensitivity, *Ruminococcus gnavus, Alistipes shahii, Streptococcus mitis group, Eubacterium hallii,* and *Escherichia coli* showed the strongest associations.
Taniguchi et al. (2018)	33 elderly men	Endurance exercise intensity increases progressively from 60% of pre-exercise VO_2 peak to 75% during weeks 4 and 5	Changes in a-diversity indices during intervention were negatively correlated with changes in systolic and diastolic blood pressure, especially during exercise. Microbial composition analyses showed that the relative abundance of *Clostridium difficile* significantly decreased, whereas that of *Oscillospira* significantly increased during exercise as compared to the control period. The changes in these taxa were correlated with the changes in several cardiometabolic risk factors.

(Allen et al., 2018; Chen, Guo, Gui, & Xu, 2018; Codella et al., 2018; Pasini et al., 2019). Some mechanisms can explain the positive effect of exercise in gut microbiota, as alterations in gut transit time, in metabolites profile, in pathways related to inflammation, and immunological regulation (Donati Zeppa et al., 2019). In individuals previously physically inactive, short-term moderate exercise caused alterations in microbiota composition in several microorganisms, but no collective alterations were detected (Moitinho-Silva et al., 2021).

In individuals with inflammatory bowel diseases, physical exercise seems to act in the improvement of quality of life and can be considered as an alternative therapy due to the role of skeletal muscle in the secretion of myokines such as irisin, interleukin (IL)-6, IL-15, and IL-1ra released during and after the exercise session. About irisin, it seems to play an important role in regulating metabolic functions in adipose tissue, which are altered in the presence of Crohn's disease and ulcerative colitis (Bilski et al., 2016).

Another substance secreted by skeletal muscle that deserves to be highlighted is IL-6 since despite being considered a pro-inflammatory factor, it induces anti-inflammatory responses such as an increase in IL-10 and IL-1ra and stimulates the secretion of the peptide-like glucagon 1 (GLP-1), which can act to repair the intestinal mucosa after damage related to the pathogenesis of inflammatory bowel diseases (Bilski et al., 2016). In addition, exercise seems to play an important role in improving the diversity of the intestinal microbiota, usually unregulated in Crohn's disease and ulcerative colitis, being one of the factors that can aggravate or predispose to the onset of these diseases (Bilski et al., 2016; Clarke et al., 2016; Codella et al., 2018).

Patients with irritable bowel syndrome (IBS) when advised weekly to increase cardiorespiratory fitness by practicing 20-60 minutes of moderate to vigorous physical activity, 3 to 5 times a week showed a significant improvement in the symptom score of these patients after 12 weeks of counseling (Johannesson, Simrén, Strid, Bajor, & Sadik, 2011). In another research, Johannesson et al. (2015) also found improvements in GI and psychological symptoms, such as fatigue, depression, and anxiety, and these alterations seemed to be modulated by gut microbiota changes. Attenuation of GI symptoms in patients with IBS after aerobic training for 24 weeks may be due to the attenuation of the inflammatory process with reduced plasma concentrations of cytokines (IL1β, IL-6, IL-8, IL-10, and TNF-α), reduction of oxidative stress markers, such as xanthine oxidase (XO), plasma malondialdehyde (MDA) and nitric oxide (NO), as well as increased antioxidant activity, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Maleki et al., 2018).

Some investigations have shown benefits associated with the practice of physical exercise in the treatment of patients with colorectal cancer, acting to improve conditioning, functional and mental capacity, stress, and life quality, improved immune function and oxidative stress, and better prognosis (Brown et al., 2018; Onerup et al., 2017; Singh et al., 2020). The performance of a supervised exercise program for 18 weeks in patients with colorectal cancer undergoing chemotherapy reduced fatigue, being safe and feasible (Van Vulpen et al., 2016).

Among the mechanisms involved in the actions of physical exercise in the better prognosis of colorectal cancer is the modulation of the intestinal microbiota, with an increase in the production of short-chain fatty acids with anti-inflammatory action, an increase in the population of *Bifidobacterium*, and a reduction in species such as *Fusobacterium nucleatum* and LPS-producing bacteria, which have a pro-inflammatory action promoting increased intestinal permeability and metastasis (Song & Chan, 2018). On the types of physical exercise, as well as studies conducted in other conditions that interfere with the GI tract, most research has focused on the benefits and protocols of aerobic exercise, and there is still a large gap in clinical trials that can investigate the effects and establish recommendations for resistance training in patients with colorectal cancer (Singh et al., 2020).

14.5 Gut microbiota changes in athletes

Is also known that athletes can have alterations in gut microbiota profile, and the extension of these changes depends on the type, duration, and intensity of exercise and can be influenced by dietary intake and be different according to local and ethnicity. This altered profile of intestinal microbiota is generally accompanied by functional changes, like short-chain fatty acid (SCFA) or other bioactive molecules production (Mailing, Allen, Buford, Fields, & Woods, 2019).

According to current evidence, adaptations of gut microbiota found in athletes may be accompanied by a greater functional capacity in tissue repair and a greater ability to harness energy from the diet with increased capacity for carbohydrate, cell structure, and nucleotide biosynthesis, and these changes are also modulated by their dietary intake (Jäger et al., 2019).

More specifically, a greater abundance of *Akkermansi*a, *Veillonella*, and *Prevotella* has been described in athletes, with increased richness, and selection advantage for lactate-utilizing bacteria in consequence of metabolic adaptations of exercise. Other bacterial species have been reported as decreased in athletes, as *Bacteroides* spp. and *Proteobacteria* spp., commonly associated with Western diet and increased fat metabolism (Marttinen, Ala-Jaakkola, Laitila, & Lehtinen, 2020).

Clarke et al. (2014) found that male elite professional rugby players had a higher diversity of gut microorganisms, representing 22 distinct phyla, which in turn positively correlated with protein consumption and creatine kinase, being the diet and use of supplements other factors that impact intestinal microbiota. Jang et al. (2019) also found gut microbiota changes in athletes at the genus and species level, where *Faecalibacterium*, *Sutterella*, *Clostridium*, *Haemophilus*, and *Eisenbergiella* were the highest in bodybuilders, while *Bifidobacterium* and *Parasutterella* were the lowest.

According to Mohr et al. (2020), a recent review of microbiota alterations in response to training levels, exercise, and the athletic classification promote a more "health-associated" gut microbiota, characterized by a higher abundance of health-promoting bacterial species, increased microbial diversity, functional metabolic capacity, and microbial-associated metabolites, stimulation of bacterial abundance that can modulate mucosal immunity, and improved GI barrier function

14.6 Exercise, dysbiosis, and intestinal permeability

Although moderate and regular physical exercise is associated with improvements in microbiota patterns, anti-inflammatory, and antioxidant effects in the GI tract, exhaustive training has been associated with negative repercussions in gut epithelial barrier and microbial composition in athletes (Donati Zeppa et al., 2019). Several athletes suffer from dysbiosis, which is a condition that occurs the loss of diversity among gut microbiota species and can contribute to immune-regulated pathological conditions and decreased performance (Mach & Fuster-Botella, 2017; Marttinen et al., 2020).

The negative effects of high intensity or exhaustive exercise in gut microbiota can be explained by several factors (Fig. 14.1). First, exercise can induce a deviation in blood flow to skeletal muscle, reducing the delivery of oxygen to the GI tract, therefore causing hypoxia

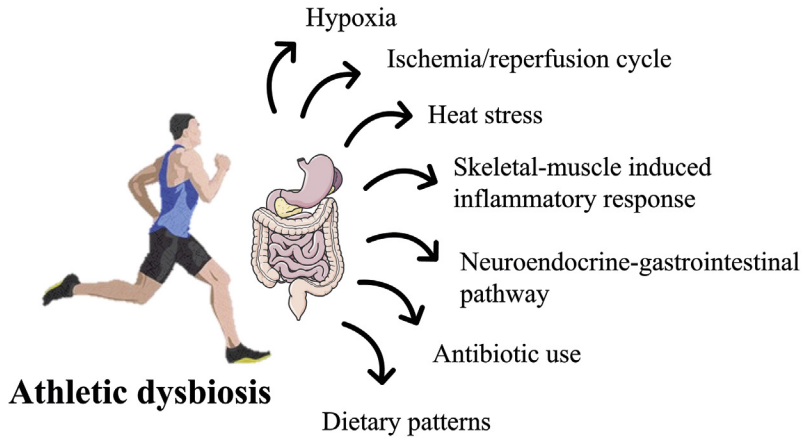


FIG. 14.1 Main causes of dysbiosis in athletes.

in these organs. Hypoxia is an imbalance between supply and demand of oxygen in tissue, leading to activation of hypoxia-inducible factor (HIF), a transcription factor that initiates cellular responses to oxygen altered levels (Mach & Fuster-Botella, 2017; McGarry, Biniecka, Veale, & Fearon, 2018). Activation of HIF in gut epithelia stimulates the synthesis of necrosis tumoral factor (TNF) and innate immune cells, as neutrophils, macrophages, and dendritic cells activity. These responses lead to inflammation and increase the generation of reactive oxygen species (ROS), therefore increasing the reactivity of immune cells and damage to the gut epithelial barrier (Kim et al., 2021).

When the intestinal blood flow returns with the reduction of exercise intensity, occurs the ischemia-reperfusion cycle. In general, cells can survive along short periods of ischemia, but some cells can die, and this led to the loss of tissue structural integrity. Moreover, during the return of blood flow and oxygen supply, there is the generation of ROS, calcium overload, activation of proteases, and inflammatory and thrombogenic cascades, damage to lipids, proteins, and DNA and cell death (Kalogeris, Baines, Krenz, & Korthuis, 2016). The ischemia/reperfusion cycle causes damage to the intestinal mucosa, producing increased gut permeability and bacterial translocation (Chen, Lui, Rooijen, & Tam, 2004).

Another possible mechanism to explain the reduced microbiota diversity in high-intensity exercise can be related to heat stress, compromising the integrity of the intestinal barrier. Intestinal epithelial cells (IECs) are connected by tight junctions proteins, such occludes zona (ZO-1, ZO-2, and ZO-3), and claudins. Heat stress may activate the phosphorylation of tight junctions proteins; thus, decreasing the interaction of occludin and claudin with the ZO. This phenomenon can facilitate endotoxin leakage and liberation of pro-inflammatory cytokines, bringing to a cascade of immune response and GI symptoms, like diarrhea, and loss of barrier integrity (Zuhl et al., 2014).

Exhaustive exercise causes an increase in the production of cytokines by skeletal muscle, such as IL-6, TNF-α, and IL-1β, and anti-inflammatory mediators. These products can interact

with the gut, in a skeletal muscle-gut pathway. However, these pro-inflammatory cytokines can act in the GI system and may induce dysbiosis and modifications of intestinal microbiome composition and their secreted products (Mach & Fuster-Botella, 2017).

A neuroendocrine-GI pathway also has been described. The physical and psychological stress due to intense or prolonged exercise training activates sympathetic drive by stress hormones liberation, altering gut transit and motility, as well as increasing liberation of cortisol. Cortisol liberation is proportional to the intensity of exercise. This hormone has been linked to increased plasma concentrations of Gram-negative derived lipopolysaccharide (LPS), a major endotoxin, immunosuppression, and alterations in microbiota abundance and diversity (Mach & Fuster-Botella, 2017).

Antibiotic use also is a common prescription for athletes, at almost twice the rate as the general population with the intent of returning athletes to play sooner (Ferry, DeCastro, & Bragg, 2020). The use of antibiotics is associated with negative effects, especially the elimination of many commensal bacterial species and reduction of antimicrobial defenses, facilitating the colonization of pathogenic antibiotic-resistant bacterial species and gut permeability (Pamer, 2016).

The athlete's dietary intake can also affect microbiota composition and is very difficult to isolate the effects of diet or exercise on the GI tract because athletes commonly have increased energy and nutrient demands, as well as the ingestion of dietary supplements (Jäger et al., 2019). Moreno-Pérez et al. (2018) show that long-term protein supplementation increased the abundance of the Bacteroidetes phylum and decreased the presence of health-related taxa including *Roseburia*, *Blautia*, and *Bifidobacterium longum*, although there was no effect in fecal pH, water content, ammonia, and short-chain fatty acids (SCFA) concentrations. The authors suggested that long-term protein supplementation may harm gut microbiota.

More specifically, beef proteins have been associated with adverse effects in microbial composition and health by increasing the number of *Proteobacteria*, and when associated to a high-fat diet can increase in *Desulfovibrionaceae*, *Firmicutes*, and *Proteobacteria*, and decreases in Lactobacillaceae and Bacteroidetes, independent of obesity. Gram-negative bacteria produce LPS, which causes endotoxemia (Albracht-Schulte, Islam, Johnson, & Moustaid-Moussa, 2021). However, in a systematic review, Albracht-Schulte et al. (2021) do not find harmful effects of beef protein supplementation in intestinal microbiota, highlighting that absence of homogeneity of the studies evaluated.

One of the repercussions of dysbiosis and intestinal permeability in athletes is the bacterial and their toxic products translocation to the systemic circulation promoting systemic inflammation. Highlighting LPS that can activate TLRs and a cascade of signals, which lead to NF-κB translocation to the nucleus and activation of gene transcription of several inflammation mediators' (Cătană et al., 2015; Ticinesi et al., 2019).

14.7 Nutritional strategies for athletes

Nutrition aims to adopt healthy eating habits that promote homeostasis in the body. Protein consumption, for example, is pointed out as an important factor for maintaining muscle

integrity and the synthesis of signaling molecules. In this sense, there was a trend toward the search for the consumption of specific nutrients, contributing to the expansion of the supplement industry (Lösch, Moghaddam, Grossschmidt, Risser, & Kanz, 2014).

The dietary supplement consists of a product intended for consumption that has a dietary ingredient to add nutritional value to the food, which may be one or more of the following substances: vitamins, minerals, herbs or other botanical products, amino acids, in the form of tablets, capsules, liquids, etc., promoting the total increase in concentrate, metabolite, constituents or extracts (FDA, 2017).

Athletes use supplements depending on the sport they practice and the results they want. Generally, the purpose of supplementation is to improve the athlete's physical performance or recovery, although, in some situations, they also aim to enhance the individual's health.

In Brazil, supplements are classified as: i) hydro-electrolytic supplement for athletes; ii) energy supplement for athletes; iii) protein supplement for athletes; iv) supplement for partial replacement of athletes' meals; v) creatine supplement for athletes; VI caffeine supplement for athletes (Brasil, 2010). The use among athletes varies between 40 and 70% (Garthe & Maughan, 2018; Outram & Stewart, 2015). The motivations for the benefit of supplements by athletes are i) to supply a nutrient deficiency; ii) improve sleep quality; iii) prevent or reduce illness or injury; and iv) enhance performance (Garthe & Maughan, 2018).

Improved performance is often the benefit most athletes expect from supplementation. This effect can be obtained indirectly by improving the athlete's body composition, increasing willingness for training, and reducing pain. The athlete's illness is a problem that can prevent him from participating in competitions, for example. The high-performance athlete is more susceptible to diseases because intense training and lower food consumption harm immunity (Bermon et al., 2017). In this context, care with food is essential for the athlete, and when not supplied by food.

Ergogenic resources for athletes are any training technique, nutritional ingredient or practice, pharmacological method, or psychological technique that can improve exercise performance capacity or training adaptations. Therefore, ergogenic resources can help prepare an individual for exercise, improve exercise efficiency, improve exercise recovery, or help prevent injuries during intense training (Leutholtz & Kreider, 2001; Kreider et al., 2009).

The proper consumption of carbohydrates ensures the necessary energy supply and better performance. In addition, the practice of physical exercise reduces the amount of muscle and liver glycogen stores, which results in fatigue, decreased work, and impaired concentration (Vitale & Getzin, 2019).

The amount of intake for athletes varies depending on the level of physical activity (Table 14.3). At the same time, the International Society for Sports Nutrition (ISSN) recommends that to maximize muscle glycogen stores, and the athlete must consume between

TABLE 14.3 Carbohydrate recommendation for athletes according to the level of physical exercise intensity.

Intensity level	The recommended amount of daily carbohydrates
Light	3–5 g/kg
Moderate	5–7 g/kg
High	6–10 g/kg
Very high	8–12 g/kg

8 and 12 g/ kg per day of carbohydrates (Thomas, Erdman, & Burke, 2016). However, there is also evidence that reducing the availability of carbohydrates in the athlete can promote specific adaptations in the muscle, in which this strategy is related to nutritional periodization (Bartlett, Hawley, & Morton, 2015; Hawley & Burke, 2010; Morrison et al., 2015; Paulsen et al., 2014).

Considering the objective of promoting mass body gain and that dietary restrictions in athletes can contribute to the loss of fat-free mass, there is a search for protein supplements by this population and someone from protein intake through diet (Rasmussen & Phillips, 2003). Furthermore, adequate protein intake stimulates protein synthesis and mass body gain, promoting positive protein balance (Atherton et al., 2010; Greenhaff et al., 2008; Volpi, Kobayashi, Sheffield-Moore, Mittendorfer, & Wolfe, 2003).

Endurance-trained athletes require 1.8 to 2.7 g/kg of body weight each day during an energy deficit (or 2.3–3.1 g protein/kg of MLG each day) (Helms, Zinn, Rowlands, & Brown, 2014). According to the ISSN (International Society of Sports Nutrition) for muscle protein synthesis and maintaining muscle mass through a positive muscle protein balance, an overall daily protein intake in the range of 1.4 to 2.0 g/kg of protein per day is sufficient for most individuals who exercise (Jäger et al., 2017). Due to the high demand for proteins, a commonly used strategy is supplementation with whey protein, BCAA, creatine, β-alanine (Kreider et al., 2017).

As for the consumption of lipids, the intake of 20 to 35% of the total energy value (TEV) is recommended. There is evidence that up to 20% of the VET consumption does not affect physical activity performance. The consumption of lipid sources is important, as they provide energy and contribute to the absorption of fat-soluble vitamins; however, high-fat diets are discouraged (Kerksick et al., 2018).

In addition to the search for supplementation that improves physical performance, there is also the search for supplements with more specific functions, such as stimulants such as caffeine, which promote the increase of endorphins and neuromuscular function, in addition to improving alertness and reducing the perception of effort (Peeling, Binnie, Goods, Sim, & Burke, 2018), as well as supplements that increase immunity such as glutamine and probiotics (Gleeson, Nieman, & Pedersen, 2004).

14.8 Supplementation with probiotics for athletes

Probiotics are live food ingredients that promote beneficial health effects (WHO, 2002), in which the most used genera are *Lactobacillus* and *Bifidobacterium* (Nichols, 2007; Pyne, West, Cox, & Cripps, 2015). Studies show that probiotics have numerous health benefits, such as improving GI symptoms (De Wolfe et al., 2018; Ibrahim et al., 2020; Ringel-Kulka et al., 2011; Sharif, Kashani, Nasri, Soleimani, & Sharif, 2017), schizophrenia (Ng et al., 2019), pain reduction due to osteoarthritis (Taye, Bradbury, Grace, & Avila, 2020), obesity (Krumbeck et al., 2018; Sanchez et al., 2014; Sergeev, Aljutaily, Walton, & Huarte, 2020), and diabetes (Kobyliak et al., 2020).

Studies with athletes or practitioners of physical exercise are still few. However, they point to a potential effect of probiotics in improving the health of these individuals, mainly considering their impact on the immune system and that exercise practitioner, depending on the

volume and intensity, show increased susceptibility to respiratory diseases (Coqueiro, de Oliveira Garcia, Rogero, & Tirapegui, 2017; Leite et al., 2019; Spence et al., 2007).

Thus, performing a prolonged or intense exercise can alter the mucosal surfaces of athletes, increasing the risk of health problems such as nausea, bloating, cramps, pain, diarrhea, or bleeding (de Oliveira & Burini, 2011; Simpson et al., 2009). In addition, the practice of high-intensity exercise is related to a three times higher incidence of infections of the upper respiratory tract (URTI) and GI alterations, which, therefore, can harm the athlete's general health and affect their performance (Spence et al., 2007).

A single session of physical exercise until fatigue increases the concentration of plasma cortisol, which in turn is capable of inhibiting lymphocyte proliferation and impairing monocyte function (Nieman et al., 2007). Likewise, a decrease in energy substrates (glucose and glutamine) for immune system cells was verified (Carvalho-Peixoto, Alves, & Cameron, 2007). Physical exercise also reduces the count and activity of immune cells, such as NK (Natural Killer) and neutrophil cells, as well as promotes an increase in the plasma concentration of pro-inflammatory biomarkers and reduced plasma concentration of anti-inflammatory cytokines (Dönmez, Kısadere, Balaban, & Kadiralieva, 2014; Lollo et al., 2012). Considering the impacts of physical exercise on the immune system, probiotics prevent upper respiratory tract diseases since intestinal modulation is essential for mediating the response to infection and homeostasis of the mucosa (Otczyk & Cripps, 2010).

The mucosal lining of the GI tract is the organism's first line of defense against the invasion of pathogens, and it can perform recognition between pathogenic and non-pathogenic microorganisms (Strober, 2011). In addition, microbiota modulation is a key to preventing aberrant inflammatory responses (Wallace et al., 2011). Notably, an athlete's gut microbiota is different from that of a sedentary individual since greater diversity and greater abundance of health-promoting bacterial species associated with exercise and increased protein intake (Jäger et al., 2019).

There is research investigating the potential of supplementation with probiotics on GI health, exercise performance, fatigue, and body composition, as seen in Table 14.3 (Batatinha et al., 2020; Cox, Pyne, Saunders, & Fricker, 2010; Gill, Teixeira, Rosado, Cox, & Costa, 2016; Gleeson, Bishop, & Struszczak, 2016; Gleeson, Bishop, Oliveira, & Tauler, 2011; Harnett, Pyne, McKune, Penm, & Pumpa, 2021; Huang et al., 2019; Huang et al., 2018; Jäger et al., 2016; Khani, Jazayeri, Ebrahimi, Younesi-Melerdi, & Farhadi, 2018; Michalickova et al., 2017; Michalickova et al., 2018; Michalickova et al., 2016; Moreira, Kekkonen, Korpela, Delgado, & Haahtela, 2007; Pugh et al., 2019; Shing et al., 2014; Strasser et al., 2016; Townsend et al., 2018).

Among the most investigated genus, *Lactobacillus*, *Bifidobacterium*, *Streptococcus*, *Bacillus*, *Lactococcus*, and *Enterococcus* stand out. Moreover, the most investigated species in physical activity practitioners are *L. acidophilus*, *L. casei*, *L. helveticus*, and *L. fermentum*. However, there is no consensus between the intervention period to promote health benefits for athletes, and studies vary between 1 to 20 weeks of treatment with probiotics. Therefore, it is important to consider the sport modality, the individual's health, the type of strain, and the number of CFU/mL or g indicated (Table 14.4).

Few studies investigate the association of supplementation with physical performance. Evidence shows that probiotics can reduce muscle pain (Harnett et al., 2021), as well as fat mass (Huang et al., 2019), increase lean mass (Huang et al., 2019), enhance performance (Huang et al., 2019; Jäger et al., 2016; Shing et al., 2014), and reduce muscle damage

(Jäger et al., 2016; Shing et al., 2014). However, to date, there are no reported effects on body composition (Townsend et al., 2018).

Systematic reviews of the impacts of probiotic supplementation on athlete performance report that there is no evidence of an effect on athlete performance (Calero, Rincón, & Marqueta, 2020; Nichols, 2007), but another more recent systematic review has shown that probiotics can indirectly influence sports performance, improving other parameters, such as the immune system, the response to respiratory infections and the decrease in oxidative stress (Díaz-Jiménez et al., 2021; Table 14.4).

TABLE 14.4 Effects of supplementation with probiotics in physically active individuals.

Reference (Country)	Study type	Population (sample)	Supplementation (dose)	Observed effect
Harnett et al., 2021 (Australia)	Double-blind randomized controlled trial	Rugby players (n = 9)	*Lactobacillus, Bifidobacterium and Streptococcus* (6×10^9 CFU/mL), and *Saccharomyces boulardii* (250 mg) for 17 weeks	Reduction of muscle pain, leg weight, and CRP levels
Schreiber et al., 2021 (Israel)	Double-blind randomized controlled trial	Cyclists (n = 11)	*Lactobacillus helveticus Lafti L10* (4.3×10^9), *Bifidobacterium animalis ssp. lactis Lafti B94* (4.3×10^9 CFU/mL), *Enterococcus faecium R0026* (3.9×10^9 CFU/mL), *Bifidobacterium longum R0175* (2.1×10^9 CFU/mL), *Bacillus subtilis R0179* (0.4×10^9 CFU/mL) for 12 weeks	Reduction in the incidence of gastrointestinal symptoms, without significant change in CRP, IL-6, and TNF-α levels
Batatinha et al., 2020 (Brazil)	Double-blind randomized controlled trial	Marathonist (n = 14)	*B. animalis subsp. Lactis* (10×10^9 CFU/mL), *Lactobacillus acidophilus* (10×10^9 CFU/mL), maltodextrin (5 g) for 4 weeks	Supplementation did not change the immune response
Huang et al., 2019 (China)	Double-blind randomized controlled trial	Individuals of both sexes, practitioners of treadmill exercise (n = 18)	*Lactobacillus plantarum* TWK10 (3×10^{10} ou 9×10^{10} CFU/mL) for 6 weeks	There was an impact on body composition, especially in the group with the highest dose, which showed a reduction in body fat and an increase in muscle mass
Pugh et al., 2019 (UK)	Double-blind randomized controlled trial	Runners (n = 12)	Lactobacillus acidophilus CUL60, L. acidophilus CUL21, Bifidobacterium bifidum CUL20e Bifidobacterium animalis subsp. lactis CUL34 (2.5×10^{12} CFU/mL) for 4 weeks	In the last 2 weeks, there was a reduction in gastrointestinal symptoms, with less severity of symptoms in the supplemented group
Pumpa, McKune, & Harnett, 2019 (Australia)	Double-blind randomized controlled trial	Athlete (n = 10)	*Ultrabiotic 60 + SB Floractiv (250 mg)* 17 weeks	Increased salivary alpha-amylase levels
Khani et al., 2018 (Iran)	Clinical trial	Sprint athletes (n = 28)	*Bifidobacterium bifidum* (2×10^9 CFU/mL) for 8 weeks	Increases in the levels of IgA, IgM, the percentage of lymphocytes and monocytes, and the CD4 count

(Continued)

TABLE 14.4 Cont'd

Reference (Country)	Study type	Population (sample)	Supplementation (dose)	Observed effect
Huang et al., 2018 (China)	Double-blind randomized controlled trial	Men undergoing treadmill exercise (n = 8)	*Lactobacillus plantarum* TWK10*for 6 weeks	Supplementation improved exercise performance
Michalickova et al., 2017 (Serbia)	Double-blind randomized controlled trial	Elite athletes (n = 15)	*Lactobacillus helveticus* Lafti L10 ($2{,}0 \times 10^{10}$ CFU/mL) for 14 weeks	Maintained total salivary IgA levels
Michalickova et al., 2018 (Serbia)	Double-blind randomized controlled trial	Elite athletes (n = 10)	*Lactobacillus helveticus* Lafti L10 (2.0×10^{10} CFU/mL) for 14 weeks	Decrease in MDA, AOPP levels
Townsend et al., 2018 (USA)	Double-blind randomized controlled trial	Baseball players (n = 13)	*Bacillus subtilis* DE111(1,2 bilhão de CFU/mL) for 12 weeks	It did not affect body composition, performance, hormonal status, or intestinal permeability, only reduced TNF-α levels
Gleeson et al., 2016 (UK)	Double-blind randomized controlled trial	Endurance and individual sports practitioners (n = 137)	*Lactobacillus casei* (6.5×10^9 CFU/mL) for 20 weeks	There was no reduction in the incidence of respiratory infection; however, it reduced CMV and EBV antibody titers in plasma
Gill et al., 2016 (UK)	Randomized blind controlled clinical trial	Endurance-trained men in hot environmental conditions (n = 8)	*Lactobacillus casei* (1.0×10^{11} CFU/mL)for 1 weeks	Improvement of immunological parameters related to the oral mucosa, such as salivary IgA and salivary antimicrobial protein
Jäger et al., 2016 (USA)	Diet-controlled crossover clinical trial.	Resistance trained men (n = 29)	*Bacillus coagulans GBI-30, 6086* (1×10^9 CFU/mL) + casein (20 g) 2 weeks	Supplementation reduced muscle damage rates, improved recovery, and maintained physical performance
Michalickova et al., 2016 (Serbia)	Double-blind randomized controlled trial	Elite athletes (n = 39)	*Lactobacillus helveticus* Lafti L10 (2×10^{10} CFU/mL) for 14 weeks	It reduced the duration and symptoms of respiratory infections but with no impact on the severity
Strasser et al., 2016 (Austria)	Double-blind randomized controlled trial	Trained athlete (n = 17)	*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Enterococcus faecium* W54, *Lactobacillus acidophilus* W22, *Lactobacillus brevis* W63and *Lactococcus lactis* W58 (1.0×10^{10} CFU/mL) for 12 weeks	Exercise-induced drops in tryptophan levels were preserved, and the incidence of respiratory infections was reduced
Dönmez et al., 2014 (Turkey)	Clinical trial	Men undergoing aerobic exercise (n = 18)	Koumiss (350 mL) for 2 weeks	Promoted an increase in the count of leukocytes and neutrophils

Reference (Country)	Study type	Population (sample)	Supplementation (dose)	Observed effect
Haywood et al., 2014 (New Zealand)	Randomized blind controlled clinical trial	Rugby union elite players (n = 30)	*Lactobacillus gasseri* (2.6×10^9 CFU/mL), *Bifidobacterium bifidum* (2×10^8 CFU/mL), *Bifidobacterium longum* (2×10^8 CFU/mL) for 4 weeks	Reduced duration and incidence of infections, but not severity
Shing et al., 2014 (Australia)	Double-blind randomized controlled trial	Runners (n = 10)	*Lactobacillus*, *Bifidobacterium* and *Streptococcus* (4.5×10^9 CFU/mL) for 4 weeks	Increased running time to fatigue and slightly reduced gastrointestinal symptoms
Salarkia et al., 2013 (Iran)	Randomized blind controlled clinical trial	Endurance swimmers (n = 23)	*Lactobacillus Acidophilus* SPP, *Lactobacillus Delbrueckii Bulgaricus*, *Bifidobacterium Bifidum*, and *Streptococcus Salivarus Thermnophilus* (4×10^{10} CFU/mL) for 8 weeks	Decreased the number of episodes of respiratory infections and the duration of dyspnea and earache
Lamprecht et al., 2012 (Austria)	Double-blind randomized controlled trial	Endurance-trained men (triathletes, runners, cyclists) (n = 11)	*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, Enterococcus faecium W54, *Lactobacillus acidophilus* W22, *Lactobacillus brevis* W63 and *Lactococcus lactis* W58 (10^{10} CFU/mL) for 14 weeks	Decreased zonulin in feces, a marker that indicates increased intestinal permeability. Beneficially affected TNF-α and protein oxidation
Gleeson et al., 2012 (UK)	Double-blind randomized controlled trial	Endurance athletes of both genders (n = 33)	*Lactobacillus salivarius* (2×10^{10} CFU/mL)for 16 weeks	There was no change in the count of blood leukocytes, neutrophils, monocytes, and lymphocytes; IgA and lysozyme
Välimäki et al., 2012 (Finland)	Double-blind randomized controlled trial	Recreational runners	*Lactobacillus rhamnosus* GG(3.0×10^8 CFU/mL)for 12 weeks	There was no effect on oxidized LDL (ox-LDL), serum antioxidant potential, and serum antioxidant levels
Gleeson et al., 2011 (UK)	Double-blind randomized controlled trial	Winter training subjects engaged in endurance-based physical activity (n = 42)	*Lactobacillus casei Shirota* (6.5×10^9 CFU/mL) for 16 weeks	It promoted a reduction in the frequency of URTI, which may be related to the maintenance of IgA levels in saliva during a period of training and winter competition
Martarelli et al., 2011 (Italy)	Clinical trial	Athletes (n = 12)	*Lactobacillus rhamnosus* IMC 501 e *Lactobacillus paracasei* IMC 502 (10^9 CFU/mL) for 4 weeks	There was an increase in the plasma levels of antioxidants, neutralizing the reactive oxygen species.
West et al., 2011 (Australia)	Double-blind randomized controlled trial	Cyclists and triathletes (n = 29)	*Lactobacillus fermentum* (10^9 CFU/mL) for 11 weeks	There was an increase in gastrointestinal symptoms, and no effect on upper respiratory tract diseases was observed.

(Continued)

TABLE 14.4 Cont'd

Reference (Country)	Study type	Population (sample)	Supplementation (dose)	Observed effect
Cox et al., 2010 (Australia)	Randomized double-blind controlled crossover clinical trial	Runners (n = 20)	*Lactobacillus fermentum* VRI-003 (1.26×10^{10} CFU/mL) for 4 weeks	Maintenance of IFN-γ levels, reduction in the number of days and the severity of respiratory disease
Kekkonen et al., 2007 (Finland)	Double-blind randomized controlled trial	Marathonist (n = 71)	*Lactobacillus rhamnosus* GG ($2,0 \times 10^{10}$ CFU/mL) for 12 weeks	There were no differences in the number of respiratory infections or episodes of gastrointestinal symptoms
Tiollier et al., 2007 (France)	Double-blind randomized controlled trial	Military cadets (n = 23)	*Lactobacillus casei* cepa DN-114 001 for 4 weeks	There was no reduction in respiratory tract infections
Moreira et al., 2007 (Finland)	Double-blind randomized controlled trial	Marathonist (n = 141)	*Lactobacillus GG* (3×10^8 CFU/mL) for 12 weeks	It did not change blood eosinophil count and total IgE concentration, nor did it reduce the incidence of asthma and allergies
Clancy et al., 2006 (Australia)	Clinical trial	Athletes (n = 9)	*Lactobacillus acidophilus* (2×10^{10} CFU/mL) for 4 weeks	It increased IFN-γ secretion by T cells to values similar to those of healthy individuals but did not change as salivary IgA

IgA: Immunoglobulin A; IgE: Immunoglobulin E; IFN-γ: Interferon-gamma; IVAS: Upper Airway Infection; PCR: C-Reactive Protein; TNF-α: Tumor Necrosis Factor-alpha; CFU: Colony Forming Units.

Physical exercise promotes numerous health benefits; however, high-intensity exercise can increase reactive oxygen species (ROS) that can damage lipids and nucleic acids, damaging the athlete's health (Teixeira, Valente, Casal, Marques, & Moreira, 2009). Therefore, some studies have focused on understanding whether supplementation with probiotics could alter oxidative stress parameters. Decreased levels of MDA (Michalickova et al., 2018), ROS (Martarelli et al., 2011), AOPP (Michalickova et al., 2018), and increased antioxidant activity (Martarelli et al., 2011) were observed; however, research also showed no antioxidant action of probiotics in recreational runners (Välimäki et al., 2012). All studies used the genus *Lactobacillus*, although there were variations between the treatment time, species, and dose.

The antioxidant effect of probiotics is due to their ability to stimulate the synthesis of bioactive peptides that collaborate to increase antioxidant enzymes and restore the intestinal microbiota (Mishra et al., 2015). The main antioxidant enzymes regulated by supplementation with probiotics are superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) (Ejtahed et al., 2012).

The main GI symptoms reported by athletes are nausea, belching, heartburn, chest pain, vomiting, cramps, bloating, need to defecate, diarrhea, flatulence, and lateral pain

(Costa, Snipe, Kitic, & Gibson, 2017; Lucía, Hoyos, Pérez, & Chicharro, 2001; Peters et al., 1999). Regarding the control of GI symptoms, studies show heterogeneous results. There is evidence of the ability of probiotics to reduce GI symptoms (Lamprecht et al., 2012; Michalickova et al., 2016; Schreiber, Tamir, Golan, Weinstein, & Weinstein, 2021; Shing et al., 2014) with reduced severity in runners (Pugh et al., 2019). However, it also promoted an increase in GI symptoms in cyclists and triathletes (West et al., 2011), while in marathon runners, there was no difference in GI symptoms (Kekkonen et al., 2007).

Exercise can promote damage to intestinal integrity leading to translocation of bacteria and bacterial toxins (LPS) from the intestinal lumen to the bloodstream, which can induce a systemic cytokine response and inflammatory response, which may interfere with athletic resistance during submaximal efforts (Costa et al., 2017; de Oliveira et al., 2014; Van Wijck et al., 2012).

Another potential benefit of probiotics that have been well investigated is controlling respiratory tract infections, a common event in athletes. Supplementation promoted a reduction in incidence in individuals undergoing resistance training (Gleeson et al., 2011; Strasser et al., 2016), endurance swimmers (Salarkia, Ghadamli, Zaeri, & Rad, 2013), while in runners, in addition to reducing the incidence, there was also a decrease in the severity of respiratory infection (Cox et al., 2010), different from that observed by Michalickova et al. (2016) and Haywood et al. (2014), in which there was a decrease in the duration and symptoms of respiratory infections, but with no impact on severity. However, in endurance exercise practitioners (Gleeson et al., 2016), cyclists and triathletes (West et al., 2011), military cadets (Tiollier et al., 2007), and marathon runners (Moreira et al., 2007), there was no reduction in the incidence of respiratory infection.

Despite the variation between the results of studies on the action on the regulation of infections in the respiratory tract, a meta-analysis provides evidence that the supplementation of probiotics, especially among professional athletes, decreases the total severity score of symptoms of URTI – Airway Infection Upper Air (Łagowska & Bajerska, 2021).

Regarding the impacts on the immune system and inflammatory process, it was observed increases in the counts of leukocytes, neutrophils (Dönmez et al., 2014), IgA, IgM, CD4, and in the percentage of lymphocytes and monocytes (Khani et al., 2018), and decreased levels of C-reactive protein - CRP (Harnett et al., 2021). In contrast, other studies have shown maintenance of IgA levels (Clancy et al., 2006; Gleeson et al., 2011; Gleeson et al., 2012; Michalickova et al., 2017), IgE (Moreira et al., 2007), leukocytes, neutrophils, blood monocytes, lymphocytes, lysozyme (Gleeson et al., 2012), eosinophils (Moreira et al., 2007), IL-6, and TNF-α (Shing et al., 2014).

The *Lactobacillus* and *Bifidobacterium* genera modulate several acute-phase proteins in inflammation and pain physiology, including C-reactive protein (Martins et al., 2009). This modulating potential justifies the anti-inflammatory effect and pain reduction in rugby players (Harnett et al., 2021). A systematic review showed that probiotic supplementation regulates respiratory tract infection, immunological markers, and GI symptoms in athletes and non-athletes, but the studies analyzed different protocols, with a small sample size, and cannot justify beneficial effects (Möller, da Cunha Goulart, Nicoletto, Alves, & Schneider, 2019).

The studies analyzed show that potential benefits from supplementation with probiotics depend on the strain used and the dose. Therefore, when choosing, it is important to consider products that have been clinically tested with validated benefits for athletes (Jäger et al., 2019). Another important aspect is that supplementation with probiotics for athletes must be tested before the competition so that there is adaptation and does not harm the athlete during

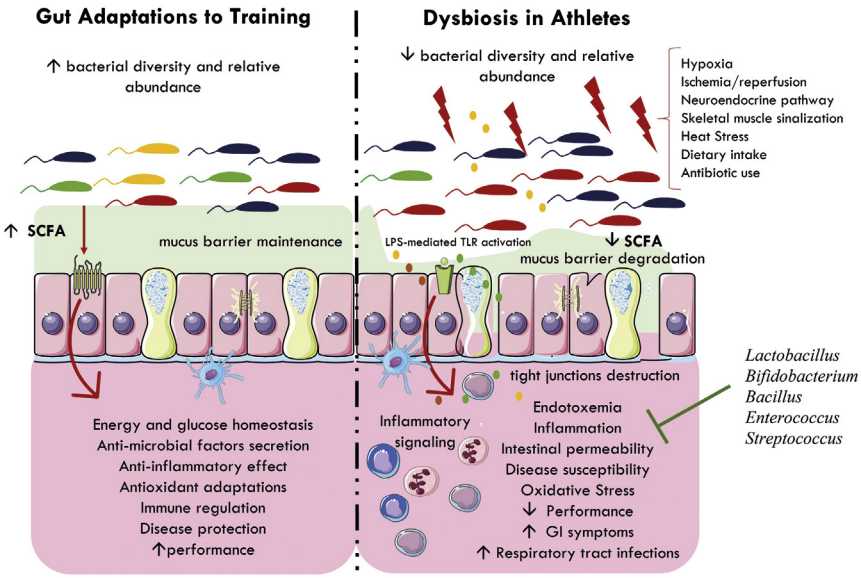


FIG. 14.2 Schematic representation of exercise-induced adaptations in intestinal microbiota and dysbiosis in athletes.

competition due to the potential effects on the GI tract (Jäger et al., 2019). Fig. 14.2 shows the relationship between exercise and microbiota focusing on the potential pathways impacted by probiotic supplementation.

14.9 Final considerations

Physical exercise has been associated with some beneficial adaptations in athletes and chronic diseases individuals. However, with increased intensity and volume some athletes can experiment a state of GI perturbations characterized by changes in gut microbiota, intestinal permeability, GI symptoms, immunological disturbance, and increase risk of infections and chronic non-communicable diseases, with decreased performance. Probiotics are a current nutritional strategy to modulate intestinal responses and have a protector effect against intestinal permeability or other undesirable repercussions. Further studies are needed to focus on defining the ideal supplementation time, quantity, viability, and probiotic strains most appropriate for the type and intensity of physical exercise.

Exercise has been related to beneficial adaptations in gut microbiota, with increased bacterial diversity and abundance and protection against chronic diseases. However, with increased volume and intensity several factors as hypoxia, ischemia/reperfusion, neuroendocrine pathways activation, skeletal muscle signaling, heat stress, antibiotic use, and dietary intake can cause disturbances in gut bacterial profile, causing inflammatory pathways activation, mucus barrier degradation, immunological response, endotoxemia, gastrointestinal symptoms, increase susceptibility to infectious and immune-based diseases, and decreased performance. Some probiotics have been studied in the perspective of attenuating or preventing these damages.

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CHAPTER

15

Probiotics and gut-brain axis modulation

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15.1 Introduction

Probiotics are live microorganisms that, when administered in sufficient amounts, can provide health benefits to the host. Although the beneficial effects of probiotics on gastrointestinal function have been generally recognized, new animal studies and clinical trials have found that changes in the intestinal microbial community can have a wide range of effects throughout the body. Non-intestinal affected areas include the immune, endocrine, cardiovascular, and central nervous systems. In particular, people are increasingly interested in the role of the gut microbiota in affecting the central nervous system (CNS)-related functions via the "microbiota-gut-brain axis." Emerging evidence suggests that probiotics have potential therapeutic benefits in a variety of CNS disorders such as anxiety, depression, autism spectrum disorders, and Parkinson's disease. Probiotics may influence the ability of the gut microbiome to affect a variety of biological processes in the host, including neurotransmitter activity, vagal neurotransmission, generation of neuroactive metabolites, and inflammatory response mediators. In this chapter, we aim to describe the current understanding in relation to the role and use of probiotics in microbiota-gut-brain axis-related dysfunction. We bring an overview of the enteric nervous system (ENS) and microbial colonization of the gastrointestinal tract. We discuss how microbiota might influence the development and function of the ENS and CNS, the role of probiotics in gut-brain axis modulation, and the influence of the intestinal microbiome on the development of neurologic disorders. Finally, we bring of the potential therapeutic benefits of using probiotics in various CNS conditions, like autism spectrum disorder, attention-deficit/hyperactivity disorder, major depressive disorder, generalized anxiety disorder, anorexia nervosa, post-traumatic stress disorder, and Alzheimer's and Parkinson's disease.

15.2 Overview of the enteric nervous system

The enteric nervous system (ENS) is a network of sensory neurons, motor neurons, and interneurons embedded in the wall of the gastrointestinal system, extending from the lower

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third of the esophagus to the rectum. First defined in 1921 by British physiologist John Langley, the term enteric nervous system was classified as a branch of the autonomic nervous system, alongside the sympathetic and parasympathetic branches (Langley, 1921). Although this classification has stood the test of time, research on ENS has revealed its unexpected complexity, which far exceeds the complexity of other branches of the autonomic nervous system (Furness & Costa, 1987). Most ENS neurons are not directly innervated by spinal cord neurons, unlike the rest of the autonomic nervous system, where each postganglionic neuron communicates with the central nervous system through preganglionic neurons (Furness & Costa, 1987). This functional independence is unique in peripheral organs and is mediated by the complex microcircuits of intrinsic primary afferent neurons, interneurons, and motor neurons found in the intestinal wall (Furness, Kunze, Bertrand, Clerc, & Bornstein, 1998).

The ENS also differs by its size, including more neurons than the entire spinal cord (Gershon, 1999). The ENS contains at least 25 different neurotransmitters, unlike the postganglionic neurons of the sympathetic and parasympathetic nervous systems, which are almost completely noradrenergic and cholinergic, respectively (McConalogue & Furness, 1994). The ENS is called the "second brain" of the human body, on account of your unique ability to function independently of the central nervous system, considerable size, and significant neurochemical diversity (Gershon, 1999).

The ENS is responsible for coordinating motor reflexes, controlling the exchange of water and electrolytes across the mucosal epithelium, regulating local blood flow, and regulating immunological processes (Costa, Brookes, & Hennig, 2000). ENS neurons are divided into two layers, the submucosal plexus and the myenteric plexus of the intestinal wall. The cell body of the neuron in each nerve plexus is divided into small, regularly spaced ganglia, which are connected to each other by bundles of nerve fibers called internode filaments. The myenteric plexus is located between the longitudinal muscle layer and the circular muscle layer and is primarily responsible for controlling digestive movements. Since the submucosal nerve plexus is inserted into the connective tissue between the ring muscle and the muscular mucosa to coordinate absorption and secretion reflexes in response to changes in the luminal environment (Furness & Costa, 1987; Gershon, 1999).

The gastrointestinal tract is also innervated by extrinsic fibers from the sympathetic and parasympathetic nervous systems (Ratcliffe, 2011). Afferent sensory fibers ascend to the CNS via the nodose and dorsal root ganglia (Blackshaw, Brookes, Grundy, & Schemann, 2007). Through these connections, the CNS is able to sense the intestinal contents and modulate the activity of the ENS. Therefore, the ENS, although capable of functioning independently of the CNS, normally works in concert with the other branches of the autonomic nervous system to coordinate digestive functions. The ENS exhibits complex coordination and exhibits plasticity and learning ability in response to changes in eating habits or gut disorders (Watson, Kirkcaldie, & Paxinos, 2010). There is growing recognition of the role of the microbiota in affecting key physiological processes, including gastrointestinal function. It is already known that the intestinal flora not only affects major components of the intestinal mucosa such as epithelial cell maturation, innate immunity, and angiogenesis (Bry, Falk, Midtvedt, & Gordon, 1996; Hooper, Stappenbeck, Hong, & Gordon, 2003; Stappenbeck, Hooper, & Gordon, 2002), but it is also important for the normal development of the enteric nervous system (Anitha, Vijay-Kumar, Sitaraman, Gewirtz, & Srinivasan, 2012; Collins, Borojevic, Verdu, Huizinga, & Ratcliffe, 2014).

15.3 Microbial colonization of the gastrointestinal tract

Traditionally, the fetus is considered to exist in a sterile environment in the womb, and birth represents the baby's first exposure to microbial species (Fanaro, Chierici, Guerrini, & Vigi, 2003). In fact, the presence of any bacteria in utero was thought to be pathologic and linked to preterm labor (Goldenberg, Hauth, & Andrews, 2000). However, recently, not only did the maternal microbial environment dynamically behave in normal pregnancy (Collado, Isolauri, Laitinen, & Salminen, 2008; Koren et al., 2012), but emerging evidence is supporting the concept that exposure to microbiota might begin in utero under nonpathological conditions. In previous studies, bacterial DNA was detected in the amniotic fluid of healthy term babies (Bearfield, Davenport, Sivapathasundaram, & Allaker, 2002; Rautava, Collado, Salminen, & Isolauri, 2012), meconium (Jiménez et al., 2008), fetal membranes (Steel et al., 2005), and several strains were cultured from the umbilical cord blood of healthy babies (Jiménez et al., 2005). Not only have bacteria been found in fetal fluids, but studies have shown that bacterial colonization of the gastrointestinal tract can begin before birth. The baby's first bowel movement is called meconium, which contains a variety of bacteria (Jiménez et al., 2008). The concept of prenatal colonization of the gastrointestinal tract is further supported by research, which shows that the composition of the meconium microbiota is very different from the composition of the microbiota that can become potential pollutants, such as the maternal vagina, rectum, and skin flora (Gosalbes et al., 2013), and that bacterial 16S rRNA can be isolated from the meconium of preterm and from full-term infants (Ardissone et al., 2014).

Although the formation of the gastrointestinal tract may begin throughout the prenatal amount, the time of birth and early postpartum period stay the foremost vital opportunities for microorganism colonization. Multiple factors are found to influence the composition of the enteric microbiota in early life (Fig. 15.1), together with physiological state age, mode of delivery, maternal contact, administration of antibiotics, and kind of alimentation (Azad et al., 2013; Fak, Ahrne, Molin, Jeppsson, & Westrom, 2008; Fanaro et al., 2003; Lamousé-Smith, Tzeng, & Starnbach, 2011; Mungovan & Ratcliffe, 2016; Penders et al., 2006). For example, the initial colonizing flora of a child can rely on whether the infant is born by vaginal delivery or by cesarean section.

When born by vaginal delivery, the early colonizing microbiota of the infant, including that found in meconium, has been found to more closely resemble the mother's vaginal flora and be dominated by *Lactobacillus* species (Dominguez-Bello et al., 2010; Salazar et al., 2014; Tidjani Alou, Lagier, & Raoult, 2016). On the other hand, infants born by Caesarean section acquire an early microbiota that more closely resembles that of their mother's skin, dominated by the common but potentially pathogenic bacterial species *Staphylococcus* and *Acinetobacter* (Dominguez-Bello et al., 2010; Salazar et al., 2014; Tidjani Alou et al., 2016).

The composition of the gut microbiota of infants evolves rapidly during the first year of life at which point the majority of species belong to the *Clostridium coccoides* group, the *Clostridium botulinum* group, the *Bacteroides* and *Veillonella* genera, the *Verrucomicrobia phylum* represented by *Akkermansia muciniphila* (Palmer, Bik, DiGiulio, Relman, & Brown, 2007). By one month of age, the enteral microbiota of cesarean delivered infants differs most from that of vaginally delivered infants by a major decrease in their levels of *Bacteroides fragilis* (Penders et al., 2006). This distinction persists up to 6 months of age, highlighting the effect of the early colonization period on the development of the intestinal flora (Grönlund, Lehtonen,

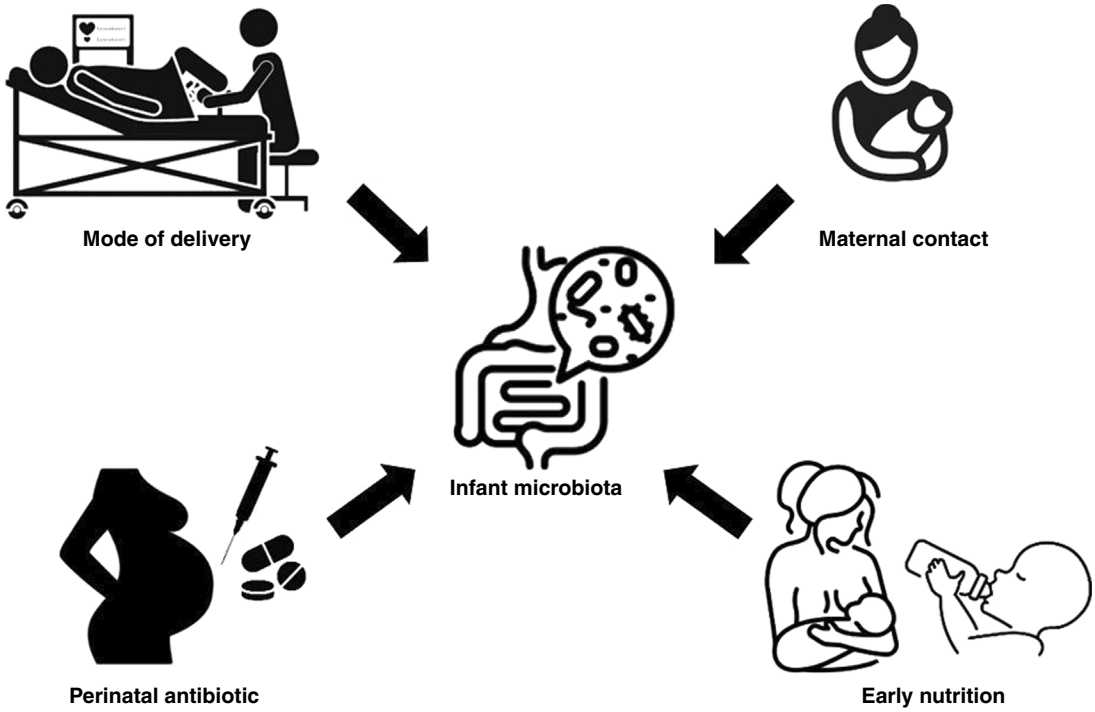


FIG. 15.1 Factors influencing the establishment of the infant microbiome. These factors include mode of delivery, maternal contact, exposure to perinatal antibiotics, and choice of early nutrition.

Eerola, & Kero, 1999). The intestinal surroundings of neonates are gas-rich, like oxygen, providing ideal conditions for invasive facultative aerobes to proliferate. As the oxygen becomes step by step depleted by around 1 week of age, the initial aerobic colonies change to facultative and obligate anaerobic, species characteristic of the adult gut flora (Guaraldi & Salvatori, 2012). *Bifidobacteria*, *Clostridia*, and *Bacteroides* are the primary anaerobic genres to colonize the gut (Isolauri, Björkstén, Salminen, Hernell, & Corthésy, 2008). After delivery, the key supply of bacterium becomes the mother's skin, via modes of transfer resembling holding, caressing, and breastfeeding (Morelli, 2008).

Breast milk contains up to 10^9 microbes/L, originating from the nipple and surrounding skin as well as the milk ducts of the breast (West, Hewitt, & Murphy, 1979). Formula-fed infants acquire bacteria from the instrumentation used to prepare the formula and the water used for suspension of the solution (Mackie, Sghir, & Gaskins, 1999). Diet also indirectly affects the composition of the gut microbiota by providing substrates that favor the expansion of certain colonies and by changing the physiology of the developing intestinal lining (Le Hurou-Luron, Blat, & Boudry, 2010). Although the types of bacteria in the digestive tract of breastfed and formula-fed infants appear to be largely the same, their relative proportions differ significantly (Mackie et al., 1999). Several studies using molecular techniques have confirmed that breastfed infants have a significantly higher proportion of bifidobacteria and a lower proportion of *Bacteroides* compared to infants fed with infant formula (Bezirtzoglou,

Tsiotsias, & Welling, 2011; Fallani et al., 2010; Harmsen et al., 2000). Therefore, the early introduction of breast milk or infant formula can have a significant impact on the composition of the intestinal microbiota.

By the end of the first year of life, when most children are being weaned from breast milk and/or infant formula with the more exclusive intake of solid foods, the intestinal microbiota begins to resemble that of the adult gastrointestinal (GI) tract (Palmer et al., 2007; Stark & Lee, 1982). A mature microflora comprises between 500 and 3000 unique bacterial species (Pfeiffer & Sonnenburg, 2011), reaching concentrations of 10^{11} to 10^{12} microbes/mL of luminal content (Whitman, Coleman, & Wiebe, 1998). Human gut microbiota presents a low diversity at the phylum level with only one archaeal phylum, *Euryarchaeota*, and seven bacterial phyla represented: *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, and *Cyanobacteria*-(Jandhyala, 2015; Maukonen & Saarela, 2015). However, this composition of the gut microbiota is achieved only at the end of the third year of life. At birth and during the first months of life, the proportion of the different phyla can differ markedly among individuals due to the influence of several factors like fetal life (antibiotic therapy, malnutrition or over-nutrition, obesity, diabetes, eczema, stress during pregnancy), and the postnatal period (duration of gestation, mode of delivery, use of antibiotics, type of feeding) (Latorre, Sternini, De Giorgio, & Greenwood-Van Meerveld, 2016; Rodríguez et al., 2015).

Healthy adult gut microbiota is dominated by three bacterial phyla, Firmicutes namely *Lachnospiraceae* and *Ruminococcaceae* families, *Bacteroidetes* namely *Bacteroidaceae*, *Prevotellaceae* and *Rikenellaceae* families and *Actinobacteria* namely *Bifidobacteriaceae* and *Coriobacteriaceae* families, and one major methanogenic archaeon, *Methanobrevibacter smithii* (Gill et al., 2006; Jandhyala, 2015; Maukonen & Saarela, 2015). In healthy humans, the concentration of microbiota increases progressively from the stomach to the large intestine, with the highest concentrations of bacteria found in the distal ileum and colon (Blaut & Clavel, 2007).

Overall, the microbial colonization of the GI tract has been shown to be critical for normal host physiology and to play a role in the developmental programming of adaptive immunity (Hooper, Littman, & Macpherson, 2012), epithelial barrier function (Bry et al., 1996), and intestinal angiogenesis (Hooper et al., 2003; Stappenbeck et al., 2002). Given that the ENS is still developing throughout the initial period of colonization, it is also potentially subject to influences from colonizing bacteria.

15.4 Influence of microbiota on the development of the enteric nervous system and central nervous system

Germ-free mice (GF), or aseptic mice, raised in sterile environments without microorganisms, have been shown to be an important tool for understanding the complex relationship between the host and its microbial inhabitants. GF mice are generally compared to specific pathogen-free (SPF) mice, which have a complex symbiotic flora and lack important pathogenic species, so they can be used as controls. Observations of newborn GF mice support the hypothesis that the gut microbiota affects the development of ENS (Collins et al., 2014). These researchers observed that on the 3rd day after birth, compared to FPS mice, the ENS of GF mice is structurally abnormal. The jejunum and ileum are thinner and the overall nerve density and

number of neurons in each ganglion are reduced. Colonizing GF mothers with a changed Schaedler flora (a simplified flora containing solely eight microorganism strains) is sufficient to restore the conventional ENS pattern of the offspring (Collins et al., 2014). These structural observations are complemented by functional data showing that GI motility is impaired within the corresponding areas of GF mice in the early postpartum period. Specifically, the frequency and amplitude of intestine contractions in the small intestine and small intestine of GF mice were considerably reduced (Collins et al., 2014).

Recent studies in GF mice have also shown that the number of enteric glial cells is reduced. Similar to enteric neurons, enteric glial cells are also derived from neural crest-derived precursors that colonize the intestine. In this work, compared to conventionally reared 8-week-old mice, the mean number and density of mucosal enteric glial cells in GF mice were significantly reduced, while the enteric glial networks in the myenteric and submucosal plexuses were not affected (Kabouridis et al., 2015). If GF mice were conventionalized at 4 weeks of age, the mucosal enteric glial cell network was found to be restored (Kabouridis et al., 2015). Therefore, the ability of intestinal mucosa glial cells to invade the intestinal mucosa and form a normal network after birth appears to depend on the existence of the intestinal microbiota.

These early-life abnormalities within the ENS of GF animals are shown to persist to adulthood. Irregularities in the patterning of the myenteric plexus of the adult GF rat bodily cavity were already delineated in the 1960s (Dupont, Jervis, & Sprinz, 1965). Additionally, GF mice at 4 weeks elderly were found to possess a major reduction in neuronic numbers in the myenteric plexus of the colon (Anitha, Vijay-Kumar, Sitaraman, Gewirtz, & Srinivasan, 2012). Furthermore, these persistent changes in ENS in adult animals with GF can manifest as functional defects. The excitability of intrinsic primary afferent neurons has been found reduced in adult GF mice compared with SPF controls, suggesting that commensal microbiota is necessary for the event of traditional electrophysiological profiles in enteric neurons (McVey Neufeld, Mao, Bienenstock, Foster, & Kunze, 2013).

As already mentioned, alterations of brain structure and function development are among the most relevant problems ascribed to modifications of the gut microbial composition because interactions between the CNS and the gut microbiota, which form the so-called gut-brain axis, appear to be already established during fetal life and continue until old age (Clarke, O'Mahony, Dinan, & Cryan, 2014). It has been demonstrated that communication between the gastrointestinal tract and the CNS occurs continuously through several routes (including hormonal, immune, and neuronal pathways) that are mostly conditioned by the microbiota composition (Latorre et al., 2016; Principi & Esposito, 2016; Yang et al., 2016).

Furthermore, it is already known that gut microbiota absence is associated with several CNS developmental problems. It was previously reported that compared with conventionally raised mice, germ-free (GF) animals had an increased expression of PSD-95 and synaptophysin in the striate nucleus (Collado et al., 2015; El-Husseini, Schnell, Chetkovich, Nicoll, & Bredt, 2000; Heijtz et al., 2011). These proteins are, respectively, a stimulator of excitatory synapse maturation and a hallmark of synaptic vesicle maturation, and therefore these findings seem to indicate that when the conventional microbiota is lacking, synaptic maturation is accelerated. This might affect motor control and anxiety-like behavior in later life, as shown in GF animals (Foster & McVey Neufeld, 2013). Researchers investigated changes in the homeostatic regulation of the neuronal transcription in GF mice within the prefrontal cortex

and found a marked, concerted upregulation of genes linked to myelination and myelin plasticity, leading to hypermyelinated axons (Hoban et al., 2016).

Colonization of experimental animals by a conventional microbiota reversed the myelin changes, confirming the necessity of intestinal flora for normal CNS development. The absence of microbiota during early life increases activity-related transcriptional pathways in the amygdala. Stilling et al. exploited unbiased genome-wide transcriptional profiling to determine gene expression in the amygdala of GF and GF mice that were colonized after weaning (Stilling et al., 2015). Using RNA-sequencing and a comprehensive downstream analysis pipeline, these researchers studied the amygdala transcriptome and found significant differences at the levels of differential gene expression, exon usage, and RNA-editing. Upregulation of several immediate early response genes such as Fos, Fosb, Egr2, or Nr4a1 in association with increased CREB signaling in GF mice was evidenced. In addition, the differential expression and recoding of several genes implicated in brain physiology processes such as neurotransmission, neuronal plasticity, metabolism, and morphology were demonstrated.

Microbes also control microglia maturation and function. GF mice display global defects in microglia, with altered cell proportions and an immature phenotype. Because microglia are formed by immune cells, this leads to impaired innate immune responses. In animals without risk factors for intestinal flora modifications, temporal gut microbiota eradication or limited microbiota complexity severely changes the microglia properties (Erny et al., 2015). Moreover, colonization with a complex microbiota partially restores microglia features, highlighting the role of gut microbiota in conditioning normal CNS development (Principi & Esposito, 2016).

15.4.1 Mechanisms of interference of the gut microbiota on the central nervous system development

Interaction between the CNS and the gut microbiota involves the gut microbes, the brain, the endocrine system, the immune system, and the autonomic nervous system. Signals from the brain can influence the motor, sensory, and secretory modalities of the gastrointestinal tract, and conversely, visceral messages from the gastrointestinal tract can influence brain function (O'Mahony, Hyland, Dinan, & Cryan, 2011). Despite the exact mechanisms by which the gut and brain reciprocally influence structure and function not being precisely defined, several data indicate that the passage of certain bacterial components through the intestinal wall plays a fundamental role in this regard. The most important bacterial components are lipopolysaccharides in the cell wall of some Gram-negative bacteria (such as *Enterobacteriaceae* and *Pseudomonadaceae*); the lipopolysaccharides may translocate from the intestinal mucosa to the systemic circulation, where they can affect immune regulation and CNS function (Bengmark, 2013). Lipopolysaccharides act upon toll-like receptor 4 to activate the systemic inflammation response with the production of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β (Theoharides, Asadi, & Patel, 2013). These cytokines, in turn, critically affect the CNS (Borre et al., 2014). By contrast, other bacteria, such as those included in the genus *Lactobacillus*, seem to exert a protective effect, probably because they secrete chemicals that limit colonization by inflammatory microorganisms (Bennett, Einarson, Taddio, Koren, & Einarson, 2004).

Microbial metabolites, mainly gut-derived tryptophan metabolites, can have critical importance. These metabolites are capable of modulating the immune system by various mechanisms, including the regulation of T cells, notably Th17 lymphocytes (Qiu et al., 2012; Veldhoen et al., 2008). Moreover, high levels of tryptophan metabolites increase kynurenine concentration, which can also engage aryl hydrocarbon receptors. This induces the differentiation of regulatory T cells and increases the levels of indoleamine 2,3-dioxygenase, an enzyme that further induces the catabolism of tryptophan and an increase in the number of circulating metabolites of this amino acid (Maes & Rief, 2012). In mice, it has been demonstrated that even when no transfer of the microbiota to the fetus from the mother has occurred, tryptophan metabolites that cross the placenta are active on the aryl hydrocarbon receptors and can significantly influence CNS development of the fetus (Gomez de Agüero et al., 2016). Finally, the level of systemic tryptophan metabolites influences the concentration of serotonin and γ-aminobutyric acid in the CNS and the production of neurotoxic molecules in astrocytes and microglia (Barrett, Ross, O'Toole, Fitzgerald, & Stanton, 2012; Nguyen et al., 2014).

Further modulation of CNS function is provided by short-chain fatty acids (SCFAs) produced by bacterial fermentation of complex polysaccharides (starches and fibers) in the colon (Wong, de Souza, Kendall, Emam, & Jenkins, 2006). Particularly, *Clostridia* and *Bacteroides* are important SCFA producers. Major effects of these compounds are the alteration of mitochondrial function via the citric acid cycle and carnitine metabolism, or the epigenetic modulation of genes controlling brain function. All these factors can produce reversible behavioral, electrographic, neuroinflammatory, metabolic, and epigenetic changes. It has been seen that when propionic acid or other short-chain fatty acids are injected into the cerebral ventricles of rats, the rats show biologic, chemical, and pathologic changes that are characteristic of autism (MacFabe, 2015).

It has also been suggested that intestinal bacteria can affect fetal neurodevelopment by influencing 5-hydroxytryptamine (5-HT) serum levels (Yano et al., 2015). Indigenous spore-forming bacteria from the microbiota promote 5-HT biosynthesis from colonic enterochromaffin cells (ECs), which supply 5-HT to the mucosa, lumen, and circulating platelets. In a previous study, select fecal metabolites were identified that are increased by spore-forming bacteria and that elevate 5-HT in chromaffin cell cultures, suggesting direct metabolic signaling of gut microbes to ECs (Yano et al., 2015). Furthermore, elevating luminal concentrations of particular microbial metabolites increases colonic and blood 5-HT in GF mice and, consequently, fetal neuronal cell division and differentiation, physiologically regulated by this compound (Gaspar, Cases, & Maroteaux, 2003). Depletions of 5-HT during development were reported to have effects on the maturation of cortical neurons (Osterheld-Haas & Hornung, 1996; Vitalis, Cases, Passemard, Callebert, & Parnavelas, 2007) and to alter barrel cortex development (Bennett-clarke, Hankin, Leslie, Chiaia, & Rhoades, 1994; Blue, Erzurumlu, & Jhaveri, 1991).

A further mechanism with which gut microbiota can influence CNS is through the endocrine pathway. Prenatal stress is accompanied by increased cortisol levels, mainly via the systemic and peripheral release of corticotropin-releasing factor (CRF) from the amygdala, which is important in the control of emotional and autonomic responses to stress and contains CRF nerve terminals, CRF cell bodies, and CRF receptors (Cratty, Ward, Johnson, Azzaro, & Birkle, 1995). Hypercortisolemia increases gut permeability and favors bacterial

lipopolysaccharide leakage across the gut wall (Rodiño-Janeiro et al., 2015). Moreover, it is associated with an increased amygdala volume (Buss et al., 2012) and with depression (Holmes et al., 2012).

Modification of the blood-brain barrier (BBB) can play a role in conditioning alterations in CNS development. The BBB acts as a gatekeeper to control the passage and exchange of molecules and nutrients between the circulatory system and the brain parenchyma. The BBB also ensures the homeostasis of the CNS. It was reported that GF mice, beginning with intrauterine life, display increased BBB permeability compared with non-GF mice with a normal gut microbial composition (Braniste et al., 2014). The increased BBB permeability is maintained in GF mice after birth and during adulthood and is associated with reduced expression of the tight junction proteins occludin and claudin-5, which are known to regulate BBB function in endothelial tissues.

Finally, the gut microbiota interacts with intestinal cells to stimulate the production of peptides that activate afferent endings of the vagus nerve. The resultant signals are transmitted to the CNS, affecting behavior and efferent neural activity. The same function appears to be exerted by pro-inflammatory cytokines. Consequently, the brain receives information regarding systemic inflammation, contributing to affective symptoms and initiating behavioral responses, including depression and other sickness behaviors (Miller, Maletic, & Raison, 2009). Vagal signals from the gut are also thought to be responsible for "gut feelings," which act as signals to the brain that an environment may be threatening or anxiety-provoking (Mayer, 2011). Activated efferent fibers of the vagus, in turn, carry anti-inflammatory signals to the periphery, reducing the production of pro-inflammatory cytokines (Vijayaraghavan et al., 2013).

If the gut microbiota plays an essential role in the modulation of brain structure and function, it is clear that when unbalance between "positive" and "negative" bacteria occurs and the latter microbes prevail, inflammation arises, the chronic immune disease can occur, and the CNS structure and function may be altered (Bengmark, 2013). Moreover, when chronic dysbiosis occurs, a systemic inflammatory state tends to persist (Principi & Esposito, 2016).

15.5 Use of probiotics as therapeutics in gut-brain axis modulation

The human intestinal microbiota is a complex community of microorganisms formed by bacteria, viruses, protozoa, and fungi whose functions, under normal homeostatic conditions, range from activating the host's innate and adaptive immune response to conducting metabolism, maintaining intestinal mucosal integrity, supporting peristalsis, producing vitamins, regulating hormone levels, and promoting neurological health (Azad, Moossavi, Owora, & Sepehri, 2017; Claesson et al., 2012; Hyland & Cryan, 2016; Zhang et al., 2015). The composition of this microbiota is site-specific and shaped by factors such as genetics, environment, health, age, gender, race, diet, and lifestyle. There has been substantial growth in research related to the microbiome over the past two decades. In particular, interest has evolved beyond the role played in local intestinal physiology and health, extrapolating to extra-intestinal organs and systems such as the central nervous system (CNS).

Considering that the brain is in constant bidirectional communication with the gut, the relationship between the host and its microbiota along the microbiota-gut-brain axis is now

considered a key area of impact for the microbial population (Cryan, O'Riordan, Sandhu, Peterson, & Dinan, 2020; Jena et al., 2020; Suganya & Koo, 2020). Emerging evidence from clinical and animal research suggests that an imbalance in intestinal microbial communities, or dysbiosis, can influence brain health or vice versa. Consequently, it recognizes the promising role of dietary and therapeutic interventions that act along the microbiota-gut-brain axis, in order to achieve a balance between these systems. Among the promising approaches to deal with the consequences of dysbiosis on the intestinal microbiota, the use of probiotics stands out.

Probiotics are live microorganisms that, when administered in adequate amounts, present health benefits to the host (FAO/WHO, 2002). These strains administered via food or in the form of nutraceuticals can alter the population of microorganisms in the intestinal microbiome and regulate the function of the intestinal ecosystem, which has implications for the health of digestion, the immune system, and the central nervous system (CNS) (Nishida, Sawada, Kuwano, Tanaka, & Rokutan, 2019; Pelsser, Frankena, Toorman, & Pereira, 2017). In particular, probiotics that, when ingested in adequate amounts, produce positive psychiatric effects on psychopathology and confer a CNS-related health benefit, have been referred to as "psychobiotics" (Dinan & Cryan, 2017a, 2017b; Sarkar et al., 2016). In an initial study, it was shown that germ-free (GF) mice showed increased physiological responses to stress compared to control mice (Sudo, 2014). The exaggerated stress reactions were reversed by bacterial recolonization after administration of probiotics, demonstrating the impact of intestinal bacteria and their manipulation by probiotics as a means of positively modulating the microbiota-gut-brain axis. Since then, many animal and human studies have suggested that probiotics could potentially be used as part of a therapeutic strategy to more effectively treat stress/mood and other neurological disorders. Reported beneficial properties range from effects on psychological stress, anxiety, depression, autism spectrum disorders, and Parkinson's disease. However, studies conducted so far tend to use a limited range of probiotics, with the Gram-positive *Bifidobacterium* and *Lactobacillus* being the most used (Sarkar et al., 2016).

Regarding the modulation of the intestine-brain axis mediated by probiotics, it is known that these can influence the ability of the intestinal microbiome to affect a variety of biological processes in the host, including neurotransmitter activity, vagal neurotransmission, generation of neuroactive metabolites, and mediators of inflammatory response. The gut and the brain are in constant communication via neuronal and systemic pathways. There is growing recognition that these communication pathways may be influenced by the state of microbial communities that reside in the host's gut, known as the microbiota-gut-brain axis. Maintaining a balance of microbial and population diversity in the gut microbiota is increasingly being recognized as important in supporting gut and brain health. The composition and health of the microbiota are shaped by genetic and environmental factors, including risk factors for disease. It is hypothesized that a change in microbiota composition to an unhealthy state (e.g., loss of microbial diversity, increased prevalence of pathogenic microbes) may negatively affect GI function, which may, through the microbiota-gut-brain axis, pose a risk for brain dysfunction (e.g., inflammation and neuronal damage). Several biological systems and pathways have been proposed as possible mediators of communication in the microbiota-gut-brain axis. Current evidence points to the involvement of the endocrine (Cussotto, Sandhu, Dinan, & Cryan, 2018), autonomic parasympathetic (e.g., vagus nerve) (Bravo et al., 2011;

Fülling, Dinan, & Cryan, 2019), and the immune system (Jaggar, Rea, Spichak, Dinan, & Cryan, 2020). Although the precise molecular pathways or mechanisms for probiotic action have not yet been fully elucidated, current evidence points to the involvement of metabolites (e.g., short-chain fatty acids), inflammatory mediators (e.g., cytokines), and various neurotransmitters (Clarke et al., 2013; Dalile, Van Oudenhove, Vervliet, & Verbeke, 2019; Gao et al., 2018; Lach, Schellekens, Dinan, & Cryan, 2018; Sadelhoff et al., 2019).

Intestinal bacteria can produce or regulate gastrointestinal levels of neurotransmitters such as γ-aminobutyric (GABA), serotonin, norepinephrine, dopamine, acetylcholine, and histamine through the metabolism of dietary amino acids (Snigdha et al., 2021). GABA is the main inhibitory neurotransmitter of the mammalian nervous system and acts to dampen the activity and function of the mature brain. Although these neurotransmitters do not cross the blood-brain barrier under normal circumstances (Dinan & Cryan, 2017a), changes in the GABAergic system are associated with psychiatric manifestations seen in schizophrenia and the pathophysiology of depression. Serotonin, on the other hand, is one of several key modulatory neurotransmitters present in the CNS, although 90% of this molecule is synthesized in the intestine (Berger, Gray, & Roth, 2009). It is noteworthy that other intestinal bacteria such as *Candida*, *Streptococcus*, *Escherichia*, and *Enterococcus* can also produce serotonin (Holzer & Farzi, 2014; Shishov, Kirovskaya, Kudrin, & Oleskin, 2009; Strati et al., 2017). Serotonin levels are closely correlated with mood, appetite, and sleep, and dysregulation can lead to anxiety and depression. The neurotransmitter dopamine is produced by the formation of *Bacillus spores* as well as *Hafnia* spp (recently being examined as a potential probiotic) (Alou, Fournier, & Raoult, 2016; Surwase & Jadhav, 2011). It is another key modulatory neurotransmitter, in addition to being a precursor to other catecholamines (norepinephrine and epinephrine), and is closely associated with motivated behaviors. Along with norepinephrine, it is also known for its role in alertness, as well as cognitive functions such as memory, learning, and attention (Borodovitsyna, Flamini, & Chandler, 2017; Strandwitz, 2018). Another neurotransmitter that can be produced by intestinal bacteria such as *Lactobacillus* is acetylcholine (Kawashima et al., 2007), which plays a key role in learning and memory, as well as motor function. Acetylcholine is also a molecular target for conditions where these cognitive elements have been impaired, such as Alzheimer's disease (Dinan & Cryan, 2017a).

An alternative means of communication along the microbiota-gut-brain axis may involve the parasympathetic autonomic system, whereas information can be communicated to the brain via primary afferents defined as neuronal spike trains in the vagus nerve. Preclinical studies have demonstrated that the vagus nerve is an important pathway for neural communication between intestinal microbes, as established by the removal of *Lactobacillus rhamnosus*-mediated effects post vagotomy (Bravo et al., 2011). Furthermore, the recent discovery of the morphology of enteroendocrine cells, which act as a sensor for luminal content, suggests a complex interaction between these cells and vagal afferent neurons, acting through an axon-like basal process known as neuropods. These structures help enteroendocrine cells communicate directly with enteric nerves, including the vagus nerve, to result in rapid neurotransmission (Kaelberer, Rupprecht, Liu, Weng, & Bohorquez, 2020). However, it has been shown that vagotomy does not disrupt the anti-anxiolytic effects of a relative increase in *Lactobacilli* abundance in innately anxious male BALB/c mice. Therefore, the vagus nerve is probably only one of the mechanisms through which intestinal bacteria can impact the brain (Bercik et al., 2011). It is likely that the microbiota effect on gastrointestinal neurotransmitter

levels can be relayed, at least in part, through modulation of vagal tone and that neuropods play a key signaling role. This evidence can be supported by the previously reported presence of GABA, serotonin, and dopamine receptors in vagal afferent nerves (Egerod et al., 2018).

There are several other ways, perhaps more direct than the neurotransmitter hypothesis, by which intestinal probiotic bacteria can alter brain activity and function, such as endocrine (e.g., cortisol), immune (e.g., cytokines), and metabolic (e.g., SCFA) (Carabotti, Scirocco, Maselli, & Severi, 2015; Sarkar et al., 2016). One of the main regulators of the human physiological stress response is the Hypothalamic-Pituitary-Adrenal axis (HPA axis). In conjunction with the autonomic nervous system, the HPA axis regulates responses to external and internal stressors (de Weerth, 2017). Activation of the HPA axis requires a cascade of endocrinological signals that include the production of corticotropin-releasing hormone (CRH) in the hypothalamus, which leads to the production of adrenocorticotropic hormone (ACTH) in the pituitary gland, which in turn triggers the production of cortisol by the adrenal glands. Cortisol prepares the body for the "fight or flight" response under stressful conditions and can increase blood sugar levels and suppress immune function by inhibiting cytokine production (de Weerth, 2017; Mayer, 2000). Most of the time, the increase in cortisol concentrations is an acute response and helps the body to deal with the stressor. This is maintained by a negative feedback loop; however, HPA axis dysregulation is found in many CNS-related pathologies (Bradley & Dinan, 2010; Faravelli et al., 2012; Guerry & Hastings, 2011; Taylor & Corbett, 2014) and can lead to feedback loop impairment resulting in a sustained increase in cortisol. Furthermore, there is evidence that the HPA axis can be activated by the intestinal microbiota due to the increased permeability of the intestinal barrier and a proinflammatory state driven by the microbiota (Farzi, Fröhlich, & Holzer, 2018). In cases like this, cytokines produced in the intestine can enter the bloodstream and signal through the BBB to influence brain areas, through different neuronal projections and extracellular routes. Through this mechanism, cytokines such as interleukin (IL)-1 and IL-6 activate the HPA axis resulting in the release of cortisol (El Aidy, Dinan, & Cryan, 2015; Farzi et al., 2018) which is one of the most potent means to activate the stress system.

Another way in which the microbiota could impact CNS function would be the process of fermentation of indigestible carbohydrate fibers, leading to the formation of short-chain fatty acids (SCFAs). Widely studied SCFAs include acetate, butyrate, and propionate, which are among the most abundant in the intestine (Dalile et al., 2019). Although these molecules can act locally, maintaining the intestinal epithelial function, they are also able to induce the production of hormones and neuropeptides, such as peptide 1 (GLP-1) and peptide YY (PYY) of intestinal enteroendocrine cells (Farzi et al., 2018). Furthermore, SCFAs are also epigenetic regulators, acting as histone deacetylase inhibitors, a mechanism involved in the processes of learning and memory (Farzi et al., 2018). It has been previously demonstrated that SCFAs can enter brain tissue, thus raising the possibility of direct action by the CNS (Oldendorf, 1973), although there is a need for studies to better demonstrate this mechanism. An indirect mode of action through the vagus may also be involved, as the administration of sodium butyrate has been shown to affect the activity of this nerve in anesthetized rats (Lal, Kirkup, Brunsden, Thompson, & Grundy, 2001). It is also conceivable that SCFAs and other metabolites produced by gut microbiota may influence CNS function by altering overall host metabolism and cardiovascular function (Snigdha et al., 2021).

Several preclinical studies have already been developed and clearly demonstrate the role of the intestinal microbiome in modulating the development and function of the CNS, and even in the behavior in rodents. However, the translation and demonstration of a causal relationship in humans remains challenging and not yet established. Research carried out in a review study with the administration of probiotics provides evidence to support the hypothesis that *Bifidobacteria* and/or *Lactobacilli* may be useful to alleviate stress, anxiety, and/or depressive behavior, in both pathological and non-pathological conditions (Snigdha et al., 2021).

In this way, it is clear the impact that probiotics have on the microbiota-gut-brain axis, changing the populations and diversity of microorganisms in the intestine and, therefore, representing a promising therapeutic approach in relation to support the treatment of gastrointestinal and brain dysfunction (Snigdha et al., 2021). Changes in the gut microbiota throughout life are hypothesized to play a role in neurological diseases that present at different ages, such as autism and attention deficit hyperactivity disorder (ADHD) in early life, multiple sclerosis (MS), and major depressive disorder (MDD) in adulthood and Alzheimer's disease (AD) and Parkinson's disease (PD) in the elderly (Cox et al., 2019). These age-specific microbiome changes are more indicative of the link between the gut microbiome and its impact on brain health and physiology. Overall, research outlining the role of the microbiota-gut-brain axis in health and disease, as well as the potential therapeutic utility of probiotics for the treatment of neurodegenerative and psychiatric disorders is an area of intense interest and should continue to grow in the future.

15.6 Probiotics and neurologic diseases

As previously reported, there has been a growing interest and appreciation of the role that the gut microbiota can play in CNS-related functions through the "microbiota-gut-brain axis." There is clear evidence of the influence of the gut microbiota on brain development and neurogenesis (Heijtz et al., 2011). Studies have already shown influences of the gut microbiota on neuropsychiatric disorders such as depression (Kelly et al., 2016), schizophrenia (Dinan & Cryan, 2015), autistic spectrum disorder (Hsiao et al., 2013), irritable bowel syndrome (Didari, Mozaffari, Nikfar, & Abdollahi, 2015), Parkinson's disease (Sampson et al., 2016), and Alzheimer's disease (Minter et al., 2016), both in rodent and human studies. Likewise, emerging evidence suggests potential therapeutic benefits of using probiotics in various CNS conditions, some of which are presented below.

15.6.1 Autism spectrum disorder

Autism spectrum disorder (ASD) is an umbrella term for several neurodevelopmental disorders, all characterized by deficits in social communication and social interactions, accompanied by restricted and repetitive patterns of behavior, interests, and/or activities. Because every patient with ASD has a wide spectrum of communicative and behavioral problems, no single therapy has proven effective. Current traditional interventions to improve communication and social behaviors in ASD patients include psychosocial and educational strategies, speech therapies, behavioral interventions, developmental therapies, and

parenting skills training programs. Altered gut microbiota in patients with ASD has been reported, and the use of probiotics to benefit children with ASD was investigated in several clinical trials (Chen & Liu, 2021). ASD affects approximately 1% of children in the United States, with a significant prevalence in men to women. Data from the US Centers for Disease Control (CDC) estimated the male-to-female ratio of ASD prevalence to be 4:1 in 2016 (Maenner et al., 2020).

Patients with ASD have deficits in social communication and interactions, and restricted repetitive behaviors and behavioral interventions for the mainstay of treatment in most cases (Chaste & Leboyer, 2012). It is hypothesized that the significant prevalence of ASD in males compared to females may be associated with the neuroprotective properties of the sex hormone estrogen, although the exact mechanism by which this protection is provided is not known. A possible potential mechanism by which estrogen can contribute to a lower prevalence of ASD disease in women would be through the modulation of the intestinal microbiome. As an example, women with higher levels of serum estradiol have been shown to have higher levels of intestinal microbial diversity (Shin et al., 2019). It has also been shown that individuals with high levels of serum estradiol had high levels of *Bacteroides* and reduced levels of *Firmicutes* (Shin et al., 2019). Similarly, children with ASD generally have a greater abundance of *Bacteroidetes* and reduced levels of *Firmicutes* and *Bifidobacterium* when compared to healthy controls (Finegold et al., 2010, 2012; Mezzelani et al., 2015). It is noteworthy that some patients with autism have a restricted diet and use antibiotics, which could explain microbial changes (Brito, Pinto, Vian, & Amaro, 2020; Cryan & Dinan, 2012; Doenyas, 2018; Louis, 2012; Mangiola et al., 2016; Vuong & Hsiao, 2017).

It is already known that the health and growth of the immune system are closely related to the interaction between the intestine, the microbiome, and its commensal bacteria. Immune cells are known to have receptors (responsible for bacterial cell wall recognition) that respond to "danger" signals. This relationship must be homeostatic so that the immune system and the normal intestinal bacterial environment coexist and interact without peripheral inflammatory processes interfering in this dynamic. There are several problems that can be caused by dysregulation of the immune response, including autoimmune diseases that present a pro-female bias (Jacobson, Gange, Rose, & Graham, 1997). In support of this notion, there is evidence to suggest that early life infection in males may "prime" microglia (e.g., possibly through lipopolysaccharide-induced activation of TLR4 or other pattern recognition receptors) in such a way that it is a challenge. Later-life immune system results in an exacerbated pro-inflammatory response increase the risk of neuronal degeneration as well as impaired cognitive function (Bilbo et al., 2005; Williamson, Sholar, Mistry, Smith, & Bilbo, 2011), an effect that does not seem to occur in females (Bilbo, Smith, & Schwarz, 2012).

There is also preclinical evidence showing that the administration of strains such as *Bacteroides fragilis* improved autism-like behaviors in a mouse model of maternal immune activation (Hsiao et al., 2013). In human studies, it was shown that the *Lactobacillus plantarum* PS128 strain positively impacted ASD symptoms in male children, possibly by inhibiting the LPS-induced production of the proinflammatory cytokines TNF-α, IL-1β, and IL-6 (Liu et al., 2019). This supports the hypothesis that the immune system is a critical component of the microbiome-gut-brain axis. Furthermore, the same study indicated that younger children (7–12 years) experienced greater benefits than older children (13–15 years), suggesting an age-related effect of the intervention (Liu, Peng, Zhang, Wang, & Luo, 2019a). It has also been

shown that these effects are mediated through reduced serum corticosterone and IL-6 levels and an increase in the level of dopamine in the prefrontal cortex, pointing to a complex microbiome-gut-immune-neurotransmitter interaction underlying the effect of probiotic administration.

15.6.2 Attention-deficit/hyperactivity disorder (ADHD)

The ADHD is a neurodevelopmental disease with about 7.2% prevalence in children (Thomas, Sanders, Doust, Beller, & Glasziou, 2015), and is characterized by a high level of inattention and/or deficit of hyperactivity and impulse control that is often inadequate. The etiology of attention deficit hyperactivity disorder is complex, and there are several factors that can affect the occurrence of the disorder, such as genetic ones [mainly associated with genes for dopamine receptors DRD4 and DRD5, as well as genes for the transmitter of dopamine and serotonin) (Bonvicini et al., 2020)] and environmental and those associated with perinatal damage; however, identifying the true causality of environmental risk factors for ADHD is highly debatable. There is a well-known genetic predisposition shown in numerous family and twin studies (Faraone et al., 2005; Grimm, Kranz, & Reif, 2020). Another new direction of focus of studies on risk factors for attention deficit hyperactivity disorder is the change and imbalance in the intestinal microbiota and their potential impacts on the occurrence and clinical course of neurodevelopmental disorders (Sandgren & Brummer, 2018).

Stimulant or non-stimulant pharmacotherapeutics are normally used for the treatment of ADHD, which targets specific systems related to dopamine, norepinephrine, and/or serotonin neurotransmission (Cortese & Coghill, 2018). Dietary intervention may also impact ADHD, and it has been suggested that, since the gut microbiota responds quickly to a change in diet, interventions such as probiotics may provide a previously underutilized treatment option for children with ADHD (Pelsser et al., 2017).

Long-term study where male infants who received *Lactobacillus rhamnosus* GG during the first 6 months of life showed a correlation between lower *Bifidobacterium* species count and the development of ADHD or Asperger Syndrome (AS) (Pärtty, Kalliomäki, Wacklin, Salminen, & Isolauri, 2015). These authors found that a lower median of *Bifidobacterium longum* at 3 months of age in children resulted in a higher prevalence of neuropsychiatric disorders (ADHD and Aspergers) compared to healthy children. At 18 months, they also had fewer *Bacteroides* and *Lactobacillus-Enterococcus* bacteria and at 24 months, fewer *Clostridium histolyticum* cells compared to children without a subsequent diagnosis. In addition, at age 13, ADHD or Asperger's Syndrome was diagnosed in 17.1% of children in the placebo and none in the supplemental probiotic group, suggesting that early intervention may help to alleviate the likelihood of developing ADHD in children. Other studies have proposed different bacterial groups as potential new ADHD markers - *Bifidobacterium* (Aarts et al., 2017; Stevens et al., 2019), *Neisseria* and *Bacteroides* (Prehn-Kristensen et al., 2018), *Faecalibacterium* (Jiang et al., 2018), *Bacteroides uniformis*, *Bacteroides ovatus* and *Sutterella stercoricanis* (Wang et al., 2020), *Ruminococcaceae* (Szopinska-Tokov et al., 2020). It is also likely that not one, but different types of bacteria could potentially be associated with ADHD, and the gut microbiome is one of many contributing factors in the etiology of ADHD.

Recently, many researchers have become interested in the influence of probiotic supplementation on mental health. The influence of probiotic supplementation can be direct or

indirect. Children with ADHD score higher on the Gastrointestinal Severity Index and report constipation and flatulence more often than healthy children (Ming et al., 2018). Reducing these symptoms leads to an improvement in the quality of life of patients (Eltokhi, Janmaat, Genedi, Haarman, & Sommer, 2020). This can be achieved by changing the composition of the microbiota, for example, by probiotic supplementation. Some studies have shown that *Lactobacillus rhamnosus* GG leads to stabilization of the intestinal permeability barrier, probably secondary to alterations in tight junctions, mucin production, and antigen-specific immunoglobulin A production (Isolauri, Kalliomaki, Laitinen, & Salminen, 2008; Pärtty et al., 2015). Furthermore, the impact of *Lactobacillus rhamnosus* on the regulation of emotional behavior and the central GABAergic system via the vagus nerve is associated with several neuropsychiatric disorders (Bravo et al., 2011; Enticott, Rinehart, Tonge, Bradshaw, & Fitzgerald, 2010; Pärtty et al., 2015), including ADHD.

15.6.3 Major depressive disorder (MDD)

The MDD is a severe mental disorder characterized by at least two weeks of low mood characteristics, such as low self-esteem, loss of interest in activities, low energy, sleep disruption, and loss of appetite. Around 16.6% of adults have at least one episode of depression during their lifetime and the chance of recurrence increases with each episode. Furthermore, rates of depression increase substantially between 13 and 18 years of age (Avenevoli, Swendsen, He, Burstein, & Merikangas, 2015). The first line of treatment for moderate major depressive disorder is antidepressant monotherapy or psychotherapy or a combination of both (Davidson, 2010).

A large body of evidence now indicates that depression is associated with a low-grade chronic inflammatory response and activation of cell-mediated immunity (Maes et al., 2012; Moylan, Maes, Wray, & Berk, 2013). Inflammatory cytokines are being considered to mediate both genetic and environmental stress (e.g., psychosocial stress, sleep disruption, unhealthy diet, physical inactivity, smoking, and medical factors) that trigger major depressive disorders (MDD) (Raison & Miller, 2011). Two meta-analyses confirmed that pro-inflammatory cytokine levels were higher in depressed patients compared to control subjects (Eyre et al., 2016; Köhler et al., 2017). Furthermore, patients with elevated inflammatory cytokine levels attributed to a variety of medical conditions have increased rates of depressive symptoms compared to healthy individuals (Yirmiya et al., 2000; Yirmiya et al., 2007). Furthermore, administration of cytokines and cytokine inducers in laboratory animals and humans can lead to behavioral changes that significantly overlap with those found in depression, including anhedonia, reduced activity, cognitive dysfunction, and altered sleep patterns (Miller et al., 2009). One of the proposed mechanisms for the effects of cytokines on symptoms of depression is the activation of inflammatory signaling pathways in the brain, which results in changes in the monoamine, glutamate, and neuropeptide systems (e.g., corticotropin-releasing hormone) and a reduction in hormone-releasing factors. growth (e.g., brain-derived neurotrophic factor) (Felger & Lotrich, 2013).

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis is also strongly implicated in the pathology of major depressive disorder (Du & Pang, 2015). According to this evidence, antidepressant medications have been shown to alter HPA axis activity (Du & Pang, 2015). Two mechanisms have been suggested to explain HPA axis hyperactivity in

depression. One mechanism is the stimulation of corticotropin-releasing hormone (CRH) released by inflammatory cytokines (Maes et al., 2009), and the second is an impairment of serotonin metabolism because serotonin increases the expression of glucocorticoid receptors in the hippocampus through the tryptophan receptor 7 of 5 hypodoxia (Heisler et al., 2007).

Significant changes in the composition of the gut microbiota have been observed in depressed individuals compared to non-depressed individuals (Jiang et al., 2015). Dysbiosis, a disturbance in the balance of the intestinal microbiota, affects the intestinal barrier function by increasing the permeability of the intestinal mucosa. As a result, translocation of gram-negative bacteria into lymph nodes and bloodstream has been observed (Maes, Kubera, & Leunis, 2008). Lipopolysaccharides (LPS) from gram-negative bacteria activate the innate immune system resulting in the production of pro-inflammatory cytokines (Berg & Garlington, 1979; Wiest & Garcia-Tsao, 2005). Probiotics have been shown to promote epithelial healing and prevent bacterial translocation across the intestinal epithelium, thereby preventing the activation of immune cells and the production of pro-inflammatory cytokines (Bravo et al., 2012).

Probiotic therapeutic intervention in animals has shown positive effects in reducing depressive behaviors such as anxiety and stress (Bravo et al., 2011; Desbonnet et al., 2010). For example, treatment with *Lactobacillus rhamnosus* JB-1 attenuated GABA receptor mRNA expression in specific brain regions to reduce stress-induced corticosterone and depression-related behavior (Bravo et al., 2011). Other species of *Lactobacillus* were also able to produce neuroactive substances such as serotonin and dopamine (Barrett et al., 2012), which can compensate for the dampening of the activity of these neurotransmitters considered to be associated with depression (Winter, Hart, Charlesworth, & Sharpley, 2018).

In clinical studies, it has been reported that healthy volunteers who received *Lactobacillus helveticus* and *Bifidobacterium longum* had decreased psychological stress and depression (Messaoudi et al., 2011). Likewise, a mixture of *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum* had beneficial effects on Beck Depression Inventory Scores (Akkasheh et al., 2016). Another important consideration in interpreting these results is the effect of antidepressants on the composition of the intestinal microbiota. In fact, some antidepressants have been shown to be against several groups of microorganisms (Munoz-Bellido, Munoz-Criado, & Garcìa-Rodrìguez, 2000).

About disturbances in the gut microbiota in patients with MDD, it was seen that women with a first depressive episode had a greater abundance of *Actinobacteria* compared to healthy volunteers. On the other hand, males showed a lower abundance of *Actinobacteria* and *Bacterodia* (Chen et al., 2018). This study showed that interactions between microbiota status and the severity of depressive symptoms can be sex-specific. However, further studies would be needed to identify whether the effect of probiotics in alleviating depressive symptoms is gender-specific. The Microbiome Projects Group revealed that adult males (18–40 years) are three times more likely to have a relative abundance of *Prevotella* and a lower abundance of *Bacteroides* (Ding & Schloss, 2014). Overall, both microbial families ferment carbohydrate structures to produce different proportions of SCFAs. However, one study has shown that having a greater abundance of *Prevotella* effectively produces 2 to 3 times more propionate and overall, more SCFAs compared to *Bacteroides* (Chen et al., 2017). This may be beneficial to the host, as higher doses of SCFA via colon

delivery resulted in greater reductions in the cortisol response to acute psychosocial stress in men, an effect that is lacking in women (Dalile, Vervliet, Bergonzelli, Verbeke, & Van Oudenhove, 2020).

15.6.4 Generalized anxiety disorder (GAD)

The GAD is a chronic psychiatric disorder characterized by disproportionate worries and symptoms such as restlessness and insomnia (Hoge, Ivkovic, & Fricchione, 2012). The expression of anxiety is often accompanied by bowel dysfunction and bowel distress, with stomach pain or nausea being commonly reported as symptoms of anxiety (Walter et al., 2013). There is some variation in prevalence between countries; while higher prevalence is observed in high-income countries (5%), middle/low-income countries have a prevalence of 1.5 to 3% (Preti et al., 2021). Pharmacotherapy (benzodiazepines and antidepressants) is the commonly accepted treatment for patients with GAD, although it has been suggested that long-term treatment with benzodiazepines should be avoided whenever possible (Andrews et al., 2018; Preti et al., 2021).

A systematic review was recently performed to provide insights into and explore the clinical intervention potential of probiotics to treat anxiety (Yang, Wei, Ju, & Chen, 2019). These researchers reported that eleven of twenty-one studies demonstrated that regulation of the gut microbiota could improve anxiety symptoms. Five of the studies used probiotic interventions, while six of the studies used non-probiotic dietary interventions. Although only 52% of probiotic studies have shown a positive effect on anxiety, this is not entirely unexpected, as the studies used different bacterial species. Furthermore, neither age nor gender was considered for these studies.

A more recent study using *Lactobacillus plantarum* DR7 also demonstrated a positive impact of probiotic administration on anxiety (but not depression) (Chong et al., 2019). In this study, it was further seen that, although probiotic administration benefited both elderly and young people about anxiety, only the younger adult population benefited from stress-related symptoms compared to placebo. Although the authors speculate that this can be attributed to greater perceived stress among young people compared to normal/elderly adults, which leaves much room for improvement through natural interventions, it is conceivable that the impact of probiotic administration is more robust in youth and points to the need for future studies to control reservations such as age.

Intestinal microbiome examination also reveals that, in GAD studies, *Prevotella* is abundant in controls and is negatively correlated with anxiety severity and positively correlated with anxiety reduction. In contrast, *Bacteroides* and *Escherichia-Shigella* are highly represented in patients with GAD and are positively associated with anxiety severity (Chen et al., 2019). However, given that healthy males have a greater abundance of *Prevotella*, it is logical that this may contribute, in part, to the higher prevalence of GAD in females. Studies in women have shown that consumption of probiotics can positively impact emotional processing (Tillisch et al., 2013) and improve self-reported mood in healthy adults suffering from negative affect (Benton, Williams, & Brown, 2007). This effect has also been shown to be mediated through dopaminergic and serotonergic pathways (Liu et al., 2019b) pointing to crosstalk between the central nervous system and the intestinal microbiota.

15.6.5 Anorexia nervosa (AN)

It is a psychiatric disorder defined by low body weight and debilitating anxiety about weight gain and concerns about body image. Lifetime prevalence is about 0.6-1%, with women having a significantly higher prevalence than men (approximately 10:1) (Galmiche, Déchelotte, Lambert, & Tavolacci, 2019; Jaggar et al., 2020; Raevuori, Keski-Rahkonen, & Hoek, 2014). Pharmacological interventions for the treatment of AN are not yet approved, although counseling and psychotherapy are recommended. It has recently been shown that disease progression is associated with intestinal dysbiosis, including low microbial diversity and taxonomic differences compared to healthy individuals and other BMI groups (Galmiche et al., 2019; Morita et al., 2015; Roubalová et al., 2020). In addition, the chronic nutritional restriction can result in impaired intestinal barrier function, which in turn contributes to a positive feedback loop and disease progression, but this has not yet been confirmed in humans (Mörkl et al., 2019; Reed, Abbaspour, Bulik, & Carroll, 2021). Metagenomic studies have revealed profound microbiome perturbations in AN, which appear to be associated with extreme weight loss, energy metabolism, and emotional imbalance (anxiety and depression) (Borgo et al., 2017; Zaplatic et al., 2020).

In AN, peptides (also known as stimulating hormones that are synthesized in the gut) alpha-melanocyte, cholecystocin, peptide tyrosine, and orexigenic peptides (ghrelins) affect the brain centers involved in the central control of hunger and satiety through the gut axis. brain. These peptides act through two intertwined pathways that involve crossing the blood-brain barrier and targeting the arcuate nucleus of the hypothalamus or acting through the vagus nerve to reach the nucleus of the brainstem solitary tract (Prinz & Stengel, 2017). The balance and interactions between these peptides influence eating behavior disorders and/or weight loss or weight regain in AN patients. Specifically, the dietary restriction that characterizes AN suggests that the phenotype associated with the condition results in the proliferation of microorganisms that can survive in a calorie-deficient environment (Igudesman, Sweeney, Carroll, Mayer-Davis, & Bulik, 2019), such as, for example, the methane-producing archeon, *Methanobrevibacter smithii*, which metabolizes the hydrogen produced during bacterial fermentation, allowing the transformation of limited nutrients into energy (Borgo et al., 2017, 2018; Million et al., 2013). Gut microbiotas from patients with AN also displayed enrichment in Bacteroidetes and a depletion of SCFA-producing bacteria in *Firmicutes*, *Roseburia*, *Ruminococcus*, and *Clostridium* - supporting the observed reduction of specific SCFA (butyrate and propionate) in patients with AN (Borgo et al., 2017; Mack et al., 2016; Morita et al., 2015). Actinobacteria levels were also increased in female AN patients at both baseline and post-weight restoration (Mack et al., 2016).

The clinical implications of the microbiota-gut-brain axis in AN remain poorly understood, despite some studies showing intestinal dysbiosis. The use of probiotics together with antibiotics may represent ancillary therapeutic options to reduce the severity of swelling and discomfort during treatment. Specifically, antibiotics can be used to eliminate pathogens that disrupt intestinal integrity, while probiotics can be used as an adjunct therapy to restore healthy beneficial species. However, the literature is absent from studies that have evaluated such an approach in AN. It appears that, although intestinal dysbiosis is prevalent in AN, mechanisms for restoring a healthy intestinal microbiome await clarification from further research (Snigdha et al., 2021).

15.6.6 Post-traumatic stress disorder (PTSD)

Another condition where women have increased disease prevalence vs. men is PTSD (Olff & van Zuiden, 2017). The lifetime prevalence of PTSD is about 10 to 12% in women compared with 5–6% in men(Charak et al., 2014; Olff & van Zuiden, 2017) and is characterized by impairments in threat processing and emotion regulation (Herringa, 2017). Behavioral therapy with a focus on trauma and reprocessing are the first line of treatment. Pharmacotherapy is used as a secondary line of defense (Buhmann & Andersen, 2017). Oxytocin, which is associated with facilitating social and behavioral connections as well as stress responses, has been shown to play a sex-specific role in the fear and stress response in recently traumatized patients (Olff & van Zuiden, 2017). In PTSD patients, sex-specific effects were observed following acute oxytocin administration, including reducing anxiety (fear learning) and fear expression. Specifically, there is increased inhibitory control of the ventromedial prefrontal cortex over the centromedial nucleus in men and fewer excitatory dorsal anterior cingulate cortex projections to the basolateral nucleus in women (Frijling, 2017). Select bacterial strains, such as *Lactobacillus reuteri*, have been found to upregulate the hormone oxytocin (Erdman & Poutahidis, 2016). In addition, the phyla *Actinobacteria*, *Lentisphaerae*, and *Verrucomicrobia* have all been shown to be decreased in PTSD in a study where over 77% of the patient group was female (Hemmings et al., 2017).

At present, there is a scarcity of evidence-based treatments to address PTSD symptoms (Bramley, Hong, Zacko, Royer, & Silvis, 2016). Moreover, while there is a compelling body of evidence to support psychotherapeutic treatment for PTSD (e.g., cognitive processing therapy, exposure therapy), many patients do not report benefits (Schottenbauer, Glass, Arnkoff, Tendick, & Gray, 2008). As a result, there is now an increased focus on exploring other strategies, including complementary and alternative medicine interventions, with a focus on associated conditions such as depression, anxiety, and gastrointestinal disorders among others (Brenner et al., 2017). Emerging research suggests that probiotics may be a viable option in this regard with the potential to decrease stress-induced inflammatory responses, as well as other associated symptoms. In preclinical work utilizing a murine model of PTSD (Reber et al., 2016), it has been demonstrated that treatment with heat-inactivated *Mycobacterium vaccae* (NCTC 11659), positively modulates the behavioral response to a psychosocial stressor and reduces the stress-induced release of proinflammatory cytokines, including interferon-gamma and interleukin (IL)-6 (Reber et al., 2016). Very few studies have looked at the impact of probiotic intervention directly on PTSD. A pilot study looking at the effect of *Lactobacillus reuteri* in veterans with PTSD showed a decrease in plasma C-reactive protein concentrations and a significantly larger increase in mean heartbeats following a stressor for the placebo group as compared with the probiotic group. However, no between-group differences were reported on a subjective measure of stress responsivity (Brenner et al., 2017). As this was a pilot study, further studies are necessary to prove this in more detail.

15.6.7 Alzheimer's disease (AD)

The AD is a progressive condition characterized by moderate to severe cognitive decline with age, with genetics being the main risk factor and with about 10% of people of age 65 and older suffering from AD (Calabrò, Rinaldi, Santoro, & Crisafulli, 2021). The

neurodegenerative condition is characterized by the gradual loss of cortical and subcortical tissue, and molecular neuropathology indicates a more abundant presence of amyloid-β plaques and tau-related neurofibrillary tangles. These pathologic changes can mediate synaptic loss, impaired immune responses, neurovascular dysfunction, inflammation, neuronal loss, and brain atrophy, leading to clinically severe dementia (Mrdjen et al., 2019). Today, increasing environmental risk factors include physical and mental stress (Ouanes & Popp, 2019), unhealthy nutrition (Power, Prado-Cabrero, Mulcahy, Howard, & Nolan, 2019), and air pollutants (Calderón-Garcidueñas et al., 2019) have been demonstrated to contribute to the development of AD (Maher, 2019). Pharmacological therapy options are limited to symptomatic management with modestly effective agents (acetylcholinesterase inhibitors, NMDA antagonist). Alterations in gut microbial communities in patients with AD may result in pathophysiological changes in the brain (Singhrao & HardingIs, 2020; Sochocka et al., 2019). In support of this hypothesis, transgenic AD mice raised under germ-free conditions have been shown to have less cerebral amyloid deposition than conventionally raised control mice (Harach et al., 2017). This suggests that gut microbiota may play role in influencing the development of amyloid pathology in AD. Recent studies have explored the impact of modulation of the microbiota-gut-brain axis by bioactive food, prebiotics, and probiotics decelerating the course of Alzheimer's disease, with positive results (Cuervo-Zanatta et al., 2021).

In humans it has been shown that for AD patients there is an increase in the phylum *Bacteroidetes*, with specific increases in *Bacteroides* at the genus level. Additionally, compared to healthy elderly subjects, AD patients exhibit decreased *Actinobacteria* levels - largely driven by changes in *Bifidobacterium*. These bacteria are associated with anti-inflammatory properties and decreased intestinal permeability. Lipopolysaccharide (LPS) is a key outer membrane component of gram-negative bacteria such as Bacteroides and is able to trigger systemic inflammation including the release of pro-inflammatory cytokines after translocation from the gut to the systemic circulation. In elderly individuals, intestinal permeability increases and has been shown to increase LPS-binding protein (a hallmark of microbial translocation). Furthermore, a study using postmortem brain tissue from patients with AD demonstrated that LPS and gram-negative *E. coli* fragments co-localize with amyloid plaques (Vogt et al., 2017) suggesting that increased levels of intestinal bacteria such as *Bacteroides* in AD may result in increased translocation of LPS from the gut to systemic circulation which may contribute towards AD pathology. Thus, a preliminary gut microbial phenotype for AD appears to be increased *Bacteroides* and decreased *Bifidobacterium*, along with a propensity for translocation of pro-inflammatory bacterial components. Furthermore, several *Bifidobacterium* species are widely used as probiotics and have been shown to have pro-cognitive benefits for subjects with AD (Akbari et al., 2016).

The probiotic therapy and improving the intestinal environment could potentially be effective in treating AD. Researchers showed that in Alzheimer's patients, the administration of probiotic milk containing *Bifidobacterium bifidum*, *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Lactobacillus fermentum* improved the mini-mental state examination score (Akbari et al., 2016). Also recently, showed that the use of *Lactobacillus plantarum* P8 in adults with high stress increased social emotional cognition and verbal learning, and memory (Liu et al., 2019a). In addition, has already been suggested that treatment with *Lactobacillus helveticus*-fermented milk in healthy older people leads to improved cognition and attention (Ohsawa, Nakamura, Uchida, Mizuno, & Yokogoshi, 2018).

15.6.8 Parkinson's disease (PD)

After AD, the next most commonly occurring neurodegenerative disorder is PD (Gazerani, 2019). The usual age of onset of PD is about 65 years and it appears slightly more frequently in men than in women. It is a condition characterized by loss of dopaminergic neurons and accumulation of alpha-synuclein (α-syn) in the remaining neurons and classical deficits in gross motor function, although the symptomatology of the condition is now increasingly being recognized as heterogeneous (Kalia & Lang, 2015). There are no current treatments that slow the neurodegenerative process.

A key feature of the heterogeneous symptoms of PD is an imbalance in inflammatory pathways and protective immune functions including neurotoxicity due to the abundance of reactive oxygen species (ROS) (Comi & Tondo, 2017; Magistrelli et al., 2019). Another symptom of PD is constipation, which affects about 70 to 80% of patients (Fasano et al., 2019). It is hypothesized that the loss of enteric dopaminergic neurons together results in an increase in dopaminergic content and diminished gastric mobility (Song et al., 2014). In addition, there is also evidence to show a direct correlation between gut microbes and disease progression in PD patients (Minato et al., 2017). PD patients show reduced levels of *Prevotellaceae* and abundance of *Enterobacteriaceae*, *Bifdobacterium*, and *Firmicutes*.

As observed in many of the conditions listed above, PD patients also present an increased intestinal permeability and higher expression of colonic pro-inflammatory cytokines (Forsyth et al., 2011). In addition to modulating inflammation through cytokines production (Nicola et al., 2016), probiotics may also impact oxidative damage through a down-modulation of ROS (Magistrelli et al., 2019). It has been proposed that increased intestinal permeability and the translocation of bacteria and inflammatory bacterial products might lead to inflammation and oxidative stress in the gastrointestinal tract, thereby initiating α-synuclein accumulation in the enteric nervous system and contributing to the progression of the disease.

Another relevant aspect of the host-microbial interaction is the role of probiotics in producing tyrosine decarboxylase (TDC) (van Kessel et al., 2019). TDC converts levodopa to dopamine in the gut, even in the presence of human decarboxylase inhibitors or tyrosine, which is a competitive substrate. In patients with PD, levels of levodopa are diminished due to an abundance of gut bacterial tyrosine decarboxylase. Thus, an increased abundance of bacterial tyrosine decarboxylases at the site of levodopa absorption can significantly impact plasma levodopa level and consequently the amount entering the brain (van Kessel et al., 2019).

Several studies have shown alterations in the gut microbiota of patients with PD when compared with healthy controls (Heintz-Buschart et al., 2018; Scheperjans et al., 2015; Wallen et al., 2020) and associations between fecal levels of specific bacteria and motor and non-motor features, such as tremor, postural instability and constipation (Scheperjans et al., 2015). Furthermore, two recent meta-analyses reported a pro-inflammatory gut microbiota in PD characterized by depletion of short-chain fatty acids (SCFAs) producing bacteria (Nishiwaki et al., 2020; Romano et al., 2020). SCFAs are metabolites deriving from intestinal microbial fermentation and exert multiple beneficial effects on human health, being able to decrease intestinal and systemic inflammation and promote normal neuronal as well as microglia maturation (Keshavarzian, Engen, Bonvegna, & Cilia, 2020). Notably, both the level of SCFAs producing bacteria and fecal levels of these metabolites are reduced in patients with PD when compared with healthy controls (Unger et al., 2016; Wallen et al., 2020).

The identification of a proinflammatory alteration of the gut microbiota in PD, and its potential role in the pathogenesis of this neurogenerative disorder, has driven the interest in investigating the use of gut microbiota-modulating interventions, such as probiotics, as possible novel therapeutic strategies for PD. Existing literature has demonstrated that probiotics can affect brain activity by reducing neuroinflammation and, consequently, increasing neurogenesis. In particular, preclinical models of rodents have shown that probiotics can decrease peripheral levels of inflammatory cytokines, such as interleukin-1beta (IL-1β) and IL-6 (Mohammadi et al., 2019; Xin et al., 2020), and ultimately prevent neuroinflammation (Shahbazi, Yasavoli-Sharahi, Alsadi, Ismail, & Matar, 2020). In fact, these peripheral cytokines are often able to cross the blood-brain barrier and access brain regions relevant for neurogenesis, especially the hippocampus, where they can exert detrimental effects on cell viability, proliferation, and differentiation (Borsini et al., 2017, 2018; Borsini, Benedetto, M., Giacobbe, & Pariante, 2020). Considering that a proinflammatory status and neurodegeneration are well-known characteristics of the PD pathology (Lim, Bang, & Choi, 2018), clinical intervention with probiotics seems a promising therapeutic approach for this condition.

While it remains unclear if changes in the gut microbiota are a cause or a result of PD pathogenesis, it is evident that there is significant neuronal loss following inflammatory insult and/or oxidative stress that may result from these microbiome changes. It also appears that probiotic bacteria, such as *Bacillus* sp. JPJ, can produce L-DOPA from L-tyrosine, which is then converted to dopamine with the aid of DOPA decarboxylase in vivo which may have some translational potential. Similarly, in vitro evidence from a recent study demonstrates the effects of probiotics (*Lactobacillus* and *Bifidobacterium* genus) in peripheral blood mononuclear cells (PBMCs) on the release of the major pro- and anti-inflammatory cytokines, in addition to the production of ROS (Magistrelli et al., 2019). Data from the study suggest that in patients with PD, *L. salivarius* LS01 and *Lactobacillus acidophilus* significantly reduced proinflammatory cytokines and increased the anti-inflammatory cytokines. It also showed that while a specific strain of *Lactobacillus* (*L. rhamnosus* LR06) may have a strong impact in cells obtained from men compared to women, a select strain of *Bifidobacterium* (*B. lactis* BS01) had a more significant impact in scavenging ROS in cells obtained from women. While both men and women show reduced bifidobacterial abundance in PD, because healthy adult females have higher levels of *Bifidobacteria*, it is possible that administration of *Bifidobacterium* would have a more beneficial impact in women with PD, while men would benefit from administration of *Lactobacillus* species. However, further studies would be required to evaluate this hypothesis.

15.7 Conclusion

Over the last decade, there has been increasing attention on the role of the gut microbiota in human health and diseases. The gut microbiota consists of a community of bacteria, viruses, protozoa, and fungi that dwell in the human gastrointestinal tract and intimately communicate with the human host, modulating intestinal barrier function, metabolism, and immune as well as nervous system activity. The gut microbiota represents, indeed, one of the main characters of the gut-brain axis, a bidirectional network between the gastrointestinal tract and the central nervous system (CNS), and is able to regulate multiple biological mechanisms

and, ultimately, brain activity. Different are the pathways of communication between the gut and the CNS, which include the autonomic nervous system (mainly the enteric nervous system and the vagus nerve), the neuroendocrine system, the hypothalamic-pituitary-adrenal axis, metabolic pathways, and the immune system.

Whilst some promising progress has been achieved in relation to investigating the role of gut microbiota and the utility of probiotic intervention in addressing mental health-related symptoms, many questions and issues remain unresolved, which will hopefully be addressed by the rise in interest in this area. Certainly, better-designed, controlled clinical trials would be able to shed light on whether there may be some difference in the outcomes of interest-based on age and sex of subjects. Looking to the future, the interest in gaining a better understanding of the role of the microbiota-gut-brain axis in human health and disease is well-positioned to grow both in terms of research and application towards the development of novel nutraceuticals and therapeutics. In the short-term, novel probiotic supplements targeting mental health are likely to emerge and may facilitate the long-term progress towards the ambition of probiotic drugs for the treatment of CNS-related disease.

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SECTION III

Probiotics in human health promotion and disease treatment

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CHAPTER

16

Probiotics for oral health and disease treatment

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16.1 Introduction

Probiotics have steadily been established as efficient means in the prevention of atopic diseases (Isolauri, Arvola, SÜtas, Moilanen, & Salminen, 2000; Kalliomäki et al., 2001; Kalliomäki, Salminen, Poussa, Arvilommi, & Isolauri, 2003; Kang et al., 2020; Schmidt et al., 2019; Wang & Wang, 2015), antibiotic-associated diarrhea (Johnston et al., 2011; Johnston, Goldenberg, & Parkin, 2016; Van Wietmarschen, Busch, Van Oostveen, Pot, & Jong, 2020), in alleviating symptoms of irritative bowel syndrome (IBS) (Andresen, Gschossmann, & Layer, 2020; Francavilla et al., 2019; Gupta et al., 2021; Martoni, Srivastava, & Leyer, 2020; Sadrin et al., 2020; Yoon et al., 2014), and improving quality of life in general (Lewis et al., 2020). Probiotics were initially targeting the gut by altering its microbiota and immune response but later the scope of probiotic applications has broadened further even affecting cognitive function, stress, and anxiety (Chong et al., 2019; Kobayashi et al., 2019; Lew et al., 2019; Rudzki et al., 2019).

Oral health and diseases of the mouth have been a subject of probiotic administration for over 20 years. Näse et al. were the first to demonstrate the role of *L. rhamnosus* GG in children's dental health (Nase et al., 2001). Human mouth, the main entry to the gastrointestinal tract, is characterized by site-specific microbiomes inherent to the particular surfaces in the mouth: teeth, gingiva, tongue, oral mucosa, and saliva. The oral microbiome is regulated by physiological and environmental factors distinct at each habitat. Currently, a total of 775 microbial species have been identified in the human aerodigestive tract, of which only 57% are officially named, while a substantial fraction (30%) are known as uncultivated phylotypes (www.homd.org). Essential requirements for oral probiotics are to integrate in oral biofilms, co-aggregate with and inhibit pathogenic species. They also interact with the underlying oral mucosa modulating the immune response. The most commonly used probiotics to combat

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oral diseases belong to the genera *Lactobacillus*, *Bifidobacterium* and streptococci. Furthermore, some species of the host-microbiome may also have probiotic characteristics (Meurman & Stamatova, 2007).

The aim of this chapter is to outline the clinical applications of probiotics in the prevention and treatment of the most common oral diseases: dental caries, periodontal disease, oral inflammatory conditions and oral candidiasis. We also discuss their means of administration and the importance in sustaining oral health.

16.2 Probiotics in caries management

Dental caries is a largely preventable disease. It is a biofilm-mediated, diet-modulated, multifactorial, non-communicable, dynamic disease resulting in net mineral loss of the dental hard tissues as recently defined by the international research community (Machiulskiene et al., 2020). Clinically, it presents with surface discoloration with demineralization, cavitation and tooth destruction accompanied by varying intensity of pain. Globally, the prevalence of caries remains high with more than 530 million children suffering from dental caries of the primary teeth (www.who.int). Caries occurs when members of the resident oral microbiota have a selective ecological advantage over other species, and the disrupted homeostasis of the plaque biofilm propels the disease process (Marsh & Martin, 1999).

The advances in molecular analyses and microbial culture techniques have shown that previously regarded caries pathogens, such as mutans streptococci and lactobacilli, are not the sole causative species but an entire range of bacteria and fungi can contribute to caries development at different stages of the disease (Koo & Bowen, 2014; Tanner, Kressirer, & Faller, 2016). Mechanical plaque removal by tooth brushing with fluoride toothpaste historically has been the standard means of caries prevention and control. However, complete biofilm eradication is not only unattainable but also essentially not necessary according to the current appreciation of the role of oral biofilms in health and disease (Philip, Suneja, & Walsh, 2018). Establishing and maintaining a healthy microbiome emerges as a contemporary approach in the prevention of oral infectious diseases in general (Kilian et al., 2016). Hence, probiotics could be considered as biological means to enhance the health-promoting microbial communities and to help in correcting the ecological imbalance in cariogenic biofilms (Philip et al., 2018).

The anticipated mechanisms of probiotics in caries prevention include pathogen inhibition, competition for binding sites, co-aggregation, and quorum sensing signaling between the biofilm species. Orally administered probiotics have shown only short-term availability in the mouth and their number rapidly diminishes after the intake terminates (Ferrer et al., 2020; Hasslöf, West, Karlsson Videhult, Brandelius, & Stecksén-Blicks, 2013; Romani Vestman et al., 2013; Taipale, Pienihäkkinen, Salminen, Jokela, & Söderling, 2012). However, prolonged and continuous probiotic milk consumption in nursery school children could reduce caries development in high-caries groups (Rodríguez et al., 2016). Stensson et al. (2014) have hypothesized that daily supplementation with *L. reuteri* from birth and during the first year of life could reduce caries prevalence and gingivitis score in the primary dentition up to 9 years of age. Possible mechanisms could be attributed to lowered *Streptococcus mutans* counts in the study population (Alamoudi et al., 2018; Chuang, Huang,

Ou-Yang, & Lin, 2011; Cortés-Dorantes et al., 2015; Jindal, Pandey, Agarwal, & Singh, 2011; Juneja & Kakade, 2012; Manmontri et al., 2020; Pahumunto et al., 2018; Stecksén-Blicks, Sjöström, & Twetman, 2009; Teanpaisan, Piwat, Tianviwat, Sophatha, & Kampoo, 2015), along with an increase in salivary human neutrophil peptides 1-3 (Wattanarat et al., 2021), and enhanced salivary buffering capacity (Glavina et al., 2012; Villavicencio, Villegas, Arango, Arias, & Triana, 2018). A probiotic *L. reuteri* lozenge administered to 2- to 3-year-olds for a 1-year period appeared an effective means of caries risk reduction when given as an adjunct to the daily use of fluoride toothpaste (Hedayati-Hajikand, Lundberg, Eldh, & Twetman, 2015). Furthermore, probiotic milk could be as effective as a fluoride mouthwash in reducing *S. mutans* and high plaque index scores (Patil, Dastoor, & Unde, 2019). Fluoridated milk could be equally effective in reversing soft and leathery primary root caries lesions as seen with milk containing *L. rhamnosus* administered for 15 months in the elderly (Petersson, Magnusson, Hakestam, Baigi, & Twetman, 2011). As regards safety issues, Keller, Larsen, Karlsson, and Twetman (2014), did not observe any alterations to the initial caries lesion area on the tooth surface when a *L. reuteri* lozenge was given for 3 months to adolescents diagnosed with caries. This was a positive finding since lactobacilli like streptococci indeed are acidogenic and aciduric microorganisms. Altogether, based on the findings cited above, probiotic lactobacilli might indeed be recommended as adjuncts to routine oral hygiene practices.

Bifidobacterium species constitute the most abundant population in infants, especially during lactation and they have been shown to modulate innate and adaptive immune responses (Ruiz, Delgado, Ruas-Madiedo, Sánchez, & Margolles, 2017). Their administration in vehicles such as in ice-cream or yogurt could alter the oral biofilm composition by reducing mutans streptococci and subsequently preventing caries lesion progression (Bafna et al., 2018; Çaglar et al., 2008; Singh, Damle, & Chawla, 2011; Zare Javid et al., 2020). Nonetheless, more studies with larger study groups and longer intake times are needed to confirm the observed outcomes with bifidobacteria.

Streptococcus thermophilus and *S. salivarius* are the streptococci utilized as commercial oral probiotics to control caries risk by suppressing mutans streptococci (Lee & Kim, 2014; Schwendicke et al., 2017). *S. salivarius* is oxidase- and catalase-negative and is among the earliest colonizers of the human mouth and nasopharynx (Poorni, Srinivasan, & Nivedhitha, 2019). Probiotic preparations containing *S. salivarius* M18 could lower mutans streptococci counts in caries-active children after 3 months of continuous administration (Burton et al., 2013). The mechanism ascribed was the production of bacteriocins targeting the cariogenic S. mutans. Additionally, S. salivarius M18 after colonizing the human oral mucosa produces the enzymes dextranase and urease that are able to counteract plaque formation and saliva acidity (Di Pierro, Zanvit, Nobili, Risso, & Fornaini, 2015).

Human oral streptococci in a healthy mouth could be an important source of potential probiotics used to colonize patients under dietary conditions of highly varying cariogenicity (Culp et al., 2020). Huang et al. (2018) have found that arginine metabolism of commensal streptococci generates ammonia that increases the pH of oral biofilms and thus lowers the growth and virulence of caries pathogens. A novel oral bacterial isolate, *Streptococcus* A12, could resist antimicrobial peptides, produce factors involved in the production of a putative antimicrobial compound, and disrupt intercellular communication of *S. mutans* (Lee et al., 2019; Lee, Kaspar, Rojas-Carreño, Walker, & Burne, 2021).

To sum up, probiotic use in caries prevention and caries management remains controversial as most probiotic species principally belong to lactobacilli and bifidobacteria, both well known for their acidogenic and aciduric properties. Despite the numerous studies showing favorable probiotic effects on dental caries, some research groups have not been able to observe significant reductions of mutans streptococci counts either in children or adolescents (Keller, Hasslöf, Dahlén, Stecksén-Blicks, & Twetman, 2012, 2014; Lexner, Blomqvist, Dahlén, & Twetman, 2010; Taipale et al., 2012; 2013). This fact further emphasizes the need for more scientific evidence in this area.

16.3 Periodontal diseases and probiotics

Periodontitis is the most common chronic inflammatory non-communicable disease of humans. Severe periodontitis represents the eleventh most prevalent condition in the world (Global Burden of Disease Study, 2017), and the prevalence of milder forms of periodontitis may be as high as 50% (Tonetti, Jepsen, Jin, & Otomo-Corgel, 2017). Periodontal infections are associated with a range of systemic diseases leading to premature death, including diabetes (Sanz et al., 2018), cardiovascular disease (Sanz et al., 2020), or adverse pregnancy outcomes (Haerian-Ardakani et al., 2013). If left untreated or inadequately treated, periodontitis leads to loss of tooth-supporting tissues and finally to loss of teeth.

Periodontal health clinically manifests in healthy appearing gingiva with no spontaneous or stimulated bleeding, no active gingival pockets and loss of tooth-supporting tissues. However, perturbed balance in oral microbiota under favorable oral conditions, the simplest being repeatedly neglected oral hygiene, could easily shift the balance towards disease progression. Periodontal health is indeed integral to general health and has a key role in maintaining a good quality of life. A strategy for achieving periodontal health is the Global Periodontal Health Project (GPHP) launched by FDI World Dental Federation in 2015 (www.fdiworlddental.org). It aims at raising the global awareness of periodontal health and helping to reduce the disease burden by integrating oral health into policies addressing general health by taking a common risk factor approach. Despite global initiatives, however, the burden of periodontal diseases remains high.

Periodontal diseases, including gingivitis and periodontitis, are mediated by the oral microbiome and host interaction with the active role of the immune system (Valm, 2019). Gingivitis is a reversible form of periodontal disease and in most cases resolves with the improvement of oral hygiene practices (Kistler, Booth, Bradshaw, & Wade, 2013). However, in immunocompromised and susceptible individuals the localized gingival inflammation may progress to chronic periodontitis (Kuru, Laleman, Yalnızoğlu, Kuru, & Teughels, 2017). The transition from health to disease is multifactorial and correlates with an increase in diversity of the microbial community with no single pathogen being the culprit for periodontal disease (Abusleme et al., 2013; Lamont et al., 2018). Various groupings of periodontal bacteria have nevertheless been suggested like the "Red Complex" pathobionts, *Prophyromonas gingivalis*, *Treponema denticola* and *Tannerella forsythia*, and the Gram-positive *Filifacter alocis*, which are generally detected in abundance in subgingival biofilm of periodontitis patients (Dabdoub, Ganesan, & Kumar, 2016; Griffen et al., 2012; Socransky, Haffajee, Cugini, Smith, & Kent, 1998). The dynamics of biochemical interactions, signaling and genetic

exchange between cells in a highly organized biofilm community could be regarded as the key mechanisms in the disease onset and progression.

Standard treatment of periodontal diseases encompasses the elimination of all infectious and inflammatory processes as short-term goals and the reconstruction of a healthy dentition fulfilling all functional and aesthetic requirements as a long-term goal (Carranza & Takei, 2015). In general practice, this includes a nonsurgical phase with the elimination of the etiologic factors of gingival inflammation by professional mechanical plaque removal. The maintenance phase, in turn, requires full compliance of the patient so that the results obtained in phase I stay and further deterioration and recurrence of the disease will be prevented.

Probiotics as means in preventing and maintaining the stable outcomes of periodontal treatment could be viewed as adjuncts in establishing healthy balance in oral biofilms. There is mounting evidence from clinical studies that probiotic administration could indeed be beneficial both in gingivitis and periodontitis of various stages. Table 16.1 and Table 16.2 show common probiotic formulations investigated in these diseases.

TABLE 16.1 Common probiotic formulations used in periodontitis.

Formulation	Probiotic	Studies
Lozenge	L. reuteri	Grusovin et al. (2020), İnce et al. (2015), Laleman et al. (2020), Pelekos et al. (2019) Szkaradkiewicz et al. (2014), Tekce et al. (2015), Teughels et al. (2013), Theodoro et al. (2019), Vicario et al. (2013)
	B. lactis HN019	Invernici et al. (2018), Invernici et al. (2020)
Tablet	L. reuteri, L. salivarius, L. acidophilus	Soares et al. (2019)
	S. oralis KJ3, S. uberi KJ2, S. rattus JH145	Laleman et al. (2015)
	L. salivarius WB21	Mayanagi et al. (2009)
Capsule	L. plantarum L-137	Iwasaki et al. (2016)
Sachet	L. rhamnosus SP-1	Morales et al. (2016); Morales et al. (2018)
Yogurt	L. rhamnosus L8020	Oda et al. (2019)

TABLE 16.2 Common probiotic formulations used in gingivitis.

Formulation	Probiotic	Studies
Lozenge/ tablet	L. plantarum, L. brevis, P. acidilactici	Montero et al. (2017)
	L. brevis CD2	Lee et al. (2015)
	W. cibaria	Kang et al. (2020)
	L. reuteri	Hallström et al. (2013), Iniesta et al. (2012), Krasse et al. (2006), Sabatini et al. (2017), Schlagenhauf et al. (2020)
Milk drink	L. casei Shirota	Slawik et al. (2011)
	B. lactis DN-173010	Kuru et al. (2017)
	L. rhamnosus	Staab et al. (2013)
Chewing gum	L. reuteri	Twetman et al. (2009)
Mouthwash/toothpaste	Bacillus subtilis, B. megaterium, B. pumulus	Alkayaet et al. (2017)

Several indices, including bleeding on probing (BOP), gingival index (GI), plaque index (PI), and probing periodontal pocket depth (PPD), are routinely measured in the clinic to assess periodontal health and disease progression of a patient. When administered to subjects with gingivitis probiotic formulations could significantly reduce BOP, GI and PI scores, respectively (Alkaya et al., 2017; Krasse et al., 2006; Kuru et al., 2017; Patyna, Ehlers, Bahlmann, & Kasaj, 2021; Sabatini, Lauritano, Candotto, Silvestre, & Nardi, 2017; Schlagenhauf, Rehder, Gelbrich, & Jockel-Schneider, 2020; Slawik et al., 2011; Twetman et al., 2009). Probiotic *L. reuteri* lozenges were shown to be a safe adjunct in controlling pregnancy gingivitis by lowering GI, PI scores and serum TNF-α concentrations when taken twice daily (Schlagenhauf et al., 2016). Reduction in the number of harbored periopathogens is among the mechanisms leading to the observed outcomes (Iniesta et al., 2012; Kang, Lee, Lee, Kim, & Nam, 2020). In healthy individuals, probiotic lozenges containing *L. rhamnosus* and *B. lactis* significantly reduced total salivary bacterial counts and lowered *A. actinomycetemcomitans*, *F. nucleatum*, and *P. gingivalis* in dental plaque (Alanzi et al., 2018). However, despite lowering PI and GI scores, in healthy subjects no reduction of periodontal pathogens has been observed probably because species like *P. gingivalis*, *P. intermedia*, and *A. actinomycetemcomitans* are generally inhabiting the mouth in very low concentrations (Toiviainen et al., 2015). Downregulating an inflammatory cascade could be another explanation to the observed effects (Lee, Kim, Ko, Ouwehand, & Ma, 2015). For example, by lowering elastase and MMP-3 activity, probiotic milk drink could prevent the development of gingivitis (Staab, Eick, Knöfler, & Jentsch, 2009).

Generally, probiotics are recommended as adjuvants to mechanical debridement and preventive treatment, not for sole treatment choice. Administering probiotics in lozenges seems to be the most preferred approach in periodontitis patients and *L. reuteri* is so far the most studied of the species (Grusovin et al., 2020; İnce et al., 2015; Tekce et al., 2015; Theodoro et al., 2019; Pelekos et al., 2020; Vicario et al., 2013; Teughels et al., 2013; Laleman et al., 2020; Szkaradkiewicz et al., 2014). Other preparations containing *Bifidobacterium lactis* NH019, *L. salivarius*, or *L. rhamnosus* have also shown clinical improvement after mechanical plaque removal (Invernici et al., 2020; Invernici et al., 2018; Mayanagi et al., 2009; Morales et al., 2016; Oda et al., 2019; Shimauchi et al., 2008).

Furthermore, halitosis or bad breath, which is a frequent concern of periodontitis patients, could be effectively mitigated by the administration of a combination of *L. reuteri*, *L. salivarius*, and *L. acidophilus* (Soares, Carvalho, & Tinoco, 2019). The beneficial effects observed after probiotic administration could be related to bacteriocins, *i.e.* reuterin, which suppresses the production of pro-inflammatory cytokines and interferes with adhesion and colonization of pathogens (Bian, Molan, Maddox, & Shu, 2011). However, Laleman et al. (2020) failed to observe significant suppressive effects on the tested periopathogens. The positive effects of probiotics on oral health could be due to immunological mechanisms and with possible interference in the expression of markers involved in regulating the immunocompetence of the epithelial barrier (Invernici et al. 2020). Pro-inflammatory markers such as TNF-α, IL-1β, IL-17, IL-8, and MMP-8 significantly decreased after *L. reuteri* administration (Szkaradkiewicz et al., 2014; Twetman et al., 2009). *B. lactis* can also trigger increased expression of beta defensin-3 in the gingival epithelium in periodontitis patients (Invernici et al. 2020). All these findings point to an immunological mechanism that could explain the partly favorable outcome of probiotic use in periodontal diseases.

Albeit probiotics have shown positive effects when applied along with mechanical plaque removal, still their effects are strongly dependent on the strain, dosage, frequency, and mode of administration (Teughels, Loozen, & Quirynen, 2011). Clinical studies with *L. reuteri* (Pelekos, Ho, Acharya, Leung, & McGrath, 2019), *L. brevis* and *L. plantarum* (Pudgar et al., 2021), *L. rhamnosus* (Morales et al., 2018; Morales et al., 2021), and *S. oralis*, *S. uberis* and *S. rattus* tablet (Laleman et al., 2015) have shown no significant differences in clinical parameters between placebo and test groups after scaling and root planning. Thus, the role of probiotics in combating or preventing periodontal diseases is not clear. The complex balance between tissue resistance to microbial attack and the manifold role of the immune system makes studies in this area highly challenging. More scientific evidence is indeed needed for the final conclusion even though there are some promising results reported in the literature.

16.4 Probiotics and candidiasis

Oral candidiasis is commonly caused by the commensal yeast *Candida albicans*, but also *C. tropicalis* and *C. glabrata* may be involved. Candidiasis is particularly common in the elderly, denture wearers, and immunocompromised patients. Predisposing factors for candidiasis are advanced age, diabetes, HIV infection and AIDS, frequent use of steroid inhalers and broad-spectrum antibiotics, and terminal illnesses (Akpan & Morgan, 2002). Overgrowth of candida clinically presents with local discomfort, altered taste and dysphagia when extending posteriorly to the esophagus.

In vitro studies have shown the inhibitory effect of probiotic species on candida either in planktonic state or in structurally well-organized biofilm models (Jiang et al., 2015, Elahi, Pang, Ashman, & Clancy, 2005; Rossoni et al., 2018, Zhao et al., 2016; Denkova, Yanakieva, Denkova, Nikolova, & Radeva, 2013; Hasslöf, Hedberg, Twetman, & Stecksén-Blicks, 2010; Jørgensen, Kragelund, Jensen, Keller, & Twetman, 2017). The downregulation of various candida genes critical to the yeast-hyphae transition, biofilm formation, tissue invasion and cellular damage may be considered the key mechanisms of probiotic effects in this area (James, MacDonald, Chanyi, Cadieux, & Burton, 2016).

Candida-associated stomatitis is indeed frequent in denture wearers. However, oral supplementation with *L. rhamnosus* or a combination of *B. longum*, *L. bulgaricus*, and *S. thermophilus* could lead to significant improvement of the severity of candida-stomatitis (Lee et al., 2019; Li et al., 2014). Colonization of denture surfaces with candida may be delayed by the administration of probiotics in a form of cheese or a capsule of lyophilized species (Ishikawa et al., 2015; Miyazima et al., 2017). Hyposalivation and yeast counts in the elderly were shown to be positively affected in the elderly consuming probiotic cheese (Hatakka et al., 2007). Used as an adjuvant, *S. salivarius* K12 could shorten the course of conventional antifungal therapy in oral candidiasis (Hu et al., 2019). *L. reuteri* tablets given to frail elderly were shown to lower high candida levels with no other side effects (Kraft-Bodi, Jørgensen, Keller, Kragelund, & Twetman, 2015).

Oral candidiasis is a common complication in patients receiving cytotoxic chemo and radiotherapy as part of the treatment of oral malignancies and leukemias. Antifungal agents are routinely prescribed to prevent superficial candidiasis in such patients with the main goal

of preventing systemic fungal infections that often are difficult to diagnose and can be life-threatening. Amphotericin B given intravenously has been a mainstay in preventing systemic candidiasis in immunocompromised cancer patients (Gotzsche & Johansen, 2002). However, a Cochrane review by Clarkson et al. (2007) has concluded that drugs that are absorbed from the gastrointestinal tract should not be prescribed for treating and preventing oral candidiasis. Hence, oral probiotics might prove beneficial in inhibiting the spread of yeasts from the mouth to the gut. This approach might even help in preventing systemic fungal infections. However, so far there is no scientific evidence on this. A meta-analysis by Mundula et al. (2019) has shown that probiotic treatment can reduce oral *Candida* spp. counts but definitely more studies are called for in this area, too.

16.5 Unsolved issues

As outlined above, probiotics have been recognized as potential adjuncts in the prevention and treatment of common oral non-communicable diseases. Probiotic formulations are commercially available and easily accessible by the consumer. However, there is inconclusive evidence as to which probiotic formula to be used for caries, periodontal disease, or candidiasis, or in fact, in any oral health indication. Lozenges and chewable tablets appear the vehicles of administration with the best potential in the scope of the oral cavity. Namely, they provide prolonged contact with oral surfaces and can be designed to deliver safe concentrations of probiotic species allowing, consequently, controlled administration. Clinical studies with probiotic gels, drops and mouthwashes have failed to demonstrate similar or better effects compared to the probiotic lozenges (Laleman et al., 2020; Pudgar et al., 2021; Sajedinejad et al., 2018). Yogurt and milk as regularly consumed food items can easily be recommended for delivering probiotics. Milk fermented with *L. rhamnosus* L8020 lowers the risk of periodontal disease in individuals with intellectual disabilities (Oda et al., 2019). Dairy products as vehicles of administration might indeed be most suitable for certain patient groups. However, fermented probiotic milk preparations also need safety assessment since if they contain sugar that may be detrimental to teeth. Surprisingly, key players in the dairy market still sell mighty probiotic shots containing 14% sugars (https://www.actimel.co.uk/yogurt-drink/original-range/multifruits). Another approach is developing mucoadhesive delivery systems that enable prolonged retention at the site of action. They seem practical in the prevention and treatment of oral candidiasis (Shinkar, Dhake, & Setty, 2012).

The daily dose needed for a probiotic effect is not known and studies are sparse in this area. Significant improvement in denture wearers was observed when the patients received three doses per day instead of two or one (Keller & Kragelund, 2018; Li et al., 2014). This finding, among others, supports the view that for an effect probiotics must be taken regularly. Similarly, the duration of probiotic supplementation to achieve stable treatment outcomes also needs to be further studied.

Live *vs.* inactivated probiotics has been a controversial issue when discussing aspects of probiotic safety. Probiotic consumption is generally safe, but in situations as with immunocompromised and critically ill patients, the translocation of bacteria to the bloodstream needs consideration (Kulkarni, 2019; Yelin et al., 2019). Here inactivated probiotics might be the choice in the future and this method would also improve the product shelf life. Heat-killed

probiotics may still support healthy immune function (Hirose, Murosaki, Yamamoto, Yoshikai, & Tsuru, 2006) and their cell wall components exert complex immune activating and present anti-inflammatory properties (Jensen, Cash, Farmer, & Keller, 2017). But even for oral diseases developing such preparations might provide hitherto better ways to control the administration. For example, heat-inactivated *L. plantarum* L-137 and *L. crispatus* KT-11 in a capsule or a tablet taken daily decreased the depth of periodontal pockets in patients undergoing supportive periodontal treatment and prevented periodontal disease through the improvement of oral conditions (Iwasaki et al., 2016; Tobita, Watanabe, Tomokiyo, & Saito, 2018). Nevertheless, more clinical studies assessing the effects of live *vs.* inactivated probiotic preparations are definitely called for.

Single species *vs.* combination of probiotics is further an area that requires more research. As cited earlier, only a limited number of studies using a combination of probiotics have been published with limited or no substantial clinical effects. Altogether, in spite of over 20-year history of probiotic applications in oral health, there are still more questions than answers. Data today nevertheless show indisputable beneficial effects in using certain probiotic bacteria for oral and dental diseases but as here discussed, selecting the best species for each indication, best ways and frequency of administration, and ensuring product safety, are still questions partly unanswered.

16.6 Conclusion

In summary, probiotic administration holds promise as an effective approach in prevention of the most common oral infectious diseases. Probiotics are able to colonize the oral cavity and thus shift the balance to a healthier oral microbiome. The interaction with the underlying oral structures and the key mechanisms of their beneficial effects, however, need further studies.

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CHAPTER

17

Probiotics for gastrointestinal health and disease treatment

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17.1 Introduction

The gastrointestinal tract (GIT) is responsible for producing and secreting digestive enzymes and absorbing essential nutrients. For this process to be effective, the involvement of the central nervous system, enteric and specialized enteroendocrine cells is necessary. In addition, the intestinal immune system and intestinal microorganisms play a maintaining effect on the physiological activities of the GIT. Gastrointestinal disorders include pathologies such as gastroesophageal reflux disease (GERD), gastritis, and inflammatory bowel diseases that can affect distinct organs, including the esophagus, stomach, and intestines (Greenwood-Van Meerveld, Johnson, & Grundy, 2017). Early evidence has shown that microbiota impairment can be found in the GIT. Thus, interventions targeting the gut microbiota could be considered to prevent or treat disorders such as irritable bowel syndrome, Crohn's disease, and constipation (Dimidi, Christodoulides, Scott, & Whelan, 2017; Greenwood-Van Meerveld et al., 2017).

The action of probiotic strains in reducing gastric acid production has been studied (Aiba, Nakano, Koga, Takahashi, & Komatsu, 2015). In addition, the adhesion of probiotics to stomach cells promotes an increase in mucin production and improves the physical gastric barrier (Urita et al., 2015). This interaction of probiotic effects on the epithelium and luminal content can contribute to the maintenance of functions and prevent pathologies that affect the upper GIT (Cheng & Ouwehand, 2020). Furthermore, probiotics may exert effects against the growth and infiltration of pathogenic bacteria (Lorea Baroja, Kirjavainen, Hekmat, & Reid, 2007), improve gut motility, as well immune, sensory, secretory, and neuroendocrine responses (Quigley, 2019).

In summary, probiotics are known for their antimicrobial, anti-inflammatory, and anticarcinogenic characteristics, suggesting a protective effect against gastrointestinal pathologies (Kim et al., 2019). Given the evidence, in this chapter, we compile experimental and clinical

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findings using probiotics to treat or prevent the main pathologies that affect the GIT. The summary of the primary studies using probiotic strains in the upper and lower GIT treatment are shown in Tables 17.1 and 17.2, respectively.

17.2 Probiotics for treatment of upper gastrointestinal tract diseases

17.2.1 Mucositis

Mucositis refers to a mucosal injury, inflammation, and associated symptoms that usually occur at the mouth's mucous lining or along the GIT. Commonly, mucositis results from cancer therapy in patients with head and neck tumors and patients submitted to hematopoietic stem cell transplantation (Bowen et al., 2019; Yarom et al., 2019). Epidemiological studies on cancers of the head and neck report a prevalence of oral mucositis of 80% for patients submitted to radiotherapy and 40% in patients receiving chemotherapy (Yarom et al., 2019). Some risk factors should also be considered such as the consequence of local infections, an allergic reaction to a food or drug, autoimmune disorders, tobacco/alcohol abuse, dental problems, and deficits in micronutrients (Cereda, Caraccia, & Caccialanza, 2018). Clinical manifestations of mucositis are heterogeneous as it could present as painful erythema and ulceration at oral mucosa, dysphagia, nausea, vomit, diarrhea, abdominal pain, bleeding, fatigue, malnutrition, and dehydration (Cereda et al., 2018; Touchefeu et al., 2014).

The development of mucositis in the GIT is marked by the upregulation of proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin one beta (IL-1b), and interleukin 6 (IL-6) (Quaresma et al., 2020). Moreover, mucositis is characterized by higher levels of lipid peroxidation products, extensive production of reactive oxygen species (ROS), and platelet-activating factor (PAF), causing more damage to the mucosa. Oxidative stress is crucial for the development of mucositis and could be associated with epithelial damage to the epithelium (Quaresma et al., 2020). Therefore, oxidative stress and pro-inflammatory conditions commonly induced by chemotherapeutic and radiotherapy treatment can reduce the integrity of the gastrointestinal mucosa.

Oncologic treatments can disrupt the natural barrier of the oral mucous membranes by affecting mucus secretion and the composition of the mucus layer (Cereda et al., 2018). The pathophysiology of mucositis is characterized by reduced crypt length, blunting and fusion of villi, enterocyte hyperplasia, and increased apoptosis in the small intestine (Quaresma et al., 2020). In addition, according to a systematic review, mucositis is strongly associated with intestinal dysbiosis (Touchefeu et al., 2014). The intestinal microbiota dysbiosis is marked by a decrease in commensals such as lactobacilli, bifidobacteria, *Clostridium* cluster XIVa, *Blautia, Roseburia,* and *Faecalibacterium*, combined with increases in *Enterobacteriaceae, Bacteroides,* and *Escherichia* and enhanced gut permeability (Touchefeu et al., 2014).

Despite available interventions, including cryotherapy and drugs (for instance, loperamide) to control mucositis, there is a need to explore further the implementation of adjunctive treatments options for mucositis and related symptoms (Thomsen & Vitetta, 2018). Over the last decades, due to advances in elucidation of the pathophysiology of this disease, a variety of new treatment options have emerged and, among them, the use of probiotics is exciting and seems to be promising (Cereda et al., 2018).

TABLE 17.1 Studies using probiotic strains in the treatment of upper gastrointestinal tract diseases.

Upper gastrointestinal tract diseases	Type of study	Strain used	Administration time	Results	References
Mucositis	Randomized clinical trial	*Lactococcus lactis*	14 days	↓mean percentage of days with oral mucositis, ↓unscheduled office and emergency room visits.	Limaye et al. (2013)
	Randomized clinical trial	*L. brevis* CD2	7 weeks	↓stay without mucositis, ↓development of grade III and IV mucositis.	Sharma et al. (2012)
	Double-blind, placebo-controlled	*L. reuteri*	30 days	↓ inflammatory cytokine levels.	Flichy-Fernandez et al. (2015)
	Triple-blind randomized clinical trial	*L. reuteri*	30 days	↓ bleeding, ↓plate index, ↓drilling pocket depth in implant level.	Galofre et al. (2018)
Gastroesophageal reflux disease	Experimental	*L. johnsonii* No. 1088	2 weeks	↓ growth of *H. pylori*, *E. coli* O-157, *Salmonella Typhimurium*, and *C. difficile*, ↓ gastric acid.	Aiba et al. (2015)
	Clinical	*B. bifidum* YIT 10347	4 weeks	↑ mucin, ↑physical gastric barrier to acidic stomach content.	Urita et al. (2015)
	Randomized controlled	*L. gasseri* LG21	12 weeks	↑pepsinogen, ↑digestion, ↓gastric residence time, ↓frequency scores for reflux.	Ohtsu et al. (2017)
	Clinical	*B. butilis* e *E. faecium* + esoprazolone	8 weeks	↓diarrhea syndrome score, ↓risk of relapse of GERD.	Sun et al. (2019)
Gastritis and Ulcer	Systematic review and network meta-analysis	*L. acidopilus*, *S. boulardii*, and *Lactobacillus* + *Bifidobacterium* + *Enterococcus*	14 days	↑*H. pylori* eradication rates	Wang et al. (2017)
	In vitro	*Lactobacillus sake*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, and *Lactobacillus brevis*	1 day	Inhibitory effect on *H. pylori* growth.	Sun, Liyuan, et al. 2018

B- Bifidobacterium; C- Clostridium; E- Escherichia; GERD- Gastroesophageal reflux disease; H- Helicobacter; L- Lactobacillus; S- Streptococcus.

TABLE 17.2 Studies using probiotic strains in the treatment of lower gastrointestinal tract diseases.

Lower gastrointestinal tract diseases	Type of study	Strain used	Administration time	Results	References
Chronic constipation	Systematic review	*B. longum*	2–24 weeks	↑ digestive transit ↓ constipation	Martinez-Martinez et al. (2017)
	Meta-analysis with clinical trials	*B. lactis*, *B. longum*, *L. casei*, and *L. rhamnosus*	10–28 days	↑ gut transit time	Miller and Ouwehand (2013)
	Clinical	*L. casei* Shirota	3 weeks	↑ stool frequency	Ford et al. (2014)
	Clinical	*B. lactis*	2 weeks	↑ stool frequency	Ford et al. (2014)
	Meta-analysis with clinical trials	*B. lactis*	2–8 weeks	↓ gut transit time ↑ stool frequency	Miller and Ouwehand (2013)
	Meta-analysis with clinical trials	*L. casei rhamnosus* Lcr35 or *B. lactis* DN-173	3–12 weeks	↓ frequency of glycerin enema use ↓ abdominal pain	Jin et al. (2018)
Crohn's diseases	Clinical	*S. boulardii*-17	3 months	↑intestinal impermeability	Garcia Vilela et al. (2008)
	Randomised controlled trial	*L.* GG	1 year	↑remission, but not reduction of lesion severity.	Prantera et al. (2002)
	Clinical	*L. rhamnosus* GG	1 week	↑remission	Schultz et al. (2004)
Ulcerative colitis	Clinical	*VSL#3	8 weeks	↑epithelial barrier function, ↑mucus production ↓ immune response	Huynh et al. (2009)
	Randomized placebo controlled clinical trial	*E. coli* Nissle 1917 + Ciprofloxacin	7 weeks	↓ pathogenic bactéria colonization, ↓ mucosal damage, ↓epithelial permeability ↑ remission rates	Derikx et al. (2016)
	Meta-analysis	**Mix of strains + aminosalicylic acid	8 weeks	↑ remission rates	Peng et al. (2019)
	Randomized controlled trial	*Bifidobacteria*-fermented milk	1 year	↑ pathology exarcebation	Ishikawa, Akedo et al. (2003)
	Meta-analysis of randomized controlled trials	*L. reuteri* ATCC 55730, *VSL#3 and other	8–12 weeks	↑ treatment responde rates ↑ remission rates	Fujiya et al. (2014)

Lower gastrointestinal tract diseases	Type of study	Strain used	Administration time	Results	References
Irritable bowel syndrome	Randomized clinical trial	*S. thermophilus*, *B. breve*, *B. longum*, *B. infantis*, *L. acidophilus*, *L. plantarum*, *L. paracasei*, and *L. delbrueckii* subsp. *bulgaricus*	4 weeks	↑ *Lactobacillus* and *Streptococcus*	Staudacher et al. (2021)
	Clinical	*L. acidophilus* DDS-1 or *B. animalis* subsp. *lactis* UABla-12	6 weeks	↓abdominal pain, ↓bloating, Improvement in bowel habits and quality of life.	Martoni et al. (2020)
	Randomized clinical trial	*B. lactis* B94	4 weeks	↓belching-abdominal fullness, ↓bloating, ↓constipation.	Basturk et al. (2016)
	Clinical	*B. longum* NCC3001	6 weeks	↓depression scores	(Pinto-Sanchez et al. (2017)
	Randomised, double-blind, placebo-controlled clinical trial	*B. bifidum* MIMBb75	8 weeks	↓IBS-associated symptoms.	Andresen et al. (2020)
	Randomized clinical trial	*L. paracasei* HA-196 and *B. longum* R0175	8 weeks	Improved quality of life, emotional well-being and social functioning.	Lewis et al. (2020)
	Randomized clinical trial	*L. acidophilus* NCFM	12 weeks	↓pain scores.	Lyra et al. (2016)
	Randomized clinical trial	*L. paracasei*, *L. salivarius*, and *L. plantarum*	4 weeks	Improvement of abdominal symptoms,	Oh et al. (2019)

B- Bifidobacterium; E- Escherichia; L- Lactobacillus; S- Streptococcus.

[*] *Prepation containing Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Lactobacillus plantarum, Streptococcus thermophiles, Bifidobacterium breve, Bifidobacterium infantus and Bifidobacterium longum.*

[**] *Preparation containing S. thermophilus, L. acidophilus, L. delbrueckii ssp. bulgaricus, L. paracasei, L. plantarum, B. longum, B. infantis, and B. breve.*

Probiotics beneficially influence mucosal integrity by the capacity of these agents to inhibit the proliferation of pathogenic bacteria, restore gut microbiota, downregulate the local inflammation, improve the immune response, and recover the mucosal barrier permeability (Cereda et al., 2018). Although with limited high-quality evidence, early systematic reviews of randomized clinical trials have investigated the role of probiotics in the prevention of intestinal adverse effects induced by radiotherapy and chemotherapy (Bowen et al., 2019; Touchefeu et al., 2014; Yarom et al., 2019).

A recent systematic review analyzed two randomized clinical trials (Yarom et al., 2019). Both studies demonstrated the efficacy of probiotics (*Lactobacillus* and *Lactococcus*) in preventing oral mucositis in head and neck cancer patients. Among included studies, it was shown that topical administration of *Lactococcus lactis* sAGX0085/AG013 at dosing schedules of 1 (2×10^{11} CFU), 3 (6×10^{11}CFU), or 6 times daily (1.2×10^{12} CFU) per day, respectively) reduced approximately in 35% the mean percentage of days with oral mucositis compared to placebo (Limaye et al., 2013). In addition, patients who received AG013 had fewer unplanned office and emergency room visits (36%) when compared to placebo (60%). The other study (Sharma et al., 2012) evaluated the effects of administering *Lactobacillus brevis* CD2 lozenges on the incidence and severity of mucositis and tolerance to chemo-radiotherapy. Grade III and IV mucositis developed in 52% of patients treated with *L. brevis* CD2 and 77% in the placebo. In addition, a more significant proportion of patients remained free of mucositis when treated with *L. brevis* CD2 (28%) compared to the placebo (7%).

A double-blind, randomized, placebo-controlled study evaluated the effect of *L. reuteri* in patients with peri-implant mucositis. After 30 days of probiotic administration, the patients showed reduced inflammatory cytokines compared to the placebo group (Flichy-Fernandez et al., 2015). More recently, in a randomized, triple-blind clinical trial using the same strain and intervention time in patients with mucositis, Garofré et al. reported improvement in general clinical parameters, to cite: reduced bleeding, plaque index, and probing depth of the implant-level pocket, as well as a reduction in bacterial load of *Porphyromonas gingivalis* (Galofre, Palao, Vicario, Nart, & Violant, 2018). Taken together, the clinical findings suggest that probiotic therapy can be a safe strategy for the treatment of mucositis.

17.2.2 Gastroesophageal reflux disease and functional dyspepsia

Gastroesophageal reflux disease (GERD) is a common digestive disorder that primarily affects the esophagus and gastro-duodenum (Cheng & Ouwehand, 2020). This disease occurs when gastric or duodenal contents refluxate pathologically into the esophagus, leading to inflammation, erosion, and ulceration of the esophageal mucosa (Cheng & Ouwehand, 2020). Typical symptoms of GERD include heartburn, regurgitation, nausea, abdominal pain, discomfort, and life quality impairment (Sun, Wang, Sun, Zhang, & Zhang, 2019).

Pathogenesis of GERD seems to linked to dysfunction of gastric microbiota (Fox & Muniraj, 2016). Patients with GERD display a distinct bacterial community than healthy subjects (Fox & Muniraj, 2016), characterized by a lower amount of *Prevotella* spp., a diverse genus of Gram-negative anaerobic bacteria found in abundance in the stomach (Cheng & Ouwehand, 2020).

Management of GERD comprises lifestyle, dietary changes, and medical treatments that suppress intragastric acid secretion (Chatila et al., 2020). Nutritional approaches include diet modifications, such as reducing the portion size per meal, cessation of chocolate, caffeine,

spicy foods, citrus, carbonated beverages, and allergens (dairy and gluten) (Chatila et al., 2020). Lifestyle modifications include weight loss, head-of-bed elevation, tobacco, and alcohol cessation. GERD's medical management comprises proton pump inhibitors (PPIs), the most commonly prescribed drug worldwide (Sun et al., 2019).

PPIs provide significant symptomatic relief, but their adverse effects on gut microbiota have gained recent attention (Cheng & Ouwehand, 2020). Long-term treatment with PPI reduced the abundance and diversity of gastric and intestinal microbiota (Cheng & Ouwehand, 2020). Thus, a therapeutic approach capable of modulating and recovering gastric and gut microbiota has gained strength in treatment and attenuating GERD symptoms.

Pre-clinical and clinical studies have demonstrated the beneficial effects of probiotic consumption on GERD symptoms (Aiba et al., 2015; Cheng & Ouwehand, 2020). An early investigation revealed that administration of *L. johnsonii* No. 1088 (10^6 CFU, for 2 weeks) inhibited the growth of *Helicobacter pylori*, *Escherichia coli O-157*, *Salmonella* Typhimurium, and *Clostridium difficile*, and also reduced gastric acid production in mice (Aiba et al., 2015).

Regarding clinical findings, administration of 1×10^7 CFU/mL of *Bifidobacterium bifidum* YIT 10347 for 4 weeks stimulated mucin production and improved the physical gastric barrier to acidic stomach content in patients with GERD (Urita et al., 2015). In addition, the study showed that *B. bifidum* YIT 10347 could alleviate visceral hypersensitivity and improve the interactions between luminal contents and host esophageal epithelium in GERD (Urita et al., 2015). A randomized controlled study verified that 1×10^9 CFU of *L. gasseri* LG21 for 12 weeks increased pepsinogen, improved digestion, shortened gastric residence time, and lowered frequency scores for reflux (Ohtsu et al., 2017). Another randomized controlled trial investigating the effectiveness of co-intervention using esomeprazole (20 mg) combined with *B. subtilis* and *E. faecium* enteric-coated capsules for 8 weeks (Sun et al., 2019) demonstrated that the combined therapy decreased diarrhea syndrome score and reduced the risk of relapse of GERD patients (Sun et al., 2019).

In summary, these findings indicated that probiotic therapy could exert beneficial properties in GERD conditions. However, according to a systematic review, limited evidence is available to compare the efficacy between probiotic strains due to the small number of available trials, co-interventions, and study heterogeneity (Cheng & Ouwehand, 2020). Moreover, most studies evaluated the gastrointestinal benefits of probiotics in the lower digestive tract, and limited data regarding the upper digestive tract have been reported, particularly for GERD. Lastly, probiotics might be helpful as an adjuvant treatment for GERD; however, further randomized, double-blind, placebo-controlled trials will be needed to confirm their effectiveness in alleviating GERD symptoms.

17.2.3 Gastritis and ulcer

H. pylori is a ubiquitous Gram-negative, flagellated, spiral bacteria reported as important causes of gastric inflammation, peptic ulcer disease, mucosa-associated lymphoid tissue lymphoma, and gastric cancer (Poonyam, Chotivitayatarakorn, & Vilaichone, 2019). It is estimated that chronic *H. pylori* infection affects approximately 50% of the world's population (Boltin, 2016). However, among infected individuals, 85% are asymptomatic or have mild gastritis, 15% may develop peptic ulcer disease, and about 1% can develop gastric cancers (Ji & Yang, 2020). Extragastric disorders can be manifest, such as urticaria, iron deficiency

anemia, and idiopathic thrombocytopenic purpura (Emara, Elhawari, Yousef, Radwan, & Abdel-Aziz, 2016).

Regarding pathophysiological aspects, *H. pylori* can penetrate the mucus layer and access the gastric mucosa by enhancing gastric pH through the secretion of urease, which reduces the viscosity of gastric mucus. Once reaching the stomach lumen, *H. pylori* can activate neutrophils and monocytes, leading to increased production of IL-1β and TNF-, which are potent cytokines inhibitors of gastric acid. The progression of *H. pylori* infection promotes apoptosis of gastric epithelial cells, gastric atrophy, and loss of gastric glands (Emara et al., 2016). Such gastric cell impairments create a favorable environment for *H. pylori* growth (Ji & Yang, 2020).

Management of gastritis and ulcer induced by *H. pylori* infection comprises pharmacological therapies with PPIs, clarithromycin, and amoxicillin (Hu, Zhu, & Lu, 2017). However, *H. pylori* eradication with pharmacological therapies is not always effective (Poonyam et al., 2019). The inadequate treatment results from several factors, including antibiotic resistance, increased bacterial virulence, improper regimens, poor patient compliance, massive gastric bacterial loads, internalizing bacteria, high gastric acidity, gene polymorphisms (IL-1B and CYP2C19), antimicrobial washout, and biofilm formation (Hu et al., 2017).

It has been demonstrated that chronic *H. pylori* infection causes dysfunction of gastric microbiota, a microbial community characterized by the abundance of *Actinobacteria* (including *Bifidobacterium*), *Bacteroidetes*, *Firmicutes* (including *Lactobacillus* spp), and *Proteobacteria* (including *Helicobacter* spp.) (Boltin, 2016). For example, patients infected by *H. pylori* exhibited a low relative abundance of *Firmicutes* and increased *Proteobacteria* (Boltin, 2016).

Over the past decade, there has been increasing interest in agents that can increase the success of *H. pylori* eradication, including probiotics (Boltin, 2016). Patients who received probiotics combined with standard therapy reported further improvements in their symptoms than patients who received only standard treatment (Emara et al., 2016). Thus, the benefits of using probiotics in combination with antibiotics to increase antibiotic drug efficacy and restore the gastrointestinal microbiota can be a suitable intervention.

According to two systematic reviews, supplementation with multi-strain probiotics combined with standard antibiotic therapy can effectively increase the eradication rate and reduce the incidence of adverse events in adults (Wang et al., 2017) and children (Feng et al., 2017) with *H. pylori* infection. Among probiotic strains, *L. acidophilus, Saccharomyces boulardii*, and *Lactobacillus* + *Bifidobacterium* + *Enterococcus* showed efficacy to increase the eradication rates when supplemented in 14-day triple therapy (Wang et al., 2017).

However, probiotic monotherapy has shown a minimal effect on *H. pylori* eradication rate, suggesting that probiotics need to be managed as adjuvant therapy (Losurdo et al., 2018). In summary, there is an agreement in the available literature that probiotics are potential adjuvant agents when added to the standard treatment.

17.3 Probiotics for treatment of lower gastrointestinal tract diseases

17.3.1 Chronic constipation

Constipation is one of the most frequent gastrointestinal disorders in clinical practice, and its prevalence increases with age and affects approximately 14% of the general population (Martinez-Martinez, Calabuig-Tolsa, & Cauli, 2017). This gastrointestinal disorder is

characterized by persistently tricky, infrequent, or incomplete defaecation (Martinez-Martinez et al., 2017). Difficult evacuation is defined by the presence of two or more of the following symptoms at least 25% of the time: straining, lumpy or hard stools, the sensation of incomplete evacuation, the sensation of anorectal obstruction or blockage, or use of manual maneuvers to promote defecation (Dimidi, Mark Scott, & Whelan, 2020). The main consequences of constipation in these patients are fecal incontinence, fecal impaction, urine retention (causing an increased probability of infection), hemorrhoids, anal fissure, rectal prolapse, and cardiovascular alterations (Dimidi et al., 2020). All these signs and symptoms promote psychological stress, reduce social interaction and work productivity, which impairs quality of life. The cause of constipation is multifactorial, including inappropriate diet (poor fiber ingestion, excessive animal protein consumption), little fluid intake, side effects induced by drugs, and a sedentary lifestyle.

Pathophysiological mechanisms of chronic constipation involve slow colonic transit time and dysregulated or deficient colonic propulsive motor patterns (Airaksinen et al., 2019). Damage in colonic function results from dysfunction of colonic smooth muscle, neural innervation, or changes in microbiota composition (Vriesman, Koppen, Camilleri, Di Lorenzo, & Benninga, 2020). Several clinical studies have demonstrated differences in the gut microbiota composition in patients with chronic constipation, suggesting that bacterial dysbiosis may have a pathogenic role in this disorder (Airaksinen et al., 2019; Yoon et al., 2018). Bacterial dysbiosis induced by chronic constipation involves a decreased concentration of bifidobacteria and lactobacilli and increased numbers of *Bacteroidetes* (Dimidi et al., 2020). Such changes in gut microbiota composition have been reported as potential causes to slow gut transit time in constipated patients.

The initial management of constipation-related symptoms focuses on lifestyle and diet changes, such as increasing fluid intake, eating more fiber-rich foods, and exercising regularly (Vriesman et al., 2020). When lifestyle and diet changes are ineffective, pharmacological treatment may be considered, such as laxative drugs and serotonin-receptor agonists or different surgical options in severe cases (Airaksinen et al., 2019). However, these medications have limited efficacy, are expensive, and may result in side effects, especially over long-term use. Thus, there is a need for alternative constipation treatments that are safe, effective, and cost-effective, such as probiotic intervention (Dimidi et al., 2020).

Clinical trials report that the administration of probiotics can improve constipation in the general population, improving gut transit time, stool frequency, and stool consistency (Airaksinen et al., 2019; Dimidi et al., 2019). These effects may be related to the capacity of probiotics to maintain gut lumen homeostasis by suspending the growth of luminal pathogens and restoring the normal flora of the gut. Another possible mechanistic action of probiotics in constipation is that some strains may increase short-chain fatty acid (SCFA) concentration, normalizing gut motility (Dimidi et al., 2017). In vitro and ex vivo experiments have shown that SCFA may affect gut motility by stimulating mucosal receptors connecting to enteric or vagal nerves, acting directly on colonic smooth muscle (Dimidi et al., 2020). Moreover, probiotics may have beneficial antioxidant and anti-inflammatory properties, potentially influencing gut motility (Dimidi et al., 2020). In addition, probiotics modulate the mucosal immune barrier and normalize dysmotility (Dimidi et al., 2020). Also, it is important to mention the role of modulation of gut-brain interactions induced by probiotics. For instance, *L. reuteri* has been shown to increase the excitability of myenteric neurons and interact with the gut-brain

axis via alterations in afferent sensory nerves that affect gut motility, suggesting that probiotics influence the enteric nervous system (Dimidi et al., 2020).

In recent years, systematic reviews have been published evaluating the effects of different strains of probiotics in reducing chronic constipation symptoms. According to a systematic review, the most commonly-tested strain was *B. longum*, singly or combined with other strains. This strain accelerated digestive transit and improved constipation in elderly individuals by 10 to 40% (Martinez-Martinez et al., 2017). In addition, a systematic review and meta-analysis of eleven randomized clinical trials demonstrated the efficacy of different strains of probiotics (*B. lactis, B. longum, L. casei*, and *L. rhamnosus*) with doses ranging from 0.48×10^9 to 97.5×10^9 CFU/d and treatment duration (about 10–28 days) decreased intestinal transit time (Miller & Ouwehand, 2013). Similarly, another systematic review and meta-analysis of fifteen randomized clinical trials showed that probiotics therapy is moderately efficacious for reducing intestinal transit time in adults (Miller, Zimmermann, & Ouwehand, 2016). A systematic review and meta-analysis of two randomized clinical trials showed that administered 6.5×10^9 CFU/d of *L. casei* Shirota for 3 weeks or 1.25×10^9 CFU/d of *B. lactis* for 2 weeks caused a significant increase in stool frequency (Ford et al., 2014).

Regarding data obtained with a children population, a systematic review and meta-analysis of randomized controlled trials evaluated the effect of probiotic supplementation on functional constipation. Based on this study, probiotic strains including *L. casei* (8×10^8 CFU/d) or *Bifidobacterium lactis* strain DN-173 010 (4.25×10^9 CFU/d) reduced the frequency of glycerin enema use and abdominal pain but had no significant effect on treatment success (Jin et al., 2018).

Despite favorable results reported, the clinical importance of probiotics in constipation remains uncertain due to several limitations of clinical trials, including small study sample sizes, the different types of probiotics administered, duration of the probiotic treatments, co-interventions with additional ingredients, and use of nonvalidated assessment tools. Therefore, further adequately powered randomized clinical trials using standardized outcome measures are needed to determine which species/strains, doses, and duration of probiotics are efficacious.

17.3.2 Crohn's diseases

Crohn's disease (CD) is a chronic inflammatory bowel disease associated with an impaired immune response, alternating between periods of activation and remission. It is a disease that can affect the GIT entirely. The most frequent symptoms are diarrhea and abdominal pain, which can seriously affect patients' quality of life. The form of presentation of CD varies according to some factors, such as the location, degree of extension, and disease pattern. The diagnosis is based on the combination of clinical, biochemical, endoscopic, histological, and/or radiological criteria (Baumgart & Sandborn, 2012; Cushing & Higgins, 2021).

CD affects men and women equally, and its incidence and prevalence have increased significantly and commonly occur in developed and urban areas. Almost 30% of patients start with perianal disease, and up to 50% have extraintestinal manifestations at the time of diagnosis, mainly articular, cutaneous, or ocular.

Treatment for CD is based on remission, aiming to prevent complications such as disease progression. The therapeutic management consists of immunosuppressive drugs and strict monitoring of the inflammatory profile of patients (Torres, Mehandru, Colombel, & Peyrin-Biroulet,

2017). Among the therapeutic options, the use of probiotics has been proposed as a potential safe strategy in reducing the intestinal inflammatory process, still without a well-established consensus for the treatment of CD (Akutko & Stawarski, 2021; Kikut, Konecka, Zietek, Kulpa, & Szczuko, 2021).

In a meta-analysis in which two randomized clinical trials were included, the evidence and safety of *Saccharomyces boulardii* for various disease indications in adults was analyzed. Significant positive effects were observed regarding CD in remission of symptoms and relapse. Furthermore, no adverse effects associated with the use of *S. boulardii* were found in any of the trials (McFarland, 2010).

An early study assessing the therapeutic potential of *S. boulardii* in patients with CD in remission demonstrated that treatment with *S. boulardii* (4×10^8 cells, for 7 weeks) improved intestinal permeability and may be an important adjunct strategy in the treatment of disease that alters intestinal permeability (Garcia Vilela et al., 2008). On the other hand, a randomized, placebo-controlled study carried with subjects operated on for CD showed that probiotic therapy with *L.casei* GG (12 billion CFU, for 12 weeks) seems neither to prevent endoscopic recurrence nor reduce the severity of recurrent lesions (Prantera, Scribano, Falasco, Andreoli, & Luzi, 2002).

A systematic review evaluated the efficacy and safety of using probiotics for CD remission (Limketkai, Akobeng, Gordon, & Adepoju, 2020). Among included studies, (Schultz et al., 2004) treated patients with corticosteroids and antibiotics, followed by randomized assignment to *L. rhamnosus* strain GG (two billion UFC/day) or corn starch placebo for one week. They found no induction of disease remission. Another study supplementing a synbiotic containing *B. longum* (2×10^{11} CFU) and 6 g of a commercial product for 7 months effectively improved clinical symptoms in patients with active CD (Steed et al., 2010). The systematic review concluded that the efficacy or safety of probiotics for induction of remission in CD is still very uncertain, and further well-designed RCTs in this area are needed to soved this question (Limketkai et al., 2020).

In conclusion, probiotics in CD show divergent results, and the more effective probiotic in attenuating DC symptoms is uncertain. Therefore, for these and other reasons, to date, the European Society for Enteral and Parenteral Nutrition (ESPEN) still does not recommend the use of probiotics in the maintenance of CD remission or active disease in clinical practice (Bischoff et al., 2020).

17.3.3 Ulcerative colitis

Inflammatory bowel disease (IBD) occurs in one of two forms, ulcerative colitis (UC) and Crohn's disease (CD), mainly in the second-to-forth decade of life and with unknown etiology (Bjarnason, Sission, & Hayee, 2019). These conditions are characterized by chronic relapsing inflammatory disorder of the gastrointestinal tract (Bjarnason et al., 2019). Their prevalence is about 37.5 to 248.6 per 100,000 in North America and 4.9 to 505 per 100,000 in Europe. It is important to mention that UC patients with long disease duration have an increased risk of developing colorectal cancer (Ou, Wang, Wang, & Shao, 2021).

The etiology of IBD remains unclear; however, the influence of environmental factors, immune dysfunction, and genetic predisposition is discussed. In this sense, it seems that genetically susceptible individuals have abnormalities of humoral and cell-mediated

immunity and a generalized increased reactivity against commensal intestinal bacteria, which predisposes them to colonic inflammation (Ordas, Eckmann, Talamini, Baumgart, & Sandborn, 2012; Palumbo et al., 2016). Clinical features involve diarrhea, abdominal pain, pus, blood in the stool, weight loss, and fatigue, which affect the quality of life (Ordas et al., 2012). Some patients develop extraintestinal manifestations including primary sclerosing cholangitis, skin lesions, and joint problems (Ordas et al., 2012).

The intestinal microbiome is significantly altered in UC, suggesting that bacterial dysbiosis may have an etiological or pathogenic role in this disorder (Derikx, Dieleman, & Hoentjen, 2016). Patients with UC display a distinct composition and decreased fecal microbiota diversity than healthy individuals (Guo, Liu, & Hao, 2020). Early evidence has reported a less abundance of *Firmicutes*, *Bacteroides*, and *Clostridium* sub-cluster XIVab and more Proteobacteria (e.g., *Escherichia–Shigella*) in the intestinal microbiota of patients with UC than healthy controls (Guo et al., 2020).

The clinical treatment of UC aims to induce and maintain the remission of inflammation and avoid the occurrence of colectomy and colorectal cancer (Ordas et al., 2012). Treatment for UC comprises mainly of mesalazine, corticosteroids, immunosuppressive drugs, and monoclonal antibodies to TNF-α (Ordas et al., 2012). Although the treatment success is dependent on some factors, such as the use of the correct medication, optimization of the dose, and maximization of drug adherence, some undesirable effects in pharmacological therapy can be pointed, such as side effects, higher risk of infectious complications, and the high costs of medical treatment (Ordas et al., 2012).

Non-pharmacological options may help to alleviate UC symptoms, such as homeopathy, stem cell therapy, acupuncture, traditional Chinese medicine, and probiotics (Parian & Limketkai, 2016). Patients with UC exhibit an intestinal microecological disorder, and strategies using probiotics have received more attention (Parian & Limketkai, 2016). The use of probiotics in combination with standard treatment improves the quality of life and life expectancy, reducing significantly clinical symptoms and minimizing side effects.

Probiotics can provide an alternative or adjuvant strategy for traditional treatment by changing the intestinal flora composition and regulating the host immune system (Derikx et al., 2016). Probiotics act into the inflamed intestine, destroying potentially pathogenic bacteria and restoring gut microbiota (Derikx et al., 2016). In addition, probiotics act promoting a downregulation of inflammatory factors, including TNF-α, IL-6, and IL-8 levels (Ou et al., 2021). Therefore, probiotic therapy can attenuate the extent of inflammation, prevent mucosal injury, and alleviate UC.

Administration of probiotic VSL#3 containing *L. acidophilus, L. bulgaricus, L. casei, L. plantarum, Streptococcus thermophilus, B. breve, B. infants and B. longum* for 8 weeks improved epithelial barrier function and increased mucus production in children with UC (Huynh et al., 2009). Another randomized placebo-controlled clinical trial demonstrated that combined daily treatment with *E. coli Nissle* 1917 (5×10^9 CFU) combined with Ciprofloxacin (1000 mg/d) for 7 weeks prevented and antagonized colonization of pathogenic bacteria and increased remission rates of disease (Petersen et al., 2014). Also, this non-pathogenic strain of *E. coli* reduces colonic mucosal damage and decreases epithelial permeability, improving colonic healing (Derikx et al., 2016). A randomized controlled trial demonstrated that bifidobacteria-fermented milk supplementation (100 mL/day, for one year) successfully maintained the remission and prevented UC relapse.

A meta-analysis showed that combined treatment with aminosalicylic acid and probiotic preparation (*S. thermophilus, L. acidophilus, Lactobacillus delbrueckii* ssp. *bulgaricus, L. paracasei, L. plantarum, B. longum, B. infantis*, and *B. breve)* for 8 weeks could prolong remission rate and effectively prevent recurrence of UC (Peng, Zhong, Wang, & Jiang, 2019).

The use of probiotics in UC could be considered a potential aid to the current conventional therapies. Thus, it is suggested that combination therapies using drugs with probiotic preparations improve the efficacy of treatment for UC patients. However, some data limitations obtained by clinical trials investigating the therapeutic potential of probiotics in UC patients have been reported. The studies analyzed co-interventions with drugs and different probiotic strains, which were compared in different dosages. The variety within study designs limits the possibility of determining the adequate treatment method using probiotics. In addition, according to a systematic review, other limitations need to be considered, to cite: lack of blinding methods, inappropriate methods of analysis, and origin of studies (majority comes from Asia). In clinical practice, the ESPEN guideline recommends that only *L. reuteri* or VSL#3 be used in patients with mild to moderate UC for induction of the remission period (Bischoff et al., 2020). This recommendation is based on studies evaluated in a meta-analysis of randomized controlled trials, which highlight that supplementation, especially with these probiotic strains, can increase the efficacy of clinical management and enhance the relapse rate of UC (Fujiya, Ueno, & Kohgo, 2014).

17.3.4 Irritable bowel syndrome

Irritable bowel syndrome (IBS) is one of the most commonly diagnosed gastrointestinal diseases. In the absence of any other causative disease, IBS is defined as the presence of abdominal pain or discomfort with altered bowel habits. There is evidence that an impaired gut microbiota can increase the IBS symptoms severity (Chey, Kurlander, & Eswaran, 2015).

IBS treatment should be individualized, and a significant factor in management remains a solid patient-physician relationship. Nonetheless, the use of probiotics can be considered in some situations, but it remains unclear about which doses and duration of treatments may be more effective in alleviating IBS symptoms (Surdea-Blaga, Cozma-Petrut, & Dumitrascu, 2021).

Knowing that the diet is both a modulator of the gastrointestinal microbiota and an important therapy in IBS, a study investigated the impact of the low fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAP) diet and co-administration of a probiotic. The treatment group received two sachets per day (11.95 log10) of probiotic-containing *S. thermophilus*, *B. breve*, *B. longum*, *B. infantis*, *L. acidophilus*, *L. plantarum*, *L. paracasei*, and *L. delbrueckii* subsp. *bulgaricus* for 4 weeks. Probiotic supplementation led to higher *Lactobacillus* and *Streptococcus* vs. placebo abundance compared with placebo. In addition, probiotic treatment attenuated *Bifidobacterium* reduction caused by a low FODMAP diet (Staudacher et al., 2021).

A randomized clinical trial conducted by Martoni, Srivastava, and Leyer (2020) investigated the clinical efficacy of two probiotic strains on abdominal pain severity and symptomology in IBS. Three hundred thirty adults with IBS were allocated (1:1:1) to receive a placebo, *L. acidophilus* DDS-1 (1×10^{10} CFU/day), or *B. animalis* subsp. *lactis* UABla-12 (1×10^{10} CFU/day) for 7 weeks. After the intervention, pain severity decreased in both probiotic groups compared to placebo and both *L. acidophilus* DDS-1 and *B. lactis* UABla-12 significantly improved abdominal pain, bloating, bowel habits, and quality of life (Martoni et al., 2020).

A randomized clinical trial carried with 71 children aged 4 and 16 years and diagnosed with IBS according to the Rome III criteria demonstrated that synbiotic (5×10^9 CFU of *B. lactis* B94 and 900 mg inulin) or probiotic (5×10^9 CFU *B. lactis* B94) therapy for 4 weeks improved belching-abdominal fullness, bloating after meals, constipation, and mucus in the feces (Basturk, Artan, & Yilmaz, 2016).

To assess the psychiatric symptoms in patients with IBS receiving probiotic therapy, a pilot study was carried with 44 adults (Pinto-Sanchez et al., 2017). Patients were randomly assigned to a probiotic group (n = 22) who received sachets of spray-dried *B. longum* NCC3001 (1.0×10^{10} CFU/1 g powder with maltodextrin) and placebo (n = 22) who received 1 g maltodextrin for 7 weeks. After the intervention, the authors identified that patients receiving the *B. longum* group had fewer depression scores than the placebo group. However, anxiety symptoms were similar between groups, suggesting that the probiotics reduce depression but not anxiety, probably due to reduced limbic activity (Pinto-Sanchez et al., 2017).

In a randomized clinical trial, the authors evaluated the effectiveness of non-viable, heat-inactivated *B. bifidum* MIMBb75 (SYN-HI-001) in treating IBS and its symptoms. Patients received either two placebo capsules or two capsules with a combined total of 1×10^9 non-viable *B. bifidum* HI-MIMBb75 cells to be taken orally once a day for 8 weeks. The findings demonstrated that *B. bifidum* HI-MIMBb75 substantially alleviates IBS and its symptoms in a real-life setting, independently of cell viability (Andresen, Gschossmann, & Layer, 2020).

A clinical study evaluated the efficacy of *L. paracasei* HA-196 and *B. longum* R0175 in reducing the gastrointestinal and psychological symptoms of IBS. Participants were randomized to one of three groups: *L. paracasei* (n = 84), *B. longum* (n = 83) or placebo (n = 81). Both probiotics contained 10×10^9 CFU of freeze-dried *B. longum* or *L. paracasei* and were administered for 8 weeks. This study demonstrated that both *L. paracasei* and *B. longum* supplementation improved the quality of life in emotional well-being and social functioning compared with baseline, mainly in adults with constipation and diarrhea (Lewis et al., 2020).

In another randomized clinical trial performed with the administration of *L. acidophilus* NCFM, the authors investigated the effects of probiotics on symptoms of IBS and quality of life. For 12 weeks, patients received orally in 1 daily capsule that contained 10^9 or 10^{10} CFU of *L. acidophilus* NCFM or placebo. After the intervention, pain scores significantly reduced in the treatment groups compared to the control (Lyra et al., 2016).

Patients with unconstipated IBS were randomly assigned to either the probiotics or placebo groups. One group received the probiotic mixture containing three strains of the L. species, *L. paracasei*, *L. salivarius*, and *L. plantarum*, in a 5:4:1 ratio, reaching a one $\times 10^9$ CFU/mL concentration, and the placebo group received capsules contained the excipient only. In this randomized clinical trial, IBS abdominal symptoms improved in subjects receiving probiotic therapy compared to placebo. In addition, there were no adverse events in either group during the study period. Thus, the results suggest that the new combination of *Lactobacilli* appears to be promising in relieving abdominal symptoms, such as abdominal pain, bloating, and altered stool frequency and consistency (Oh, Jang, Kang, Chang, & Min, 2019).

Overall, the effects of probiotics on reducing symptoms in IBS have been demonstrated in the literature, increasing the possibilities for treating these patients. However, other clinical trials and meta-analyses are needed to elucidate the practical paths to be followed by health professionals. It is expected that these new studies will contribute to funneling scientific evidence and support a more specific practical action.

17.4 Conclusions

Studies with probiotics in the treatment of GIT diseases have been promising. Given the works listed here, it is clear that it is difficult to establish a strain, quantity, and time of use, both for prevention and effective pathology treatment in clinical practice. Furthermore, despite the promising results, pathologies such as inflammatory bowel diseases require greater caution in using probiotics as adjuvant therapy due to their pathophysiological complexity and difficulty in controlling.

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CHAPTER

18

Probiotics in gastrointestinal surgery

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18.1 Introduction

Gastrointestinal tract (GIT) surgeries significantly affect the intestinal microbiota. It has already been shown that, after surgical insult, the microbiota undergoes profound changes in terms of density and activity (Earley et al., 2015). In recent years, the role of intestinal microbiota modulation has been investigated, aiming to reduce the adverse outcomes associated with GIT surgeries. Postoperative infection, for instance, is the most common cause of hospital readmission, contributing to the increase in healthcare expenses (Guyton & Alverdy, 2017).

In addition to the surgical procedure itself and the pre-existing disease, some approaches used in preparing for surgery can also contribute to changes in the intestinal microbiota, producing a higher expression of virulence by microorganisms and intense inflammatory response, such as prolonged fasting; use of parenteral nutrition; administration of vasoactive drugs, gastric acid inhibitors, and opioids; mechanical cleaning of the intestine; and prophylactic administration of antibiotics (Guyton & Alverdy, 2017; Lapthorne et al., 2015). The last two procedures are based on the fact that the stress imposed by the surgery makes the individual more susceptible to infection; therefore, the aim is to eliminate as many pathogens as possible in the skin and intestine (Nelson, Gladman, & Barbateskovic, 2014). Fortunately, these practices are continually revised to minimize the harm caused to patients. Currently, the adoption of preoperative fasting abbreviation protocols is encouraged (Tesauro et al., 2021).

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Although surgical infections have been historically associated with inadequate infection control techniques, it is now known that most hospital infections originate from the intestinal microbiota itself, usually after the suppression of beneficial microbial communities, in the process known as dysbiosis (Guyton & Alverdy, 2017). Bacterial translocation caused by intestinal dysbiosis is the main risk factor for infections in the postoperative period. The intestinal microbiota comprises an important component of resistance against infection, competitively eliminating pathogenic microorganisms and contributing to the development of local immune cells, influencing the systemic immune response (Kamada, Chen, Inohara, & Núñez, 2013).

In this sense, the interest in compounds that aim to maintain the overall structure and composition of the intestinal microbiota has been growing, intending to prevent complications associated with GIT surgeries, especially infectious complications. Probiotics are live microorganisms that, when administered in adequate amounts, confer benefits to the health of the host, improving the balance of the intestinal microbiota (Gibson et al., 2017; Linares, Ross, & Stanton, 2016).

The use of probiotics in the perioperative period has shown beneficial effects, such as maintaining the integrity of the mucosa, reducing colonization by *Enterobacteriaceae*, *Clostridium difficile*, and *Pseudomonas*, and reducing the occurrence of bacteremia (Mizuta et al., 2016). In addition, there are also reports of reduced rates of infectious complications, reduction of inflammatory markers, improvement in postoperative diarrhea, decreased rates of pneumonia, surgical site infections, and anastomotic dehiscence (Kotzampassi et al., 2015; Liu et al., 2013; Mizuta et al., 2016; Theodoropoulos et al., 2016). In this sense, understanding the host-microbiota interaction is important in establishing approaches to mitigate the risk of complications in gastrointestinal surgeries.

18.2 Changes in microbiota induced by surgical stress

The intestinal microbiota performs several functions in the organism, such as nutrient metabolism, maintenance of the intestinal barrier; stimulation and activation of the immune system, ensuring the development of innate immunity and T helper cell function, resistance to colonization by pathogens, and production of antimicrobial peptides. Therefore, it is likely that the absence of a healthy microbial community enables pathogenic bacteria to cause post-surgical complications, including generalized infection, anastomotic dehiscence, and *C. difficile* infection (Abt & Pamer, 2014).

Several factors, including diet, contribute to changes in the intestinal microbiota, with notable changes being observed in short periods. Despite these constant fluctuations, the general structure and activity of the intestinal microbiota of healthy individuals return to a stable baseline. However, after a physiological insult, as in surgery, the microbial structure undergoes profound changes, impacting both its density and functionality (David et al., 2014; Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012).

Changes in the intestinal microbiota composition in the postoperative period were demonstrated, with a reduction in bacterial richness, increased potentially pathogenic bacteria, and decreased lactobacilli and bifidobacteria. On the other hand, individuals that received probiotics had lower rates of infectious complications, faster return of intestinal peristalsis,

and lower occurrence of diarrhea, and abdominal cramps (Ferrie, Webster, Wu, Tan, & Carey, 2021; Lederer et al., 2017).

In a study with rats submitted to colectomy, displacement of luminal bacteria associated with the mucosa was observed, with an increase of 500 and 200 times in the abundance of *Enterococcus* and *Escherichia* and/or *Shigella*, respectively (Shogan et al., 2014). Another study showed that mice undergoing small intestine resection showed changes in the ileal microbial community, which lasted for up to 90 days after surgery. Resection exposes the intestinal lumen to oxygen, changing the partial pressure in this anaerobic environment, which contributes to changes in the composition of the resident microbiota (Sommovilla et al., 2015).

The interruption of blood flow (ischemia) in the intestine is another factor that affects the local microbiota. In an animal model of intestinal ischemia and reperfusion-induced by occlusion of the superior mesenteric artery, it was observed a relative increase in the abundance of *Escherichia coli* and a decrease in *Lactobacillus* in the ileum and colon (Wang et al., 2013a; Wang, Li, Wang, Tang, & Li, 2012). According to the authors, these changes are clinically relevant as the intestinal microbiota contributes to the prevention of tissue damage after ischemia. Supplementation with the probiotic bacteria *Bifidobacterium longum*, *B. bifidum*, and *B. adolescentis* was able to attenuate tissue damage induced by intestinal ischemia-reperfusion lesions in germ-free mice (Perez-Chanona, Mühlbauer, & Jobin, 2014; Wang et al., 2013a).

Intestinal reconstructions, important to reestablish enteric flow in cases of intestinal obstruction, ischemia, or resection of injured areas, may compromise the processes of digestion and absorption of nutrients. In addition, as a result of resection, downstream microbial communities are altered, which may affect digestion, absorption, and immune function (Guyton & Alverdy, 2017).

Preoperative procedures, such as prolonged fasting, bowel cleansing, oral or intravenous administration of antibiotics, parenteral nutrition, administration of vasoactive drugs, gastric acid inhibitors, opioids, and surgical manipulation itself, can disrupt the host-microbiota balance, producing a higher expression of virulence by microorganisms and an intense inflammatory response (Lapthorne et al., 2015).

Preoperative procedures such as mechanical preparation of the intestine, performed to remove fecal material, induce transient changes in the intestinal microbiota, specifically a decrease in the abundance of *Bifidobacterium* and *Lactobacillus,* and an increase in the abundance of *E. coli* and *Staphylococcus*. On the other hand, oral antibiotics reduce the luminal bacterial load, eliminating species associated with the mucosa, while intravenous antibiotics exert additional selective pressure on bacterial composition and function (Ferrer, Santos, Ott, & Moya, 2014; Ubeda & Pamer, 2012). However, data in the literature about the influence of these procedures on the intestinal microbiota are extremely scarce.

The underlying disease and other treatments performed before surgery contribute to changes in the microbiota. For example, changes in the intestinal microbiota composition and metabolite production have been demonstrated in individuals with colorectal cancer that received neoadjuvant chemotherapy (Tang et al., 2017). Other comorbidities, such as diabetes mellitus, rheumatoid arthritis, obesity, and Crohn's disease, may also affect the surgical patients' microbiota and subsequent surgery response (Woting & Blaut, 2016).

It is noteworthy that intestinal bacteria have developed adaptation mechanisms to respond to changes in the microenvironment where they live, detecting local environmental signals. This ability allows them to increase their adaptation and their survival (Leung, Dufour, &

Lévesque, 2015). It is suggested that both commensal and hospital-acquired microbiota detect molecular signals released in the intestine during surgical injury (such as opioids, cytokines, and ischemic end products) and respond with increased pathogenicity (Wu et al., 2005).

When interrupted early by appropriate interventions, such as immediate surgical repair, administration of fluids, medications, antibiotics, and nutrition, this adaptive progression results in successful recovery, with the reestablishment of the commensal microbiota (Ojima et al., 2016). On the other hand, prolonged stress or severe injuries may create an environment in which the reestablishment of the microbiota does not occur properly, leading to the development of higher pathogenicity and adaptation of microorganisms (such as *E. faecalis*, *P. aeruginosa*, and *C. difficile*) in adverse environmental conditions (Chauv et al., 2016).

It is estimated that the recovery of the intestinal microbiota begins six hours after the injury. In the presence of continuous stress, the time for recolonization increases, being associated with a higher frequency of surgical complications (Wang et al., 2013b). Adequate postsurgical recolonization is a relevant aspect for intestinal healing, especially in the anastomotic areas. It has been suggested that the aggregated microbiota stimulates the healing of intestinal anastomosis, which is not observed in germ-free mice (Okada, Bothin, Kanazawa, & Midtvedt, 1999). Also, animals that presented anastomotic tissues with dehiscence were colonized by *Enterococcus faecalis* strains with high collagenase activity. These collagenase-producing bacteria can cleave collagens I and IV, converting tissue matrix metalloproteinase 9 into its active form, contributing to the occurrence of dehiscence (Shogan et al., 2015).

18.3 Therapeutic peptides with a probiotic or synthetic origin

Today, more than ever, the balance of the intestinal microbiome is sought to avoid colonization by increasingly resistant pathogens, preventing infections, and reducing the abusive use of antibiotics. The lactic acid bacteria (LAB) are suitable to act as probiotics due to their ability to modify the environment where they are, producing different metabolites by inhibiting the growth of enteropathogenic bacteria by competitive exclusion, including antibiotic-resistant strains, being widely used as a natural alternative to food preservation and/or also as an adjuvant to the use of antibiotics (Hols, Ledesma-García, Gabant, & Mignolet, 2019).

Peptides are small proteins with less than 50 acidic amino acids. These compounds perform a variety of functions in the human body and are capable of modulating epigenetic mechanisms. Different reports discuss gene regulation and the effects of peptides (Cicenia et al., 2014; Hill et al., 2014). Depending on the peptide, these effects are direct or indirect by binding to receptors and activating intracellular signaling cascades. Peptides from different sources (endogenous, food-derived, environmental, and synthetic) can influence different aspects of epigenetic regulation. For example, endogenous short peptides, resulting from proteolytic cleavage of proteins or after translation of transcripts, can block DNA methylation and, through this, regulate gene expression.

Bacteriocins are peptides or proteins ribosomally synthesized by intestinal bacteria, with an antimicrobial activity that contributes to the stability of homeostasis of the human bacterial community. They are usually associated with the immunity protein encoded in the genome. They are produced by all major eubacterial strains and by some Archaea. Bacteriocins produced by Gram-negative bacteria include some higher molecular-weight proteins

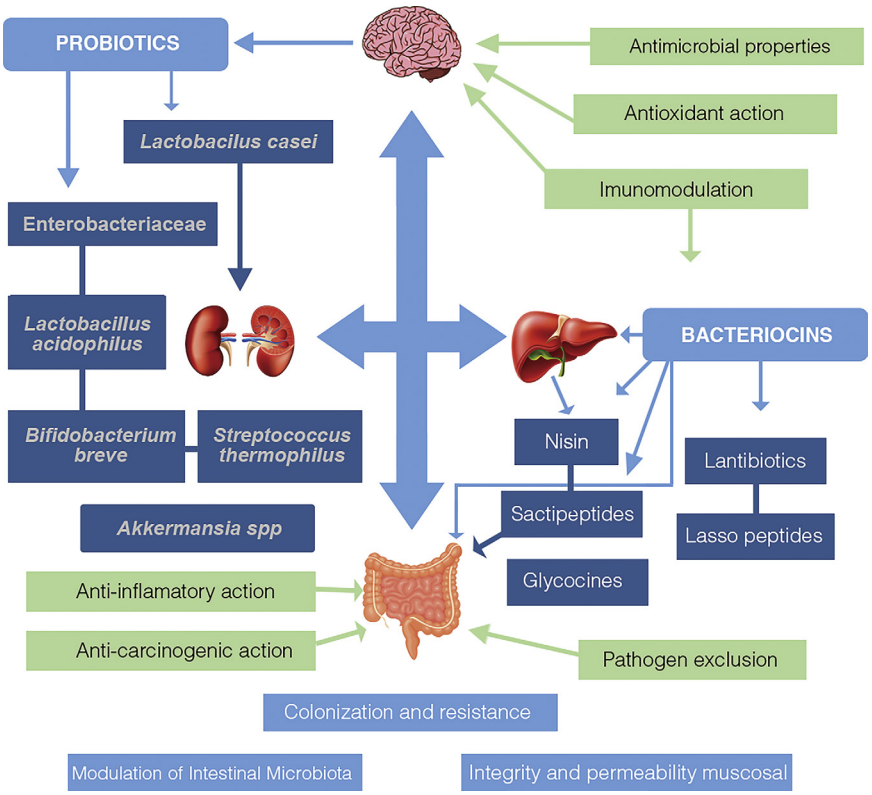


FIG. 18.1 Layout of the performance of probiotics and bacteriocins in the different systems of the organism.

(for instance, colicins from 449 to 629 amino acids), and bacteriocins from Gram-positive bacteria are peptides with less than 70 amino acids. Bacteriocins are also classified into four classes according to the structure of the peptide. It is worth noting that class I contains polycyclic amino acids, lanthionine thioether, or methyllanthionine, which, in turn, are subdivided into different types: glycocines, lantibiotics, sactipeptides, and lasso peptides (Egan et al., 2016).

Nisin, for example, produced by Gram-positive bacteria, such as *Lactococcus lactis* and some *Streptococcus* spp, is the most studied lantibiotic, used as a food preservative, and was listed in the category recognized as safe by the US Food and Drug Administration – 2017 (FDA). Furthermore, nisin and other lantipeptides have been proven to be cytoneural concerning several eukaryotic cell lines, even at doses 100 times higher than the lethal concentration, exerting their antimicrobial activity by binding to lipid II of the bacterial cell wall. Furthermore, the created complex induces the formation of pores and changes in permeability, inhibiting the synthesis of glycan peptides. Thus, it has wide application in clinical treatments, especially in local and external applications (Kassaa et al., 2019).

Fig. 18.1 summarizes the performance of some species of probiotics and some classes of bacteriocins involved in processes related to the microbiome and host and their systemic integration.

18.4 Probiotics in gastrointestinal tract surgeries

After surgical procedures (especially large ones) that result in anatomical and physiological changes, the intestinal microbiota and the GIT itself gradually adapt to reach a new state of equilibrium. However, postoperative complications can occur when this balance is not achieved, such as impaired intestinal motility, malabsorption syndrome, failures in the healing process, and increased risk of infection.

The postoperative intestinal microbiota may not have microbial components that guarantee intestinal health, and the role of microorganisms that colonize the GIT in recovery after surgery is increasingly evident. There is, therefore, a need to produce a deeper understanding of the effects of the microbiota that develops after the surgical procedure to optimize patient outcomes (Guyton & Alverdy, 2017).

The adoption of microbiota elimination techniques to suppress the number and virulence of potential pathogens is extremely common. However, current strategies, based on growing evidence, seek to keep the resident microbiota. In this sense, the effects of probiotic supplementation in the perioperative period have been investigated.

Regarding GIT surgeries, it was shown that probiotics might be effective in maintaining microbial diversity, improving the immune response, reducing the systemic inflammatory response after surgery, and improving quality of life. Indirectly, they can influence the duration of hospitalization, ventilatory support, and infectious complications in general (Kotzampassi et al., 2015; Zhang et al., 2012). However, the results of the studies are still conflicting and may be justified, at least in part, by the wide variety of methods used, such as the period of administration, the number of viable cells used, use of multi- or single-strain probiotics, number of participants in studies, among others. In addition, the surgical technique itself may favor bacterial contamination in the peritoneal cavity.

Among the main benefits associated with the use of probiotics in GIT surgeries, the reduction in the number of infections is one of the most explored. It is proposed that probiotics could improve both gastrointestinal and systemic microbial balance, reducing colonization by potentially pathogenic microorganisms, such as *Pseudomonas aeruginosa* (Siempos, Ntaidou, & Falagas, 2010).

A meta-analysis of randomized clinical trials, published by He et al. (2013), included 361 patients with colorectal cancer undergoing elective surgery and demonstrated that perioperative administration of probiotics reduced the incidence of total operative infections (OR 0.39, 95% CI: 0.22–0.68, $p = 0.0010$) and pneumonia infection (OR 0.32, 95% CI 0.11–0.93, $p = 0.04$). There was also an increase in the abundance of Lactobacillus (MD 2.66, 95% CI: 2.13–3.18; $p < 0.00001$) and a reduction in Enterobacteriaceae (MD -1.52, 95% CI: -1.93 to -1.11, $p < 0.00001$).

The response to the use of probiotics seems to differ depending on which segment of the GIT undergoes surgery. A meta-analysis including 15 randomized clinical trials (1201 patients) demonstrated a 38% reduction in the risk of developing postoperative sepsis. However, when the risk was evaluated according to the type of surgery, a 35% risk reduction was observed for colon surgery (RR = 0.65, 95% CI 0.53–0.80, $p < 0.001$), 73% for hepatopancreatic-biliary surgery (RR = 0.27, 95% CI 0.09-0.80, $p = 0.019$), and 56% for liver transplantation (RR = 0.44, 95% CI 0.23–0.85, $p = 0.015$) (Arumugam, Lau, & Chamberlain, 2016).

There is still no consensus in the literature on which probiotic species would be associated with the greatest benefits. Liu et al. (2017) performed a systematic review with meta-analysis,

in which nine studies were included, totaling 1146 patients (556 received a multi-strain probiotic, and 590 received a single-strain probiotic). The authors observed that the use of multi-strain probiotic significantly reduced total infections (OR = 0.30, 95% CI: 0.15–0.61, p = 0.0009), surgical site infection (OR = 0.48, 95% CI: 0.25–0.89, p = 0.02), and non-surgical site infection (OR = 0.36, 95% CI: 0.23–0.56, p < 0.00001). However, these results were not obtained with the use of single-strain probiotics (except for non-surgical site infection).

Despite the potential benefits of probiotics in gastrointestinal surgeries, the safety of using live microorganisms in these situations is still widely discussed due to a possible bacterial translocation. One of the main causes of mild bacteremia is bacterial translocation induced by changes in the intestinal microenvironment and increased permeability of the intestinal epithelium, known as occult bacterial translocation (Yokoyama et al., 2021).

Some studies using symbiotics (combination of probiotic and prebiotic) demonstrated benefits in controlling bacterial translocation (Kanazawa et al., 2005; Sugawara et al., 2006). For example, patients with esophageal cancer were randomized to receive the symbiotic (*Lactobacillus casei* Shirota, *Bifidobacterium breve,* and galactooligosaccharides) or placebo for 7 days before surgery. A higher detection of microorganisms in mesenteric lymph nodes was observed in the placebo group. Moreover, bacteremia was also higher in the placebo group, as was the neutrophil count on days one, two, and seven after the surgery (Yokoyama et al., 2014).

18.5 Specific surgeries

18.5.1 Bariatric and metabolic surgery

Bariatric surgery is a gastroplasty used to treat severe obesity. After the surgery, deficiencies in some nutrients and other disorders, such as excessive growth of bacteria in the small intestine, weight gain, and abdominal symptoms, are common (Karbaschian et al., 2018; Ramos et al., 2021; Wagner et al., 2021).

Gastric bypass is a common technique that promotes about 65 to 70% of weight loss and reduces associated comorbidities; however, it presents risks and side effects, such as gastrointestinal symptoms resulting from anatomical changes in the GIT. These changes include inadequate secretion of enzymes and lower production of hydrochloric acid in the stomach, and changes in intestinal peristalsis contributing to the imbalance in the microbiota (Boerlage et al., 2019; Dolan et al., 2021).

Small Intestinal Bacterial Overgrowth (SIBO) is common after a gastric bypass and may cause several gastrointestinal symptoms due to an increase in small intestinal bacteria. Symptoms are variable and include bloating, flatulence, abdominal discomfort, nausea, and changes in intestinal habits (Dolan et al., 2021).

Two aspects are involved in weight loss induced by Roux-en-Y gastric bypass (RYGB): the neurohumoral mechanisms, which affect the control of hunger and satiety, and changes in the intestinal microbiota. In obese individuals, the *Firmicutes/Bacteroides* ratio is increased, while in post-RYGB patients, there is a notable reduction in *Firmicutes* phylum bacteria and an increase in *Bacteroidetes* (Chakravartty, Tassinari, Salerno, Giorgakis, & Rubino, 2015). Other changes, such as increased *Akkermansia spp.* and reduction of *Bifidobacterium spp.*, are also observed after RYGB (Jones, Martoni, Ganopolsky, Labbé, & Prakash, 2014).

According to Wagner et al. (2021), the use of probiotic FloraVantage (5 billion *Lactobacillus acidophilus* NCFM and 5 billion *Bifidobacterium lactis* Bi-07) for 90 days after RYGB was able to reduce swelling, abdominal pain, heartburn, and nausea in individuals. In addition, patients supplemented with the same probiotic (FloraVantage) for a shorter period, that is, three consecutive months after RYGB, showed increased levels of vitamins D and B12, reduced triglycerides, less weight gain, and improved glycemic profile (Ramos et al., 2021). Furthermore, it was also reported that patients treated with a probiotic supplement (Puritan's Pride) composed of 2.4 billion live cells of *Lactobacillus* species for 6 months after RYGB showed a reduction in the occurrence of SIBO and less weight gain, which may be related to modulation of the microbiota, in addition to the increase in vitamin B12 levels, which in turn may indicate a lower number of B12 catabolic bacteria (Woodard et al., 2009).

Therefore, probiotics can contribute to reducing weight gain and improving gastrointestinal symptoms after bariatric surgery. Moreover, particular species of *Lactobacillus* and *Bifidobacterium* can produce some vitamins, such as folates and vitamin B12. They may influence the status of vitamin D, which is important because, after bariatric surgery, there may be a higher deficiency of these micronutrients, even with an intake of multivitamin supplements (Karbaschian et al., 2018).

18.5.2 Gastrectomy

Gastrectomy is surgery for the partial or total removal of the stomach that causes the loss of place for food storage. Thus, a smaller amount of food is allowed into the small intestine at one time, leading to symptoms, such as dysphagia, heartburn, and disorders. Furthermore, an imbalance in microbial diversity is observed. Therefore, improving the patient's quality of life after gastrectomy is important (Portanova, 2010; Tegels, Hulsewé, Sosef, & Stoot, 2015).

In this context, a study investigated the effect of the treatment in reducing the severity of physiological disorders and microbial diversity in feces after partial gastrectomy. Within 3 to 5 days after surgery, patients resumed feeding together with the probiotic *Bifidobacterium* Tetravaccine Tablets ($>10^6$ CFU/tablet *B. infantile*, *L. acidophilus*, *E. faecalis*, *B. cereus*, and total bacteria) for seven days (three capsules, thrice daily). The combination of probiotics promoted a significant reduction in leukocyte inflammation indices and an increase in lymphocyte immunity indices and nutritional indices, such as albumin and total protein. Therefore, there was an improvement in the inflammatory state and an increase in the patients' immunity. Moreover, patients in the probiotic group showed a considerable reduction in the *Firmicutes*/*Bacteroidetes* ratio and the abundance of *Streptococcus*, and a concomitant increase in the richness of the beneficial bacteria *Bacteroides* and *Faecalibacterium* in the feces, indicating modulation of the intestinal microbiota through probiotics (Zheng et al., 2019).

18.5.3 Intestinal resection

Surgical site infection (SSI) is caused by factors, such as administration of antibiotics, stress resulting from invasive surgery, reduction in intestinal peristaltic movements, atrophy of the intestinal mucosa due to perioperative fasting, and ischemia in the intestinal tract. In

addition, the imbalance in the intestinal microbiota may be another important risk factor for the development of SSI (Aisu et al., 2015; Zhang et al., 2012). Thus, the administration of probiotics can reduce SSI risk by modulating the microbiota and systemic inflammatory responses (Pitsillides, Pellino, Tekkis, & Kontovounisios, 2021; Zhang et al., 2012).

In this context, Aisu et al. (2015) investigated the effects of the preoperative treatment with probiotics in reducing SSI in patients undergoing elective colorectal cancer (CRC) surgery. Patients in the probiotic-treated group received six tablets a day of BIO-THREE (Toa Pharmaceutical Co., Ltd., Tokyo, Japan) orally, three to 15 days before surgery, and restarted on the same day they resumed drinking water. Each tablet contained 2 mg of *Enterococcus faecalis* T110, 10 mg of *Clostridium butyricum* TO-A, and 10 mg of *Bacillus mesentericus* TO-A. It was observed that 27 of the 156 patients in the study had superficial SSI, being 16 individuals from the untreated group and only five from the probiotic group. Among those with profound SSI, four belonged to the placebo group. Also, the probiotic group showed an increase in *Bifidobacterium*, faster recovery of peristalsis, and increased immune function through ATP activity in $CD4^+$ lymphocytes. Thus, probiotics in the preoperative and consecutive postoperative period contributed to a reduction in the incidence of superficial incisional SSI and increased beneficial bacteria in feces.

Another study observed that the use of probiotics *B. longum*, *L. acidophilus*, and *Enterococcus faecalis* for three days preoperatively (colorectal cancer) significantly increased the *Bifidobacterium* count, while the *Escherichia* count decreased. Also, concentrations of endotoxins, D-lactic acids, interleukin-6 and C-reactive protein, and increased IgG and IgA, were reduced compared to the placebo group. The incidence of postoperative infectious complications was significantly lower in the probiotic group (Zhang et al., 2012).

In the study by Yang et al. (2016), a group of patients undergoing a CRC resection was treated with combined probiotics (2 g of powder) containing *Bifidobacterium longum* ($\geq 1.0 \times 10^7$ CFU/g), *Lactobacillus acidophilus* ($\geq 1.0 \times 10^7$ CFU/g), and *Enterococcus faecalis* ($\geq 1.0 \times 10^7$ CFU/g) (Sine Pharmaceuticals, Shanghai, China) for 12 consecutive days, five days before and seven days after surgery. The use of probiotics promoted short-term clinical benefits, such as the faster recovery of intestinal function, because patients had a shorter time for the first flatus and defecation, a lower incidence of diarrhea, and a slightly lower rate of bacteremia.

Maintenance of intestinal barrier function is an important mechanism to reduce the risk of postoperative infectious complications. In a randomized clinical trial, 150 patients with colorectal carcinoma were randomized (n = 75/group) to receive probiotics (*Lactobacillus plantarum*, *Lactobacillus acidophilus*, *Bifidobacterium longum*) orally, six days preoperatively, and ten days postoperatively or placebo. It was observed that the probiotic group had a lower rate of postoperative infectious complications, in addition to a reduction in serum zonulin (early sepsis biomarker), duration of postoperative pyrexia, and antibiotic therapy (Liu et al., 2013).

Probiotics can prevent postoperative infections in patients with colorectal cancer by protecting the epithelial barrier function and maintaining the balance and diversity of the microbiota because of surgery-induced injuries, improving host defenses, especially in the recovery period. In addition, possible mechanisms of action of probiotics may be associated with early evacuation, restricting bacterial translocation in the intestine, or through modulation of innate immune responses (Chen, Wen, & Zhao, 2020).

18.5.4 Resection of colorectal liver metastases

The liver is a common organ for metastases in individuals with CRC (Zhao et al., 2013). Thus, resection of colorectal liver metastases (CLM) is considered the best choice in the treatment of patients with CRC and liver metastases (Shindoh et al., 2012; Yin et al., 2013). However, the risk of infectious complications in the postoperative period has become increasingly common and may increase the lethality rate of these individuals (Liu et al., 2013). Thus, strategies aimed at reducing these complications are explored, such as the use of preoperative and postoperative probiotics to reduce intestinal permeability, the incidence of complications related to infection, bacterial translocation, and promote balance in the intestinal microbiota.

To verify the influence of probiotics on CLM postoperative period, a group of patients was treated with 2 g/day (2.6×10^{14} CFU) of an encapsulated mixture of three probiotic bacteria (Institute of Life Science of Only, Shanghai Jiao Tong University, Shanghai, China), composed of LP (CGMCC No. 1258, cell count $\geq 10^{11}$ CFU/g), LA -11 (cell count $\geq 7.0 \times 10^{10}$ CFU/g) and BL-88 (cell count $\geq 5.0 \times 10^{10}$ CFU/g) for 16 days, 6 days before and 10 days after surgery. After the intervention, it was reported that the group treated with probiotics reduced the incidence of infectious complications, such as sepsis, urinary tract infection, diarrhea, cumulative duration of antibiotic therapy, postoperative hospital stay, abdominal cramps, abdominal distention, in addition to low levels of plasma endotoxin. Furthermore, the serum zonulin concentration was lower in the probiotic group after 10 days of postoperative treatment, being related to a lower rate of septicemia in this group (Liu et al., 2015). It is reported that when serum zonulin levels are elevated in septic patients, it can serve as a mechanism for increased intestinal permeability in sepsis and systemic inflammatory response syndrome (Fasano, 2012; Klaus et al., 2013).

18.6 Conclusions

Considerable advances have been made in recent years regarding the knowledge of the performance of probiotics used to maintain human health, involving the intestinal microbiota balance or even their role in the treatment of diseases. Studies including systematic reviews and meta-analyses, in addition to experimental investigations, have contributed to providing convincing evidence of their benefits. However, because the consensus statement on the scope and appropriate use of the term probiotic, carried out by a panel of experts in 2013 with the International Scientific Association for Probiotics and Prebiotics (ISAPP), many questions remain unanswered, such as the safe dose for use according to each microbial species or each specific disease situation or even in interventions, such as postoperative ones.

Individuals undergoing surgery in the gastrointestinal tract are susceptible to a high risk of infectious complications in the postoperative period, in addition to significant changes in the intestinal microbiota. Therefore, to optimize these patients' results, there is a need to produce a deeper understanding of the effects of the microbiota that develops after the surgical procedure. Furthermore, the use of probiotics to reduce postoperative complications has shown promising results, such as reducing gastrointestinal symptoms, reducing the risk of infections, and maintaining the intestinal barrier. Furthermore, probiotics can be effective in

modulating the intestinal microbiota and systemic inflammatory responses, improving the immune function of individuals, and increasing resistance to postoperative infections.

The use of probiotic supplements administered in the preoperative period came as an attempt to boost the host's immune function and increase resistance to infections since systemic inflammatory responses. Thus, the microbial imbalance may be positively modulated by treatment with probiotics. However, researchers are still searching for important pieces in this puzzle to establish behaviors to prevent bacterial translocations, better knowledge of the species to be used, decrease inflammation rates, and improve the inflammatory state with increased immunity of the patients.

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CHAPTER

19

Probiotics for obesity and metabolic syndrome prevention and treatment

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19.1 Introduction

The global obesity epidemic is associated with environmental exposures, such as diet and a sedentary lifestyle, as well as epigenetic changes, with susceptibility differing according to sex, age, race, ethnicity, and socioeconomic level (Jensen et al., 2013). According to the World Health Organization, obesity is classified using the body mass index (BMI), so that cutoff points higher than 25, 30 and 40 kg/m^2 represent overweight, obesity, and severe obesity, respectively (Jensen et al., 2013). Additionally, central obesity is defined as waist circumference 102 cm for males and 88 cm for females (National Institutes of Health, 2002; National Institutes of Health, 2000). The prevalence of obesity in the United States reached a plateau in males between 2009 and 2012, starting to rise again in 2013, while in females it has steadily increased since 1999, and it is projected that by 2030, 81% of men and 74.9% of American women will be overweight or obese (Wang et al., 2020).

Obesity increases the risk of several debilitating and deadly diseases, and obesity-related comorbidities vary greatly among individuals, especially dyslipidemia, hypertension, and diabetes (Zupancic et al., 2012). These comorbidities, when associated with central obesity, are characteristics of a complex condition called metabolic syndrome (Dommermuth & Ewing, 2018). Several definitions of metabolic syndrome have been used; however, they generally involve central obesity, hypercholesterolemia, HDL reduction, arterial hypertension, hypertriglyceridemia, and insulin resistance (Rader, 2007). From the analysis of the National Health and Nutrition Examination Survey (NHANES 2011 to 2016), the prevalence of metabolic syndrome in the United States was 32.5% in 2011-2012 and 36.9% in 2015-2016, being higher in individuals over 60 years of age (Hirode & Wong, 2020).

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The gut microbiota has been linked with the development of obesity and metabolic syndrome since 2007. The consumption of a high-fat diet, rich in refined carbohydrates, can increase intestinal permeability, enabling activation of toll-like receptors by bacterial lipopolysaccharides (LPS), a component of the cell wall of gram-negative bacteria, resulting in a chronic inflammatory process and, consequently, which can lead to the development of insulin resistance (Rohr, Narasimhulu, Rudeski-Rohr, & Parthasarathy, 2020). Additionally, the limited effectiveness of current dietary treatments and the difficulty in maintaining the weight loss have been related to persistent changes in the gut microbiota, which in turn contributes to metabolic changes (Thaiss et al., 2016). Therefore, in addition to lifestyle changes, modulating the gut microbiota may be a promising strategy for preventing obesity and metabolic syndrome (Alard et al., 2021).

The administration of probiotics is one of the strategies that can be used to modulate the gut microbiota. Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (WHO, 2001). These microorganisms can modulate (Foligne et al., 2007) the immune system, strengthen the intestinal barrier (Alard et al., 2018), regulate lipid metabolism (Martín et al., 2016), and alter the enteroendocrine pathways (Delzenne, Neyrinck, Bäckhed, & Cani, 2011), helping treat individuals with obesity and metabolic syndrome. In this chapter, we will discuss the gut microbiota's influence on the development of obesity and metabolic syndrome, as well as the potential effects of probiotics in the prevention and treatment of these conditions.

19.2 Gut microbiota and its relationship with obesity and metabolic syndrome

The gut microbiota is an extremely complex ecosystem, comprised of 10^{11} to 10^{12} microorganisms per mL of luminal content (Preveden, Scarpellini, Milić, Luzza, & Abenavoli, 2017). Advanced bacterial DNA sequencing techniques and bioinformatics tools have contributed to identifying a large part of these bacterial communities. Currently, we recognized thousands of bacterial species composing this ecosystem, interacting among themselves and with their human host, and forming a "*de facto* microbial organ" that has an impact on almost every physiological aspect of the human body. Breaks in this symbiotic relationship between microbial and human cells are often called dysbiosis: an unbalanced change in the microbiota composition associated with the development of diseases (Nyangale, Mottram, & Gibson, 2012).

One of the first studies to investigate the relationship between gut microbiota, obesity, and metabolic syndrome identified that obese individuals had a decreased proportion of the phylum Bacteroidetes and a greater proportion of the phylum Firmicutes within their gut microbiomes (Ley et al., 2005). More recent data have indicated that the Firmicutes/Bacteroidetes ratio was lower in overweight and obese individuals than eutrophic individuals (Schwiertz et al., 2010), and it is argued that the link between the gut microbiota and obesity is likely more dependent on the profile of genera and species than just on bacterial phyla (Armougom, Henry, Vialettes, Raccah, & Raoult, 2009).

The identification of mechanisms connecting the gut microbiota with the development of obesity and metabolic syndrome are the result of studies initially carried out with germ-free mice and conventionalized mice (a germ-free mouse that is colonized with the fecal or gut

microbiota from a non-germ-free mouse or human). Colonization of germ-free mice with the microbiota of conventionalized mice resulted in a 60% increase in body fat, higher hepatic triglyceride synthesis, and the development of insulin resistance, independently of food intake and energy expenditure (Bäckhed et al., 2004). Since then, numerous publications have put forward proposed mechanisms for how the gut microbiome may act on the development of these conditions, such as stimulation of lipogenesis, suppression of the expression of fasting-induced adipocyte factor (FIAF) (Mandard et al., 2006), reduction of AMP-activated protein kinase (AMPK) activity (Samuel et al., 2008), increased energy extraction from food (Turnbaugh et al., 2006), appetite modulation (Tolhurst et al., 2012), increased intestinal permeability, metabolic endotoxemia and epigenetic modifications (Cani et al., 2007; Bhat & Kapila, 2017).

19.3 Metabolic endotoxemia and insulin resistance

Obesity and metabolic syndrome are associated with the westernization of dietary patterns (Rodriguez-Monforte, Sanchez, Barrio, Costa, & Flores-Mateo, 2017). The Western dietary pattern is characterized by excessive consumption of refined carbohydrates and saturated fatty acids (Rodriguez-Monforte et al., 2017). A high-fat diet has been shown to increase the adhesion of gram-negative bacteria to the intestinal mucosa, favoring LPS translocation into circulation (Moreira, Texeira, Ferreira, Peluzio, & Alfenas, 2012). It has been previously identified that this type of diet alters the gut microbiota composition, with associations in the performance of intestinal barrier components, goblet cells, Paneth cells, and tight junctions (junctions between enterocytes), leading to increased intestinal permeability (Levy, Kolodziejczyk, Thaiss, & Elinav, 2017). On the other hand, maintaining a healthy gut microbiota improves tight junction function (Willemsen, 2003), and enables an adequate production of mucus by goblet cells (Paone & Cani, 2020), and antimicrobial substances by Paneth cells (Schoenborn et al., 2019). The proper functioning of these components controls the translocation of bacterially derived molecules, mainly through specialized M cells located in structures called Peyer's patches, which are part of the gut-associated lymphoid tissue (GALT) (Schoenborn et al., 2019).

An imbalance in the gut microbiota contributes to an alteration of the intestinal barrier function, increasing overall intestinal permeability (Thaiss et al., 2016), which results in the translocation of LPS and its migration to mesenteric lymph nodes (Levy et al., 2017; Hooks & O'Malley, 2017; Tremaroli & Bäckhed, 2012). In mice, a high-fat diet has been associated with increased intestinal permeability, which may be related to gut microbiota alterations. Some intestinal bacteria can induce the expression of tight junction proteins, contributing to a decrease in permeability, on the other hand, other bacteria can secrete proteases that degrade tight junction proteins, and yet other bacteria can disrupt tight junctions by instigating enterocytes to down-regulate or internalize tight junction proteins. Additionally, the simultaneous use of a high-fat diet and antibiotics decreased gut permeability, suggesting that this is not a direct effect of lipid intake but rather a result of an alteration in the gut microbiota (Cani et al., 2008).

LPS is also known to be absorbed through chylomicrons, being transported to the liver and adipose tissue. The increase in plasma LPS concentration is called metabolic

endotoxemia and is related to insulin resistance (Cani et al., 2008). LPS binds to toll-like receptors 4 (TLR-4), a receptor expressed on immune cells belonging to the myeloid lineage (monocytes, macrophages, and dendritic cells), endothelial cells, and adipocytes. The binding between LPS and TLR-4 depends on the presence of LPS binding protein (LBP), cluster of differentiation 14 (CD14), and myeloid differentiation protein-2 (MD-2) (Miyake et al., 2002), involving the acyl chains and phosphate groups of LPS lipid A (Park et al., 2009). The hexa-acylated and diphosphorylated LPS (present for example in *Escherichia coli*) is a potent activator of TLR-4, while under-acylated and dephosphorylated LPS (present in commensal bacteria such as *Bacteroides thetaiotaomicron*) have low TLR-4 activation capacity (Steimle, Autenrieth, & Frick, 2016).

Activated TLR-4 will initiate two signaling pathways dependent on myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-beta (TRIF), which will result in translocation of nuclear factor kappa B (NF-kB), activation of transcription factors activator protein-1 (AP-1) and cyclic AMP response element-binding protein (CREB) (Lu, Yeh, & Ohashi, 2008). These pathways induce the expression of genes that encode pro-inflammatory mediators such as tumor necrosis factor α (TNF-α) and IL-6 (Kawai & Akira, 2011). Activation of TLR-4 by LPS also results in activation of the nucleotide-binding domain and leucine-rich repeat protein-3 (NRLP3) inflammasome, which promotes autoproteolysis and activation of caspase-1. Caspase-1 cleaves pro-IL-1 β and pro-IL-18 into their secreted forms, which have a pro-inflammatory action (De Souza et al., 2005; Swanson, Deng, & Ting, 2019).

The activation of intracellular proinflammatory pathways increases the expression of signaling proteins such as JNK, PTP1B, and IKK (De Souza et al., 2005; Zabolotny et al., 2008; Zhang et al., 2008). These signaling cascades can induce insulin resistance by altering the activation and expression of the insulin receptor, and by increasing the phosphorylation of IRS-1 in serine residues, which impairs insulin signal transduction (Hirosumi et al., 2002). In animal models, short-term low-grade chronic inflammation induced by endotoxemia results in hypothalamic insulin resistance without changes in body weight (Rorato et al., 2017). Additionally, animal model experiments with longer endotoxemia exposure can result in the development of obesity and diabetes. Insulin, when properly functioning, inhibits food intake and weight gain. These effects are decreased when hypothalamic insulin resistance occurs, generating a vicious cycle between obesity and insulin resistance (Woods, Lotter, McKay, & Porte, 1979). Therefore, the reduction of low-grade chronic inflammatory processes by decreasing the metabolic endotoxemia could be a strategy to restore insulin sensibility and, consequently, help in the treatment of metabolic syndrome (Cani et al., 2007).

An experimental study carried out with germ-free mice observed that LPS infusion increased body fat and that the ingestion of a high-fat diet caused metabolic endotoxemia associated with dysbiosis (Cani et al., 2007). Mice fed a diet containing 37% of the calories from lard showed an increased proportion of Firmicutes and a decreased proportion of Bacteroidetes, when compared to mice fed a low-fat diet. On the other hand, mice fed a diet containing 37% fat from milk showed an opposite effect in the proportion of Firmicutes and Bacteroidetes phyla, and an increase in the proportion of *Bilophila wadsworthia*. The increase in the proportion of this species was attributed to a higher concentration of taurocholic acid in bile, resulting in the availability of sulfur components for this bacterium, enabling its proliferation (Devkota et al., 2012). This imbalance was also associated with a higher

concentration of LPS in the bloodstream (Natividad et al., 2018) and intestinal inflammation (Devkota et al., 2012).

Several studies have evaluated the effect of probiotic administration on metabolic endotoxemia, to test the hypothesis that the intestinal microbiota can interfere with LPS translocation. In mice fed a high-fat diet, dosages of 10^7 to 10^{10} CFU/day of *Lactobacillus rhamnosus*, *Lactobacillus sakei*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Bifidobacterium longum*, *Bifidobacterium infantis* and *Bacillus cereus*, for 14 weeks, were able to decrease the concentration of LPS or LBP with an improvement in glycemic control, obesity, and dyslipidemia (Chan et al., 2016). Additionally, oral administration of *Bacteroides vulgatus*, *B. dorei* (Yoshida et al., 2018) and *Akkermansia muciniphila* (Chelakkot et al., 2018) have been shown to up-regulate the expression of tight junction genes, decreasing intestinal permeability, while *Faecalibacterium prausnitzii* has shown the same effect in vitro models (Carlsson et al., 2013).

The administration of *Roseburia intestinalis* (Shen et al., 2018), *Bacteroides fragilis* (Chang et al., 2017), *Akkermansia muciniphila* (Greer et al., 2016), *Lactobacillus plantarum* (Li et al., 2016), and *L. casei* (Chen et al., 2014) increased IL-10 concentration which may contribute to the prevention of insulin resistance (Dagdeviren et al., 2017). *Roseburia intestinalis* (Zhu et al., 2018) and *Bacteroides thetaiotaomicron* induced the expression of the regulatory T cell, which may also contribute to the reduction of the inflammatory process (Hoffmann et al., 2016). Furthermore, *L. plantarum*, *L. paracasei* and *L. casei* can lower the expression of IL-1β, monocyte chemotactic protein-1 (MCP-1), and C-reactive protein (Liu et al., 2018). *L. paracasei* and *B. fragilis* decreased IL-6 expression (Sun et al., 2017), while *Lactobacillus* (Li et al., 2016), *Bacteroides* (Chang, Ching, Chiu, Liu, & Hung, 2017), and *Akkermansia* decreased TNF-α; *L. paracasei* (Sun et al., 2017), *F. prausnitzii* (Breyner et al., 2017), *Roseburia* and *Faecalibacterium* inhibited NF-kB activation (Kinoshita, Suzuki, & Saito, 2002); *Lactobacillus casei* and *Roseburia intestinalis* reduced IFN-γ (Zhu et al., 2018). Some bacteria also appear to directly improve insulin sensitivity and glucose uptake. *Bifidobacterium lactis* HY8101 and *Lactobacillus gasseri* BNR17 (Kang et al., 2013) increased the expression and translocation of the glucose transporter GLUT-4 in an animal model, which improves glucose uptake by adipocytes. *Lactobacillus casei* CCFM419 also increased the action of intermediate substrates in the insulin signaling cascade, improving its functioning (Li et al., 2016). We also highlight *Akkermansia muciniphila*, which inhibits the action of the alpha-glucosidase enzyme, preventing the breakdown of complex carbohydrates and reducing postprandial hyperglycemia (Zhang et al., 2018).

Although the use of probiotics in animal and in vitro models has shown great promise, the results from human clinical studies have been quite heterogeneous. For instance, a clinical trial identified that the supplementation of 1×10^{10} CFU/day of *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19 for 12 weeks, decreased LPS concentration (Szulińska, Łoniewski, Hemert, Sobieska, & Bogdański, 2018). However, supplementation of 2×10^{10} CFU/day of *Lactobacillus casei* Shirota for three months increased LBP concentration in metabolic syndrome patients, which would likely indicate an increase in LPS.

In healthy subjects, the administration of 4×10^9 CFU of *Lactobacillus salivarius* UBL S22 for 6 weeks decreased insulin resistance. In diabetes patients, both the administration of a

mix of *Lactobacillus acidophilus*, *Lactobacillus casei*, *Lactobacillus lactis*, *Bifidobacterium bifidum*, *Bifidobacterium longum* and *Bifidobacterium infantis*, in a dosage of 10^{10} CFU per strain (Firouzi, Majid, Ismail, Kamaruddin, & Barakatun-Nisak, 2017), or the administration of a mix of probiotics containing 2×10^9 CFU of *Lactobacillus acidophilus*, *Lactobacillus casei*, and *Bifidobacterium bifidum*for 12 weeks improved insulin sensitivity. An improvement in insulin sensitivity was also obtained from the administration of 10^{10} CFU of *A. muciniphila* for 12 weeks in individuals with insulin resistance (Soleimani et al., 2017). In contrast, supplementation of 10^9 CFU of *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* BB-12 for 6 weeks (Tonucci, Santos, Oliveira, Ribeiro, & Martino, 2017) and 4×10^9 CFU of *Lactobacillus reuteri* ADR-1 for 24 weeks did not affect insulin resistance (Hsieh et al., 2018). A systematic review highlighted the paucity and heterogeneity of human studies that analyzed the effect of probiotic supplementation on insulin resistance, as sample characteristics, types of probiotics, dosages, duration of interventions, and sensitivity assessment methods/insulin resistance differed greatly between studies, which makes it difficult to define a standardized clinical approach (Salles, Cioffi, & Ferreira, 2020). Although the effects of using traditional probiotics on plasma LPS concentration are conflicting, recent work using next-generation probiotics (also called live biotherapeutic products), using bacterial strains isolated from the intestinal system with specific goals in mind, has shown promising results in insulin resistance and glucose metabolism control (Madempudi, Ahire, Neelamraju, Tripathi, & Nanal, 2019). Table 19.1 shows the positive results observed from the administration of probiotics on metabolic endotoxemia, insulin resistance, and glycemic control in humans.

19.3.1 Food intake and extraction of energy from food

One of the paramount factors related to the development of obesity is the maintenance of a positive energy balance, a condition resulting from the excessive consumption of calories mainly associated with a sedentary lifestyle. In mice, the gut microbiota can contribute to the increased extraction of calories from the diet, characterizing an obesogenic microbiota profile (Bäckhed et al., 2004). A westernized diet can contribute to a gut microbiota profile that favors the extraction of calories from food (David et al., 2014). High-refined-carbohydrate and high-fat diets may increase the uptake of calories by increasing the production of α-amylases and amylomaltases (Jumpertz et al., 2011).

Gut microbiota bacteria are capable of fermenting non-digestible dietary components, forming short-chain fatty acids (SCFA): acetate, butyrate, and propionate. These SCFA can contribute up to 10% of daily energy requirements and 75% of energy for intestinal epithelium metabolism. Obese individuals have a greater abundance of bacteria capable of fermenting carbohydrates, increasing SCFA production, which provides an extra energetic substrate for the host (Bäckhed et al., 2004).

In addition to the extraction of calories, the gut microbiota can also communicate with the central nervous system, with the potential to change the feeling of satiety and, consequently, food intake. The enteric nervous system associated with the intestine is formed by motor neurons, interneurons, and sensory neurons, which transfer information from the gut to the central nervous system through the vagus nerve (Furness, Callaghan, Rivera, & Cho, 2014). Vagal afferent neurons have receptors for several peptides

TABLE 19.1 Beneficial effects of probiotics administration on endotoxemia, insulin resistance, and glycemic control in obese, diabetics and individuals with metabolic syndrome.

References	Sample	Intervention	Time	Results	Gut microbiota
Szulińska et al. (2018)	81 obese Caucasian postmenopausal women	1×10^{10} CFU of *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19, and *Lactococcus lactis* W58	12 weeks	↓ LPS↓ glucose↓ insulin↓ HOMA-IR	Not evaluated
Soleimani et al. (2017)	60 type 2 diabetes patients on hemodialysis	2×10^{9} CFU/g *Lactobacillus. acidophilus, Lactobacillus casei*, and *Bifidobacterium bifidum*	12 weeks	↓ glucose↓ insulin↓ HOMA-IR↓ HbA1c↑ insulin sensitivity check index	Not evaluated
Sudha, Ahire, Jayanthi, Tripathi, & Nanal (2019)	79 type 2 diabetes mellitus patients	5 billion CFU of *Lactobacillus salivarius* UBLS22, *Lactobacillus casei* UBLC42, *Lactobacillus plantarum* UBLP40, *Lactobacillus acidophilus* UBLA34, B. *breve* UBBr01, *Bacillus coagulans* Unique IS2 each and 100 mg of fructo-oligosaccharides	12 weeks	↓ HbA1c	Not evaluated
Perraudeau et al. (2020)	76 type 2 diabetes mellitus patients	1.2×10^{9} *Akkermansia Muniniphila* WB-STR-0001, 2×10^{9} *Bifidobacterium infantis* 1.9×10^{8} *Anaerobutyricum hallii* WB-STR-0001, 1.6×10^{10} *Clostridium. Beijerincki* WB-STR-0005, 3.3×10^{9} *Clostridium butyricum* WB-STR-0006 and inulin	12 weeks	↓ glucose↓ HbA1c	No changes
Sabico et al. (2019)	150 type 2 diabetes mellitus patients	2g freeze-dried powder of the probiotic mixture (2.5×10^{9} CFU/g) *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19 and *Lactococcus lactis* W58	6 months	↓ LPS↑ insulin sensitivity	Not evaluated
Palacios et al. (2020)	60 subjects with prediabetes and early type 2 diabetes mellitus	6×10^{9} CFU *Lactobacillus plantarum* Lp-115, 3×10^{9} CFU *Lactobacillus bulgaricus* Lb-64, 18×10^{9} CFU *Lactobacillus gasseri* Lg-36, 7.5×10^{9} CFU *Bifidobacterium breve* Bb-03, 8×10^{9} CFU *Bifidobacterium animalis* SBSP lactis Bi-07, 7×10^{9} CFU *Bifidobacterium bifidum* Bb-06, 450×10^{6} CFU *Streptococcus thermophilus* St-21 and 45×10^{6} CFU *Saccharomyces boulardii* DBVPG 6763	12 weeks	↓ glucose↓ HOMA-IR in participants takin metformin	No differences in the bacterial beta diversity at the species level

(*Continued*)

TABLE 19.1 Cont'd

References	Sample	Intervention	Time	Results	Gut microbiota
Hsieh et al. (2018)	74 type 2 diabetes mellitus patients	4×10^9 of CFU *Lactobacillus reuteri* ADR-1	6 months	↓ HbA1c	↑ *Bifidobacterium spp.*
Khalili et al. (2019)	40 type 2 diabetes mellitus patients	10^8 CFU of *Lactobacillus casei*	8 weeks	↓ glucose↓ insulin↓ HOMA-IR	Not evaluated
Sabico et al. (2017)	78 type 2 diabetes mellitus patients	2g freeze-dried powder of the probiotic mixture (2.5×10^9 CFU/g) *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19 and *Lactococcus lactis* W58	12 weeks	↓ HOMA-IR	Not evaluated
Asemi, Zare, Shakeri, Sabihi, & Esmaillzadeh (2013)	54 type 2 diabetes mellitus patients	2×10^9 CFU *Lactobacillus acidophilus*, 7×10^9 CFU *Lactobacillus casei*, 1.5×10^9 CFU *Lactobacillus rhamnosus*, 2×10^8 CFU *Lactobacillus bulgaricus*, 2×10^{10} CFU *Bifidobacterium breve*, 7×10^9 CFU *Bifidobacterium longum*, 1.5×10^9 CFU *Streptococcus thermophilus*, and 100 mg fructo-oligosaccharide	8 weeks	Prevented a rise in fasting glucose	Not evaluated
Asemi, Khorrami-Rad, Alizadeh, Shakeri, & Esmaillzadeh (2014)	62 type 2 diabetes mellitus patients	1×10^7 CFU *Lactobacillus sporogenes* and 0.04 g inulin	6 weeks	↓ insulin	Not evaluated
Ejtahed et al. (2012)	64 type 2 diabetes mellitus patients	300 g/d of probiotic yogurt containing 1.79×10^6 CFU/g of B. *lactis* Bb12 and 1.85×10^6 CFU/g of *L. acidophilus* La5	6 weeks	↓ glucose↓ HbA1c	Not evaluated
Tonucci et al. (2017)	45 type 2 diabetes mellitus patients	120 g/day of probiotic fermented goat milk containing 10^9 CFU of *Lactobacillus acidophilus* La-5 and 10^9 CFU *Bifidobacterium animalis* subsp. *lactis* BB-12	6 weeks	↓ HbA1c	Not evaluated
Barreto, Colado, A., & Morimoto (2014)	24 patients with MetS	1×10^9 de CFU *Lactobacillus plantarum* Lp 115	12 weeks	↓glucose	Not evaluated

↓, decreased; ↑, increased; CFU, colony-forming unit; HbA1c, hemoglobin glycated; HOMA-IR, homeostatic model assessment for insulin resistance; LPS, lipopolysaccharide; MetS, metabolic syndrome.

produced in the intestine, such as cholecystokinin (CCK), ghrelin, peptide YY (PYY), glucagon-like peptide-1 (GLP-1), and serotonin (5-HT) (Lund et al., 2018), and carry information to the central nervous system, resulting in responses related to food consumption (Grabauskas & Owyang, 2017). GLP-1, PYY, and CCK are anorectic hormones released after meals to induce satiety, reducing food intake. Ghrelin, on the other hand, is an orexigenic hormone, released during the fasting period to induce hunger and stimulate food consumption (Sun, Martin, Young, & Keating, 2018). These hormones stimulate vagal afferent nerve fibers, activating receptors that directly stimulate appetite-control nuclei in the brainstem (Sun et al., 2018). Additionally, gut hormones can act through an endocrine pathway, reaching the arcuate nucleus of the hypothalamus (Cone et al., 2005), the nucleus of the solitary tract, and the postrema area in the brainstem (Murphy et al., 2004). In the arcuate nucleus of the hypothalamus, ghrelin activates agouti/neuropeptide Y (AgRP/NPY) gene-related peptide neurons, which will stimulate food intake, while GLP-1, PYY, and CCK inhibit AgPR/NPY neurons (Beutler et al., 2017).

The gut microbiota has been recognized as a key player in the relationship existing between the brain and intestine, forming the so-called microbiota-gut-brain axis (Obrenovich et al., 2017). Despite not coming into direct contact with afferent vagal fibers (Bonaz, Bazin, & Pellissier, 2018), the gut microbiota can regulate the production of intestinal peptides via enteroendocrine cells. Enteroendocrine cells have several receptors that recognize metabolites produced by the gut microbiota. Among these receptors, we have the G protein-coupled receptors (GPCRs) that recognize SCFA, which regulate the release of gut-derived satiety hormones, such as GLP-1 and PYY (Ulven, 2012).

In addition to SCFA, the intestine can also communicate with the brain through secondary bile acids. Secondary bile acids are gut microbiota metabolites generated by modifying the primary bile acids produced in the liver and secreted to the intestinal lumen during digestion (Chiang et al., 2017). These secondary bile acids are recognized by receptors present in enteroendocrine cells, such as the nuclear Farnesoid X receptor (FXR) (Chiang et al., 2017) and the cell membrane Takeda G protein-coupled receptor 5 (TGR5) (Katsuma, Hirasawa, & Tsujimoto, 2005), which can also stimulate the secretion of GLP-1 by these cells (Thomas et al., 2009). Both SCFA (Reigstad et al., 2015) and secondary bile acids also link the gut microbiota with the production of intestinal serotonin (Yano et al., 2015). Intestinal serotonin is secreted by enterochromaffin cells, and it acts by regulating gastrointestinal motility and fluid secretion (Bellono et al., 2017). Germ-free mice, and mice depleted of gut bacteria by antibiotic treatment, have been shown to have lower serotonin production than that observed in normally colonized mice (Ge et al., 2017).

In humans, circulating SCFA concentrations were positively associated with GLP-1 concentration (Müller et al., 2019) and these circulating microbiota-derived SCFA could stimulate the production of GLP-1 on the basolateral visceral side of enteroendocrine L cells, as has already been observed in mice. Additionally, the administration of 10^{10} viable *L. reuteri* SD5865 cells for 4 weeks increased GLP-1 concentration in glucose-tolerant individuals (Simon et al., 2015).

Table 19.2 shows the positive results observed from the administration of probiotics on food intake and concentrations of orexigenic and anorectic hormones in individuals with obesity and metabolic syndrome.

TABLE 19.2 Beneficial effects of probiotics administration on food intake, anorectic hormones, body fat and lipid profile in obese, diabetics and individuals with metabolic syndrome.

References	Sample	Intervention	Time	Results	Gut microbiota
Szulińska et al. (2018)	81 obese Caucasian postmenopausal women	1×10^{10} CFU of *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19, e *Lactococcus lactis* W58	12 weeks	↓ waist circumference↓ fat mass↓ subcutaneous fat↓ total cholesterol↓ triglycerides↓ LDL	Not evaluated
Sanchez et al. (2017)	105 obese individuals	3.24×10^{8} CFU of *Lactobacillus rhamnosus* CGMCC1.3724	24 weeks	↑ weight loss in woman	Not evaluated
Sanchez et al. (2014)	125 healthy overweight men and women	1.6×10^{8} CFU of *Lactobacillus rhamnosus* CGMCC1.3724	24 weeks	↑ weight loss in woman↓ fat mass↓ leptin	↓ Lachnospiraceae family
Osterberg et al. (2015)	Twenty non-obese males	Two sachets of VSL#3 (450 billion bacteria per sachet -*Streptococcus thermophilus* DSM24731, *Lactobacillus acidophilus* DSM24735, *Lactobacillus delbrueckii ssp. bulgaricus* DSM24734, *Lactobacillus paracasei* DSM24733, *Lactobacillus plantarum* DSM24730, *Bifidobacterium longum* DSM24736, *Bifidobacterium infantis* DSM24737, and *Bifidobacterium breve* DSM24732) + high-fat hypercaloric diet	4 weeks	Attenuated the increase in body mass and fat mass	Not evaluated
Pedret et al. (2019)	135 abdominally obese individuals	1 capsule containing 10^{10} CFU of *Bifidobacterium animalis* subsp. *lactis* CECT 8145 or those of its heat-killed form (h-k Ba8145)	3 months	CECT 8145: ↓ waist circumference, waist circumference/height ratio, and Conicity indexh-k Ba8145: ↓ BMI, visceral fat area	CECT 8145: ↑ *Akkermansia spp.*
Lim, Moon, Shin, Jeong, & Kim (2020)	114 adults with a BMI ≥ 25 kg/m^2	5×10^{9} CFU of *Lactobacillus sakei* (CJLS03)	12 weeks	↓ fat mass and waist circumference	Not evaluated
Agerholm-Larsen et al. (2020); Agerholm-Larsen et al. (2000)	70 healthy weight stable individuals	Yoghurt with 4.8×10^{11} CFU of *Enterococcus faecium*, and *Streptococcus termophilus*	8 weeks	↓ LDL	Not evaluated
Chung et al. (2016)	37 overweight and obese adults	1×10^{9} of CFU *Lactobacillus* JBD301	12 weeks	↓ body weight and BMI	Not evaluated
Higashikawa et al. (2016)	41 Healthy volunteers	1×10^{11} CFU of *Pediococcus pentosaceus* LP28	12 weeks	↓ BMI, body fat percentage, fat mass, and waist circumference	Not evaluated
Kadooka et al. (2010)	87 healthy adults	1×10^{11} CFU of *Lactobacillus gasseri* SBT2055 (LG2055)	12 weeks	↓ abdominal visceral and subcutaneous fat areas, body weight, waist circumference	Not evaluated

References	Sample	Intervention	Time	Results	Gut microbiota
Kadooka et al. (2013)	139 healthy adults with large visceral fat areas	Fermented milk with 1.6×10^{10} CFU of *Lactobacillus gasseri* SBT2055 (LG2055)	12 weeks	↓ abdominal visceral fat areas, BMI, waist and hip circumferences, body fat mass	Not evaluated
Madjd et al. (2016)	89 healthy, overweight, or obese premenopausal women	Yoghurt with 4×10^9 CFU of*Lactobacillus acidophilus* LA5, and *Bifidobacterium lactis* BB12	12 weeks	↓ total cholesterol, low-density lipoprotein cholesterol	Not evaluated
Minami et al. (2015)	44 adult volunteers	5×10^{10} CFU *Bifidobacterium breve* B-3	12 weeks	↓ fat mass	Not evaluated
Simon et al. (2015)	21 generally healthy subjects	2×10^{10} CFU of *Lactobacillus reuteri* SD5865	4 weeks	↑ GLP-1	No changes
Zarrati et al. (2014)	50 overweight and obese subjects	Yoghurt with 6×10^9 CFU of *Lactobacillus acidophilus* LA5, *Bifidobacterium lactis* BB12, and *Lactobacillus casei* DN001	8 weeks	↓ fat percentage and BMI	Not evaluated
Gomes et al. (2017)	43 overweight and obese subjects	2×10^{10} CFU of *Lactobacillus acidophilus* LA-14, *Lactobacillus casei* LC-11, *Lactococcus lactis* LL-23, *Bifidobacterium bifidum* BB-06, and *Bifidobacterium lactis* BL-4	8 weeks	↓ waist circumference waist-height ratio, conicity index	Not evaluated
Sanchez et al. (2017)	105 obese individuals	3.24×10^8 CFU *Lactobacillus rhamnosus* CGMCC1.3724	24 weeks	↓ hunger sensations in women	Not evaluated
Tulk, Blonski, Murch, Duncan, & Wright (2013)	65 healthy adults	200 g/day of yogurt with *Bifidobacterium lactis* Bb12, *Lactobacillus acidophilus* La5, *Lactobacillus casei* CRL431, and 4 g inulin	15 days	↓ energy, fat, and protein intakes	Not evaluated
Jung et al. (2015)	120 overweight adults	5×10^9 CFU of *Lactobacillus curvatus* HY7601, and *Lactobacillus plantarum* KY1032	12 weeks	↓ body weight, waist circumference, and fat mass	Not evaluated
Sharafedtinov et al. (2013)	40 obese patients with MetS	7.5×10^{12} CFU of *Lactobacillus plantarum* TENSIA	3 weeks	↓ BMI, Cholesterol, LDL, and triglycerides	Not changes
Takahashi et al. (2016)	137 overweight patients	8×10^{10} CFU of *Bifidobacterium animalis ssp. lactis* GCL2505	12 weeks	↓ visceral fat area	↑Bifidobacterium
(Naito et al. (2018)	98 obese patients	1×10^{11} CFU of *Lactobacillus casei Shirota*	8 weeks	↓ LDL	Not evaluated
Stenman et al. (2016)	225 obese patients	1×10^{10} CFU of *Bifidobacterium animalis ssp. lactis* 420	26 weeks	↓ waist circumference	Not evaluated
Tripolt et al. (2013)	60 patients with MetS	2×10^{10} CFU of *Lactobacillus casei Shirota*	12 weeks	↓ BMI	Not evaluated
Bernini et al. (2016)	51 patients with MetS	2.7×10^{10} CFU of *Bifidobacterium lactis* HN019	6 weeks	↓ BMI, cholesterol, LDL, triglycerides	Not evaluated
Ahn et al. (2015)	120 overweight patients	1×10^{10} CFU of *Lactobacillus curvatus* HY7601, and *Lactobacillus plantarum* KY1032	12 weeks	↓ BMI, body weight, fat mass, and LDL	Not evaluated
Barreto et al. (2014)	24 Patients with MetS	1×10^9 CFU of *Lactobacillus plantarum* Lp 115	12 weeks	↓ cholesterol	Not evaluated

↓, decreased; ↑, increased; BMI, body mass index; CFU, colony-forming unit; HbA1c, hemoglobin glycated; HOMA-IR, homeostatic model assessment for insulin resistance; LPS, lipopolysaccharide; MetS, metabolic syndrome.

19.4 Adipose tissue and lipid profile

Obesity is characterized by the accumulation of white adipose tissue, which undergoes an abnormal expansion process through hyperplasia and hypertrophy (Rosen & Spiegelman, 2006). During the hypertrophy process, the adipose tissue presents an increase in the volume of adipocytes, accompanied by an infiltration of macrophages and necrosis by hypoxia, dysfunctions related to the development of insulin resistance, and metabolic syndrome (Sun, Kusminski, & Scherer, 2011).

The colonization of germ-free mice with the microbiota of conventional mice increased their body fat content and the production of SCFA (Bäckhed et al., 2004). This observation aroused interest in unraveling the possible relationship between intestinal microbiota and body fat accumulation. Acetate is a precursor for the synthesis of cholesterol and fatty acids in the liver, propionate is a substrate for gluconeogenesis, and butyrate is considered anti-obesogenic, as it improves insulin sensitivity and increases the expression of the leptin gene (Chakraborti, 2015). Additionally, butyrate can promote fatty acid oxidation through the activation of AMP-activated protein kinase (AMPK). AMPK inhibits acetyl-CoA carboxylase, reducing the synthesis of malonyl-CoA, which activates carnitine:palmitoyl-CoA transferase-1, increasing the oxidation of long-chain fatty acids in the mitochondria of the liver and muscles (Kahn, Alquier, Carling, & Hardie, 2005).

SCFA are also related to increased expression of factors associated with the fasting-induced adipose factor (FIAF) (Carvalho & Saad, 2013), a hormone produced by white adipose tissue, liver, heart, skeletal muscle, and intestine. This hormone inhibits the activity of lipoprotein lipase, an enzyme that hydrolyzes triacylglycerol associated with lipoprotein, enabling its storage in white adipose tissue (Koliwad et al., 2009). After colonization with conventional mice microbiota, germ-free mice showed a decrease in FIAF expression, contributing to body fat accumulation (Bäckhed et al., 2004), further indicating that the composition of gut microbiota is related to an increase in adipose tissue.

Obese individuals have greater activation of the endocannabinoid system. This system is composed of neurotransmitters that bind to cannabinoid receptors (CB1 and CB2), and cannabinoid proteins expressed in the central and peripheral nervous system (Pertwee, 1997). CB1 receptors are also expressed in the central and peripheral nervous system, gastrointestinal tract (Coutts & Izzo, 2004; Liu et al., 2005), adipose tissue (Cota et al., 2003), liver (Osei-Hyiaman et al., 2005) and pancreas (Howlett, 2002) while CB2 receptors are expressed in immune system cells, spleen, thymus, pancreas, and in the nervous system central (Howlett, 2002). Activation of the CB1 receptor in adipose tissue stimulates adipogenesis and lipogenesis, which contributes to the accumulation of body fat and, consequently, to the development of alterations in insulin functioning (Di Marzo, 2008). Studies carried out with mice observed that gut microbiota controls the activity of the endocannabinoid system in the colon and the adipose tissue (Geurts et al., 2011). The activation of the peripheral endocannabinoid system is also under the control of the gut microbiota, and the greater activation observed in obese individuals stimulates adipogenesis not only by direct action on the adipose tissue but also indirectly, through circulating LPS concentration. The activation of the endocannabinoid system in the intestine by gut microbiota increases permeability and consequently plasma concentration of LPS. This process results in a dysregulation of adipogenesis and maintenance of a vicious cycle of overactivation of the endocannabinoid system and increased

concentration of LPS (Mehrpouya-Bahrami, Chitrala, Ganewatta, Tang, & Murphy, 2017; Muccioli et al., 2010).

Some criteria for the selection of probiotic strains with potential for the treatment of obesity have been proposed using in vitro screening and an animal model (Alard et al., 2021). A total of 4 (*L. salivarius* LA307, *B. longum* PI10, *L. salivarius* PI2, and *Lactococcus lactis* PI23) of the 23 strains decreased the intracellular accumulation of lipids in adipocytes, indicating a possible lipolytic and anti-adipogenic activity. From this screening, the strains were subsequently tested in an animal model, identifying that *B. longum* PI10, even when administered alone, was able to decrease the accumulation of lipids in adipocytes. Interestingly, even though *B. animalis* subsp. *lactis* LA804 and *L. gasseri* LA806 did not lead to a significant effect during in vitro experiments, their administration combined with *B. longum* PI10 resulted in limiting weight gain and adiposity increase, showing that the mixture of some bacteria can result in a benefit not observed from their isolated intake (Alard et al., 2021). In this case, the combination of these strains seems to have been responsible for the reduction in gene expression of the jejunal fatty acid-binding protein 1 (FABP-1), which facilitates the transport of lipids, enabling their storage (Furuhashi & Hotamisligil, 2008).

Table 19.2 shows the positive results observed from the administration of probiotics on the accumulation of body fat and lipid profile in individuals with obesity and metabolic syndrome.

19.5 Conclusions and perspectives

Several mechanisms link the gut microbiota imbalance with the development of obesity and metabolic syndrome, and probiotics seem to have a beneficial effect on dysbiosis, adiposity, and related metabolic disorders (Fig. 19.1). However, the benefits obtained from probiotics are dependent on the strains used, dosages, vehicle, route of administration, and period of intervention (Ouwehand, 2017). However, many clinical trials did not provide all information, not allowing us to make comparisons between different types of strains. Additionally, only a few reports analyzed changes in the gut microbiota composition after the probiotic intervention, making it difficult to establish clear beneficial pathways and to develop intervention protocols (Forssten & Ouwehand, 2020).

From the clinical trials that evaluated the effects of probiotic supplementation on obesity and metabolic syndrome, the most used species were *Lactobacillus acidophilus*, *L. plantarum*, *L. casei*, *L. rhamnosus*, *Bifidobacterium lactis*, *B. breve*, *B. longum*, and *B. infants*, although the strains are often different between studies. Intervention length and dosage varied greatly, between 3 and 24 weeks of treatment, and with doses ranging in several others of magnitude (from 1×10^8 CFU/day to 1.35×10^{15} CFU/day) (Moreno, Suárez, Avanzi, Monteoliva-Sánchez, & Aguilera, 2020).

There are still many limitations on any effects that probiotics may have in obesity and metabolic syndrome patients. Some of these limitations include trials with a small number of participants, high heterogeneity regarding strains, dosages, gut microbiome analysis (when this is performed), and the fact that not all studies assess the food intake of participants. Therefore, further research is needed to establish more standardized usage guidelines,

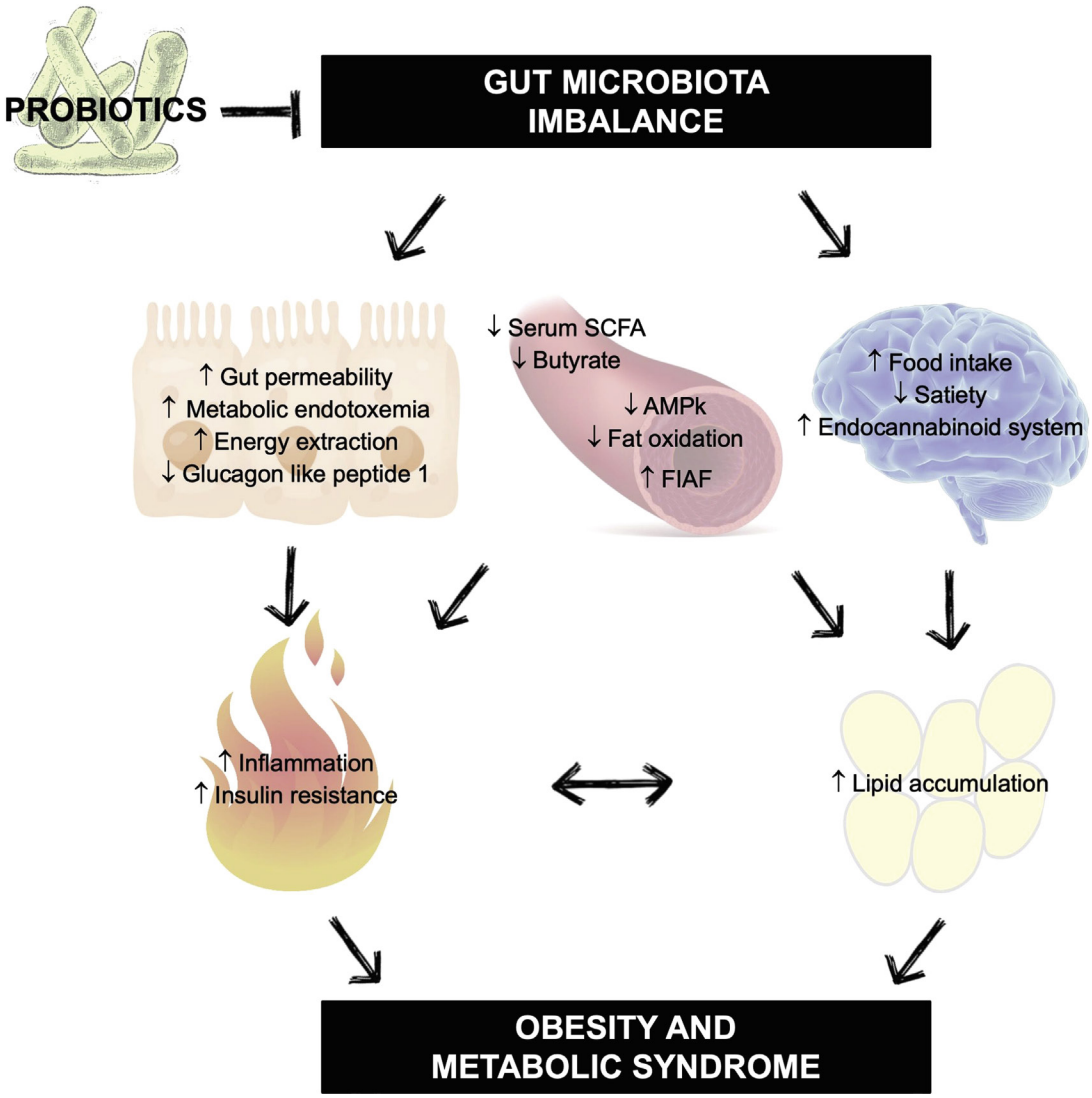


FIG. 19.1 The impact of gut microbiota imbalance and probiotic administration in obesity and metabolic syndrome.

as well as to identify the pathways associated with the benefits of using probiotics in obesity and metabolic syndrome patients.

Gut microbiota imbalance increases gut permeability and thereby translocation of bacterial components into the circulation, resulting in metabolic endotoxemia. Metabolic endotoxemia causes insulin resistance due to the activation of intracellular pro-inflammatory pathways. Changes in the gut microbiota composition are also associated with an increase in the extraction of calories from food and a decrease in the production of glucagon-like peptide-1, which

significantly impact satiety and food intake. The decrease in serum concentrations of SCFA and the activation of AMPk, combined with an increase in the activation of FIAF and the endocannabinoid system, will promote greater stimulus to the accumulation of body fat. The excess of body fat maintains a chronic inflammatory process, contributing to the decrease of insulin signaling. The administration of probiotics contributes to the maintenance of a healthy microbiota, which inhibits these pathological disorders and can be used as an adjuvant in the treatment of obesity and metabolic syndrome. AMPK: AMP-activated protein kinase; FIAF: fasting-induced adipocyte factor; SCFA: short-chain fatty acids.

Abbreviations

5-HT	serotonin
AMPK	AMP-activated protein kinase
AP-1	factors activator protein-1
CB	cannabinoid receptors
CCK	cholecystokinin
CD14	cluster of differentiation 14
CFU	colony-forming unit
CREB	cyclic AMP response element binding protein
FABP-1	fatty acid binding protein 1
FIAF	fasting-induced adipocyte factor
FXR	Farnesoid X receptor
GALT	gut-associated lymphoid tissue
GLP-1	glucagon-like peptide-1
HbA1c	hemoglobin glycated
HOMA-IR	homeostatic model assessment for insulin resistance
IKK	inhibitor of nuclear factor-κB kinase
IRS-1	insulin receptor substrate 1
JNK	c-Jun N-terminal kinase
LBP	LPS binding protein
LPS	lipopolysaccharide
MCP-1	monocyte chemotactic protein-1
MD-2	myeloid differentiation protein-2
MetS	metabolic syndrome
MyD88	myeloid differentiation factor 88
NF-Kb	nuclear factor kappa B
NRLP3	nucleotide-binding domain and leucine-rich repeat protein-3
PTP1B	protein tyrosine phosphatase 1B
PYY	peptide YY
SCFA	short-chain fatty acids
TGR5	Takeda G protein-coupled receptor 5
TNF-α	tumor necrosis factor alpha
TLR-4	toll like receptors 4
TRIF	TIR-domain-containing adapter-inducing interferon-beta

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CHAPTER

20

Probiotics for diabetes mellitus: prevention and treatment

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20.1 Introduction

Diabetes is a chronic disease mainly characterized by both an impairment of the pancreas triggering in a low production of insulin or when the body cannot properly use the insulin produced. Thus, hyperglycemia is the common characteristic of the disease and its incidence is increasing exponentially nowadays. Indeed, diabetes accounted for almost 10% of adult people and is the direct cause of 1.5 million deaths worldwide (World Health Organization, 2021).

Diagnosis of diabetes is based on the plasma glucose levels, fasting plasma glucose (FPG) value or the 2-h plasma glucose (2-h PG) value during a 75-g oral glucose tolerance test (OGTT), or hemoglobin A1c (HbA1c) criteria. The American Diabetes Association recommends three criteria for diabetes diagnosis: either a level of fasting plasma glucose (FPG) >126 mg/dL (7.0 mmol/L), or the 2-h plasma glucose (2-h PG) value >200 mg/dL (11.1mmol/L) during an oral glucose tolerance test (OGTT), or the concentration of HbA1c >6.5% (48 mmol/mol), as well as in a patient with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose ≥200 mg/dL (11.1 mmol/L) (Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2021, 2021). Once a diagnosis has been established, the treatment of these diabetic pathologies is mainly pharmacological with insulin and metformin as the two drugs more widely used (Pharmacologic Approaches to Glycemic Treatment: Standards of Medical Care in Diabetes-2021, 2021).

There are different types of diabetes with some characteristics in common like hyperglycemia, but with important differences among them. Type 2 diabetes (formerly called non-insulin-dependent, or adult-onset) and pre-diabetes state, Type 1 diabetes (previously known as

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insulin-dependent, juvenile or childhood-onset), gestational diabetes that occurs during pregnancy, and other specific types of diabetes due to other causes such as monogenic diabetes syndromes, diseases of the exocrine pancreas, or drug- or chemical-induced diabetes (Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2021, 2021). As it can be observed, although, with a common pattern, diabetes is a heterogeneous disease with different clinical presentations and a variable disease progression. In this process, there are multiple factors to consider: family history, age, lifestyle, other disturbances, environmental factors, and gender.

During the last two decades, a new important factor has burst onto the scene, the gut microbiome. The human intestinal microbiome is composed for the trillions of microorganisms found in the intestine, especially in the colon. The human intestinal microbiome has been described as a new "virtual" endocrine organ that participates in the homeostasis of the host, including glucose and lipid metabolisms.

In this manner, the implication of the gut microbiome in the etiology and pathogeny of diabetes is being widely studied. The knowledge relative to the implication of the gut microbiome in hyperglycemia and diabetes types is exponentially increasing. Type 2 diabetes (T2D) is the diabetes type with the highest number of reports. In a recent meta-analysis with 42 human studies, a particular set of genera were related negatively to T2D: *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, *Akkermansia*, and *Roseburia*; as well as positively: *Ruminococcus*, *Fusobacterium*, and *Blautia* (Gurung et al., 2020).

One of the greatest interests in disentangling the implication of the gut microbiome in health disturbances, and particularly in diabetes, is the fact that the gut microbiome can be modified. There are different strategies to make it possible, but the use and development of probiotics have always been a priority for the interest of the markets and the ease of use for patients. From the meta-analysis reported before, *Bifidobacterium* was the genus that more consistently supports protection against T2D (Gurung et al., 2020). According to that report, *Bifidobacterium* has not been used alone as probiotics for T2D, but animal studies strengthen the idea to introducing *Bifidobacterium* as probiotics could play a protective role in T2D. Among the characteristics of *Bifidobacterium spp*. production of lactic and acetic acids as the main products of carbohydrate utilization is noteworthy.

In this chapter, we are going to discuss the evidence about the use of probiotics in the management of diabetes mellitus. We will deepen into the possible mechanisms, the different types of diabetes, and the future of the use of probiotics in this metabolic disease.

20.2 General mechanisms of action of probiotics in diabetes

Probiotics have demonstrated significant potential as therapeutic options for a variety of diseases, but the mechanisms responsible for these effects have not been fully elucidated yet. Moreover, the mechanisms of action of probiotics, which are diverse and heterogeneous, are strain specific. But, in general, molecular studies suggest that probiotics exert their beneficial effects through four main mechanisms (Olvera-Rosales et al., 2021):

1. Modification of the gut microbiome.
2. Competitive adherence to the mucosa and epithelium.
3. Strengthening of the gut epithelial barrier.
4. Immunomodulation on the host.

The gut microbiome seems to be a cornerstone in the probiotics' mechanisms. Probiotics play important roles when contacting the rest of the microbial communities, influencing the metabolism of other members of the host's microbiome. In fact, probiotics can directly influence the colonization of the microbes and their posterior maintenance. Contrary to expected, probiotics impact does not reside in their ability to graft in the microbiota but rather in sharing genes and metabolites, supporting challenged microbiota, and directly influencing epithelial and immune cells (Wieërs et al., 2020), in line with the maturation of the gut microbiome that is closely linked to the development of the host immune system (Belkaid & Hand, 2014). Thus, the administration of probiotics, as well as prebiotics, has been reported as one of the main approaches to modulate intestinal microbiota, even in the mucosal community structure and the intestine, and this has been the premise in which currently most of the studies are working on, in reaching the eubiosis modulating the gut microbiome, the moment when pro-inflammatory mediators' secretion is regulated controlling local and systemic inflammation, what results in decreasing intestinal permeability, enhancing the immune system, and providing the well-known benefits for the host. Although there is an important question to consider, this impact is transient and individualized (Zmora et al., 2018), sometimes hindering the elucidation of the real mechanisms.

Within the context of diabetes, probiotics have been suggested to play an important role in its management. Although the exact mechanism of action for their beneficial effects on glycemia is not fully elucidated, it seems to be related to their immunoregulatory properties. The beneficial effect of probiotics could be related to the betterment of the metabolism through the amelioration of the impaired glucose tolerance, regulation of the lipid metabolism, the improvement of the antioxidant status, and the modulation of the gut microbiome and its short-chain fatty acids (SCFA) production. In these betterments, some mechanisms are through the improvement of the gut integrity, the decrease of the systemic levels of gut microbiota-derived lipopolysaccharide (LPS) as well as endoplasmic reticulum stress, the increase of incretins, and the improvement of peripheral insulin sensitivity, besides modulating gut microbiota, adjust energy metabolism, and influence immune response (Sun et al., 2020).

In particular, preliminary studies have indicated that alteration of the gut microbial composition by probiotics supplementation might improve T2D by reducing pro-inflammatory cytokines, intestinal permeability, and oxidative stress and that this could be related to the importance of the SCFA producing bacteria in glycemia regulation (Kim, Keogh, & Clifton, 2018). In this manner, the release of incretins such as glucagon-like peptide-1 and peptide YY, through the activation of G-protein-coupled receptors on L-cells by SCFA, results in an increase of insulin and a decrease of glucagon secretion, suppressing appetite. On the other hand, SCFA can also decrease intestinal permeability as well as the circulating endotoxins. Indeed, alterations in glucose metabolism are associated with low-grade inflammation promoted by gut microbiome LPS or endotoxin in mice. Indeed, Cani et al. demonstrated that metabolic endotoxemia (elevation of the LPS concentration) dysregulates the inflammatory tone and triggers body weight gain and diabetes (Cani et al., 2007). One study found that probiotic *Lactobacillus paracasei* NL41 decreases LPS-induced inflammation by improving the gut microbiota and preserving intestinal integrity (Zeng, Guo, Zhang, Yuan, & Chen, 2021). Therefore, lowering plasma lipopolysaccharide concentration through the modulation of the gut microbiome could be one strategy for the control of diabetes.

Additionally, oxidative stress due to the accumulation of free radicals leads to the damage to multiple tissues including the ß-cells of the pancreas besides the liver and kidney, among others. In this line, a study in rats demonstrated that the probiotic strain NL41 prevented T2D by decreasing insulin resistance and oxidative stress status and protecting beta-cell function, demonstrating through histochemistry that the probiotic inhibited beta-cell loss and alpha-cell expansion, indicating that pancreatic islets were the targeted tissues for the primary ameliorative effect of the probiotic strain (Zeng et al., 2019). In this line, the administration of the commercial multi-strain probiotic ProbiogluTM (*Lactobaccilus salivarius subsp. salicinius* AP-32, *L. johnsonii* MH-68, *L. reuteri* GL-104, and *Bifidobacterium animalis subsp. lactis* CP-9) increased the β-cell mass in STZ-induced diabetic animal models, while reducing the levels of proinflammatory cytokines (TNF-α, IL-6, and IL-1β) (Hsieh et al., 2021).

Other mechanisms have been proposed, but also others have not been elucidated yet. These mechanisms are the most reported, but in the next years, the knowledge will grow as the interest in the development and regulation of the probiotic market.

20.3 Examples of results of the probiotics use in diabetes

This chapter does not pretend to be a systematic review of the use of probiotics in diabetes, but rather to serve as a guide of the knowledge about their use within this disease context. As diabetes is a multifaceted disease, with different types, then we introduced some knowledge in the four main diabetes types according to the recent ADA classification:

20.3.1 Type 1 diabetes

Type 1 diabetes (T1D) is referred to that diabetes because of the autoimmune β-cell destruction. T1D usually leads to an absolute insulin deficiency. Within this disturbance is included the latent autoimmune diabetes of adulthood (LADA). Most of the cases are diagnosed during childhood, and most of the investigations have been focused on those ages. Three phenomena happen in early childhood when T1D mainly occurred: the development of the immune system, the maturation of the gut microbiome, and the appearance of the first T1D-associated autoantibodies, and in this manner, a microbial dysbiosis has been reported, but causal relationships between them have not been established yet (Siljander, Honkanen, & Knip, 2019). The amelioration of the gut dysbiosis with specific probiotics and prebiotics has been found to be associated with the decrease of inflammation and the autoimmune response, as well as a loss of the gut integrity through the tight-junction proteins in the intestinal epithelium.

It is known that environmental microbial exposure could be implicated in the protection against immune-mediated diseases such as T1D. In fact, exposure to an agricultural environment in early life is inversely associated with the risk of T1D (Nurminen et al., 2021). Probiotics are thought to affect immunologic responses to environmental exposures and could be used to prevent the development of T1D. Thus, The Environmental Determinants of Diabetes in the Young (TEDDY) study found that early supplementation with probiotics may reduce the risk of islet autoimmunity in children at the highest genetic risk of T1D (Uusitalo et al., 2016).

Within T1D, probiotic supplementation leads to a decrease in the pro-inflammatory cytokines that are responsible for the damage to the β-cells of the pancreas, the improvement of the gut barrier function, as well as the induction of immune tolerance. These results are translated into better glycemic control and a decrease in insulin requirements in newly diagnosed T1D children with the use during 3 months of a high dose multi-strain probiotic (Visbiome with 8 strain) together with the usual treatment (Kumar et al., 2021). However, the administration of *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12 for 6 months had no significant effect in maintaining the residual pancreatic beta-cell function in children with newly diagnosed T1D (Groele et al., 2021).

Sometimes primary outcomes are not well-chosen, and consequently, there is a lack of positive results. In this trial, the primary outcome was the increase of tryptophan levels by the probiotic, because the levels of kynurenine, which is the first byproduct of tryptophan degradation via IDO1, are significantly lower than in nondiabetic controls. Thus, daily administration of *Lactobacillus rhamnosus* GG significantly affected circulating levels of tryptophan as well as the qualitative pattern of metabolite formation in the patients with T1D, while it decreased inflammatory cytokine production by the patients (Mondanelli et al., 2020).

20.3.2 Type 2 diabetes and pre-diabetes

T2D is a prevalent chronic disease characterized by hyperglycemia and insulin resistance. T2D is popularly known as adult diabetes and has its source in insulin resistance producing a progressive loss of adequate β-cell insulin secretion. This is the diabetes pathology with the highest incidence nowadays associated with other comorbidities, with obesity leading the conditions. Indeed, T2D is usually associated with metabolic syndrome. In general, within the context of metabolic diseases, the intake of probiotics resulted in minor but consistent improvements in several metabolic risk factors. These improvements have been mostly related to the use of bifidobacteria (*Bifidobacterium breve*, *B. longum*), *Streptococcus salivarius* subsp. Thermophilus, and lactobacilli (*Lactobacillus acidophilus*, *L. casei*, *L. delbrueckii*) (Koutnikova et al., 2019).

Probiotic intervention in T2D is a promising strategy, but there are still many important issues unsolved. Efficacy and Change of Gut Microbiota in Patients with Newly Diagnosed Type 2 Diabetes (PREMOTE) trial failed to find significant metabolic improvement with probiotic supplementation in T2D patients (Zhang et al., 2020). However, the use of a multistrain microbial cell preparation with three strains from the genus *Lactobacillus*: *L. acidophilus*, *L. casei*, and *L. lactis*; and three strains from the genus *Bifidobacterium*: *B. bifidum*, *B. longum*, and *B. infantis*, were used in a randomized, double-blind, parallel group, controlled clinical trial. These authors found that this multistrain probiotic modestly improved HbA1c (−0.14 % in the probiotics and + 0.02 % in the placebo group) and fasting insulin (+ 1.8 μU/mL in the placebo group and − 2.9 μU/mL in probiotics group) in people with T2D (Firouzi, Majid, Ismail, Kamaruddin, & Barakatun-Nisak, 2017).

One meta-analysis showed that supplementation with probiotics significantly reduces HbA1c (−0.38, 95% CI [−0.62 to −0.14]), fasting insulin levels (−0.38, 95% CI [−0.59 to −0.18]), and HOMA-IR (−0.99, 95% CI [−1.52 to −0.47]) in T2D (Yao et al., 2017). Another recent meta-analysis of 15 RCT with a total of 902 participants showed a significantly greater reduction

in HbA1c (−0.24, 95% CI [−0.44, −0.04]), fasting blood glucose (−0.44, 95% CI [−0.74, −0.15]), and HOMA-IR index (−1.07, 95% CI [−1.58, −0.56]) with the use of probiotics (Tao, Gu, Mao, Zhang, & Pei, 2020). In another meta-analysis with 32 trials studying the different metabolic variables, the authors found a significant effect over the levels of the total cholesterol, triglyceride, c-reactive protein (CRP), HbA1c, fasting plasma glucose, fasting insulin levels, and both systolic and diastolic blood pressure values, as well as increasing the HDL-cholesterol level (Kocsis et al., 2020). However, the clinical heterogeneity, the different probiotic strains, and the duration of treatment deserve the development of more randomized placebo-controlled trials with large sample sizes to clarify the real role of probiotics.

Additionally, within this section, ADA has added the Pre-diabetes state, which is defined as impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and/or elevated HbA1c levels (Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2021, 2021), which are intermediate states between normal glucose homeostasis and diabetes. Although there are a lower number of trials, results are more diffuse than in T2D, although in the same direction. In a systematic review in this state of prediabetes, the authors did not find sufficient evidence to show significant benefits on glucose or lipid metabolisms or body composition, although suggested that a combination of probiotics and prebiotics in a synbiotic supplementation is more effective than probiotics alone for glycemic control (Wang et al., 2021).

20.4 Gestational diabetes

Gestational diabetes mellitus (GDM) is known as diabetes diagnosed in the second or third trimester of pregnancy that was not clearly overt diabetes prior to gestation. As with any other intervention, the use of probiotics during pregnancy is controversial, although there is no evidence of taking probiotics or prebiotics during pregnancy either increases or decreases the risk of preterm birth or other infant and maternal adverse pregnancy outcomes (Jarde et al., 2018). About GDM the pursued outcomes are usually to improve fasting plasma glucose and/or insulin resistance (HOMA-IR), as well as other variables like LDL-cholesterol or body weight (mother/baby).

A systematic review and meta-analysis of randomized controlled trials with 288 participants concluded that probiotic supplementation during 6 to 8 weeks was not effective in decreasing fasting blood glucose levels or LDL-cholesterol in women with GDM, although got a significant reduction in HOMA-IR (-0.69; 95% CI -1.24, -0.14), with no significant differences in gestational weight gain, delivery method or neonatal outcomes in comparison to the control groups (Taylor et al., 2017).

Another point is that probiotics administered during pregnancy might prevent gestational diabetes mellitus (GDM). Even a small reduction in maternal glucose in non-diabetic women, and particularly in women at high risk for GDM, may have significant benefits for pregnancy results and the future health of offspring. In a large clinical trial with 411 women, probiotics (*Lactobacillus rhamnosus* and *Bifidobacterium animalis* subspecies *lactis*) were used in order to prevent GDM in overweight and obese pregnant women. However, the main conclusion was that probiotics did not prevent GDM, although women increased their weight at a lower rate and babies presented a better body weight (Callaway et al., 2019).

The lack of benefits lowering the risk of GDM or the improvement of glucose metabolism has been also reported in women with overweight or obesity with the use of the particular strains *Lactobacillus rhamnosus* HN001 and *Bifidobacterium animalis* ssp. lactis 420 (Pellonperä et al., 2019).

20.5 Other specific types of diabetes

Cystic fibrosis-related diabetes (CFRD) is the most common comorbidity in individuals with cystic fibrosis, occurring in about 20% of adolescents and 40 to 50% of adults (Moran et al., 2018). As it has been established that people with cystic fibrosis have intestinal dysbiosis as well as inflammation, probiotics could be used for improving health outcomes in children and adults with cystic fibrosis. A recent meta-analysis established that probiotics significantly reduced fecal calprotectin (a marker of intestinal inflammation) in children and adults with cystic fibrosis (Coffey, Garg, Homaira, Jaffe, & Ooi, 2020), although little has been reported about CRFD. A study found that cystic fibrosis patients have an increase in the abundance of *Alistipes* related to an impairment of the glucose homeostasis through succinate metabolism (Coffey et al., 2019).

After transplantation, hyperglycemia is very common, usually related to the use of immunosuppressive therapies. The use of probiotics in post-transplantation diabetes mellitus has been scarcely studied. Only one study in Sprague-Dawley rats subjected to doses of tacrolimus and sirolimus in order to induce hyperglycemia and an insulin-resistant state has been found. Here bacterial diversity was significantly decreased, something reversed with the use of probiotics (*Lactobacillus plantarum* at 1×10^9 cells) (Bhat et al., 2017). Thus, modulation of the microbiome through the use of probiotics could help in minimizing the hyperglycemia effects of immunosuppression.

Monogenic Diabetes Syndromes are those monogenic defects that cause β-cell dysfunction. The most studied are neonatal diabetes and Maturity-Onset Diabetes of the Young (MODY), although only represent a small fraction of patients with diabetes (<5%). To the best of our knowledge, there are no trials on the use of probiotics in MODY patients, although, in previous work, we reported a different gut microbiota profile and functionality of MODY2 children with respect to T1DM and control children, related to inflammation and permeability outcomes (Leiva-Gea et al., 2018). This fact may suggest that the use of probiotics could alleviate, at least in part, the severity of the disease.

Finally, although not the last type of diabetes, the called "type 3c diabetes", which is characterized by hyperglycemia due to general pancreatic dysfunction. Again, there is a lack of knowledge about this rare type of diabetes. Recently, a different profile of gut microbiota has been reported in comparison to T2D and T1D. Thus, *Nesterenkonia* sp. AN1, *Clostridium magnum*, *Acinetobacter lwoffii*, *Clostridium septicum*, *Porphyromonas somerae*, *Terrabacter tumescens*, and *Synechococus* sp. were only found in Type 3c diabetes patients (Talukdar et al., 2021), indicating that the use of probiotics could change these profiles and their related metabolic outcomes.

Taking into account the little research done about the use of probiotics in this miscellaneous section of diabetes, but with the focus on the differences found in their gut microbiota profiles with respect to other diabetes or control groups, further research is deserved.

20.6 Probiotics as adjuvants in pharmacological treatments

One important factor to take into account in the use of probiotics for diabetes conditions is that pharmacological treatments should not be replaced by probiotics, although their consumption could be incorporated into the diet during disease management to provide their well-documented beneficial effects. Once this is considered, pharmacological treatments also should be taken into account as a variable or even a confounder in the results. In fact, any study done on diabetes must consider drugs, and more when the gut microbiome is one of the primary outcomes.

The most widely used anti-diabetic for T2D is metformin. Metformin has been reported as one of the main confounders in microbiome studies. Metformin has strong effects on the gut microbiome, in fact, these alterations mediate some of metformin's antidiabetic effects (Wu et al., 2017). In this manner, some researchers hypothesized that probiotics could enhance metformin's effect on glycemia. A randomized controlled pilot study was conducted in 60 adults with prediabetes or T2D. The participants took a multi-strain probiotic (*L. plantarum*, *L. bulgaricus*, *L. gasseri*, *B. breve*, *B. animalis* sbsp. *lactis*, *B. bifidum*, *S. thermophilus*, and *S. boulardii*) or a placebo for 12 weeks. The authors realized that only a subgroup of participants who used metformin showed a decrease in their main variables: fasting plasma glucose, HbA1c, insulin resistance, and zonulin. These changes were directly associated with butyrate te in the probiotic group but not in the placebo group. Indeed, the authors concluded that probiotics may act as an adjunctive to metformin by increasing the production of butyrate, which may consequently enhance glucose management (Palacios et al., 2020). Moreover, in another clinical trial in T2D patients treated with metformin, the inclusion of a multistrain probiotic (UB0316: *L. salivarius* UBLS22, *L. casei* UBLC42, *L. plantarum* UBLP40, *L. acidophilus* UBLA34, *B. breve* UBBr01, and *B. coagulans* Unique IS2) decrease in HbA1c levels (7.70 ± 0.79% vs. 8.30 ± 1.35% in placebo). But, more interestingly, the probiotics group significantly improved its quality of life (Madempudi, Ahire, Neelamraju, Tripathi, & Nanal, 2019).

The work of Sabico et al. also concluded that multi-strain probiotics (EcologicBarrier: *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W52, *Lactobacillus acidophilus* W37, *Lactobacillus brevis* W63, *Lactobacillus casei* W56, *Lactobacillus salivarius* W24, *Lactococcus lactis* W19, and *L. lactis* W58) were a promising adjuvant of the anti-diabetes therapy, although the authors did not register the anti-diabetes drug treatment of the patients (Sabico et al., 2019). This can be related to the fact that metformin has been associated with multiple secondary effects, mostly gastrointestinal, even developing a metformin intolerance because of the heavy diarrheas. Thus, the intake of probiotics for the amelioration of these symptoms has been introduced. In fact, it has been proposed that the ideal probiotic for the alleviation of metformin intolerance could be related to the production of folate (vitamin B9) (Olgun, 2017).

20.7 Probiotics as adjuvants in lifestyle treatments

As we have mentioned before, probiotics are key members involved in the host metabolism, and in this manner, their use as adjuvants of other interventions may be a potential use in

diabetes. In the previous section, we have discussed their use together with anti-diabetic drugs, but in diabetes, lifestyle interventions, meaning recommendations about diet, exercise, or even control of depression are mandatory, and normally the first line of intervention.

Nutritional management of blood glucose levels is a strategic target in the prevention and management of T2D. Current studies have shown the beneficial effects of probiotics in the dietary management of T2D. Most of these studies are focused on the interaction with the gut microbiome, providing a proper environment. It has been shown that intervention with probiotics to treat dysbiosis may be a useful approach to improve metabolic variables in diabetes. Indeed, in an interesting study, *Lactobacillus paracasei* HII01 improved hyperglycemia and inflammatory markers by favorably modifying gut microbiota and ameliorating the leaky gut and endotoxemia, indicating a potential role as an adjuvant treatment in T2D (Toejing, Khampithum, Sirilun, Chaiyasut, & Lailerd, 2021). In fact, this dietary approach is the starting point for many functional foods.

Together with diet, exercise is one of the most effective lifestyle interventions for maintaining healthy glucose levels and weight. As there is a growing interest in dietary supplements for sports players, the studies about the use of probiotics in exercise are increasingly growing. It has been observed that the combination of regular exercise together with the inclusion of the strain *Bifidobacterium longum* OLP-01 improved the blood glucose levels and body composition as well as the physical activity capacities (Hsu, Wu, Lee, & Huang, 2021). Thus, the combination of physical activity with a probiotic like OLP-01 might be used for preventing and treating diabetes.

Finally, depression is increasing within various life-threatening conditions such as neurodegenerative anomalies, cardiovascular diseases, and diabetes. Thus, the development of psychobiotics, probiotics with an effect on the brain, is paying attention, although lacking in clinical trials. However, a study in diabetic rats found that *Lactobacillus plantarum* together with inulin had anti-inflammatory and anxiolytic effects possibly through glial cells in the gut-brain axis (Hosseinifard, Morshedi, Bavafa-Valenlia, & Saghafi-Asl, 2019).

20.8 Next-generation probiotics

As noted until here, the most widely used probiotic strains belong to the safety list of generally recognized as safe (GRAS) microorganisms, mainly from *Bifidobacterium*, *Lactobacillus* or *Streptococcus* species. However, in the evolution of the probiotic's development with the new knowledge about the gut microbiome, one logical approach to developing new probiotics to benefit a particular condition is to administer bacteria from taxa whose reduced abundance is associated with that particular disease. Indeed, the associations between gut microbiota, microbial metabolites, and diabetes, open a new perspective for potential novel therapeutics in the fight against diabetes.

Nowadays, the named "next-generation probiotics" (NGPs) are a field of continuous improvement. Within metabolic diseases, related to inflammatory and diet-related disorders, three candidates based on the many reports suggesting a possible role in diabetes have been proposed for their development: *Akkermansia muciniphila*, *Faecalibacterium prausnitzzi*, and *Eubacterium hallii* (Almeida et al., 2020), all reduced in diabetes conditions.

A. muciniphila is a symbiotic bacterium that is present in the mucus layer and is able to use mucin as its sole carbon, nitrogen, and energy source. *A. mucinphila* is the only member of the phylum *Verrucomicrobia* of mammals, so it has been very easy to detect it with 16S rRNA gene sequence. For that reason, its variation has been reported in multiple states of health and disease. Several studies provided evidence that *A. muciniphila* plays an important role in the metabolic regulation of the host. *A. muciniphila* administration resulted in a reduced body and fat mass gain declined serum triglyceride and fasting glucose levels and enhanced insulin sensitivity in mice (Everard et al., 2013). The mechanism of *A. muciniphila* in glucose and lipid metabolism regulation may be explained by its regulation role in barrier function and immune response (Zhai, Feng, Arjan, & Chen, 2019), a randomized, double-blind, placebo-controlled pilot study in overweight/obese insulin-resistant volunteers with *A. muciniphila*. After 3 months of supplementation, volunteers improved insulin sensitivity and reduced insulinemia, plasma total cholesterol, body fat mass, hip circumference, and level of blood markers associated with liver dysfunction and inflammation while the overall gut microbiome structure was unaffected (Depommier et al., 2019). But, moreover, *A. muciniphila* has been found to exert beneficial metabolic and immune signaling effects in a NOD mouse model of T1D, controlling islet autoimmunity (Hänninen et al., 2018) and indicating the multiple options to use *A. muciniphila* within a diabetes context.

Following the great expectative of *A. muciniphila*, *F. prausnitzzi* by its part, although promising, has not been fully developed as probiotics. Martín et al. isolated some strains and performed a deep molecular and metabolic characterization finding some interesting characteristics such as the lack of epithelial cells' adhesion, plasmids, anti-microbial, and hemolytic activity as well as the presence of DNAse activity. Moreover, their SCFAs capacity production was assessed due to their influence on intestinal homeostasis. These authors reinforced the hypothesis that *F. prausnitzii* is able to drive a complex anti-inflammatory pathway (Martín et al., 2017). Indeed, this team tested *F. prausnitzii* as a prophylactic treatment for pelvic radiation disease, finding that *F. prausnitzii* treatment protected the epithelial colonic barrier from colorectal irradiation (Lapiere et al., 2020). Although no reports have been found in the use of *F. prausnitzii* in diabetes, its anti-inflammatory role deserves further research within this disease. Moreover, it has been proposed that *F. prausnitzii* could serve as a biomarker to assist in diseases diagnostics (Lopez-Siles, Duncan, Garcia-Gil, & Martinez-Medina, 2017).

As new strategies are being developed, new non-previously cultivated bacteria are emerging. This is the case of the newly isolated *Dysosmobacter welbionis* J115^{T}, a butyrate-producing bacterium from the human gut both in the general population and in subjects with metabolic syndrome. The abundance of *Dysosmobacter* genus has correlated negatively with body mass index, fasting glucose, and glycated hemoglobin, when supplemented in mice, partially counteracted diet-induced obesity development, fat mass gain, insulin resistance, and white adipose tissue hypertrophy and inflammation. Thus, it directly and beneficially influences host metabolism and is a strong candidate for the development of next-generation beneficial bacteria targeting metabolic diseases (Le Roy et al., 2021).

Another butyrate producer bacterium that has been tested as a candidate for an NGP is *Subdoligranulum*. The anti-diabetic drug metformin produces an increase in *Subdoligranulum* abundance (de la Cuesta-Zuluaga et al., 2017). Moreover, *Subdoligranulum* genus has been correlated negatively with HbA1c van (Cani, Moens de Hase, & Van Hul, 2021). The unique

cultivated strain of *Subdoligranulum* (*Subdoligranulum variabile* DSM 15176^T) was administrated to mice while inducing obesity by feeding them with a high-fat diet. There were no significant differences in any of the variables related to obesity or diabetes measured at the end of the treatment (Van Hul et al., 2020).

However, technological issues such as the fact that the growth conditions of these non-conventional bacteria are unknown, together with their extreme sensitivity to O_2 traces, as well as the regulatory obstacles may limit the use of strains from these non-conventional bacteria as probiotics. Thus, the easiest solution could be to use prebiotics and other dietary components to selectively enhance their growth *in situ* (Lordan, Thapa, Ross, & Cotter, 2020), but other approaches, like the intake of dead organisms, their metabolites, or only a structural part of them could also work. Indeed, the International Scientific Association of Probiotics and Prebiotics (ISAPP) has recently proposed a consensus definition for postbiotic: "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021).

Following this assumption, in a mice model, Dr. Cani's team demonstrated that a purified membrane protein from *A. muciniphila* or the pasteurized bacterium also was able to improve metabolism in obese and diabetic mice. Thus, the Amuc_1100, a protein isolated from the outer membrane of *A. muciniphila*, interacts with Toll-like receptor 2 (TLR2) and improved the gut barrier partly recapitulating the beneficial effects of the bacterium pasteurized (Plovier et al., 2017). After that, this team found that pasteurized *A. muciniphila* improved insulin sensitivity and insulinemia, as well as reduced plasma total cholesterol in humans (Depommier et al., 2019).

20.9 Discussion

As can be seen from the foregoing, gut microbiome and probiotics are profoundly related. In fact, the administration of probiotics and prebiotics has been reported to be one of the most promising approaches to modulate the intestinal microbiome. Therefore, probiotics are a promising and safe therapy for the prevention and management of diabetes, or at least, to use as an adjuvant together with the anti-diabetics or lifestyle interventions in order to prevent delayed diabetes incidence. Our view is summed in Fig. 20.1.

As the knowledge about microbiome was growing, together with the development of better culturing methodologies and more affordable genome and metagenome sequencing, new therapeutic strategies based on the development of this knowledge are opening up. Probiotics have been used since ancient ages, firstly as part of the fermented food, and after that with the development of ready-for-consumed pills with a more standardized number of live bacteria. These products have usually been produced with microorganisms from species of the GRAS list or as Qualified Presumption of Safety (QPS) at the species level by the European Food Safety Authority (EFSA), something that limited the development of new probiotics to new isolations of those species, usually found in fecal samples. But, while traditional probiotics generally show marginal ameliorative effects, emerging next-generation probiotics start to reveal as true preventive and therapeutic tools and open new and infinite opportunities in this field.

One important question to consider about NGPs is that these new probiotics must have a demonstrated clinical impact, through well-defined mechanisms, and specific functions

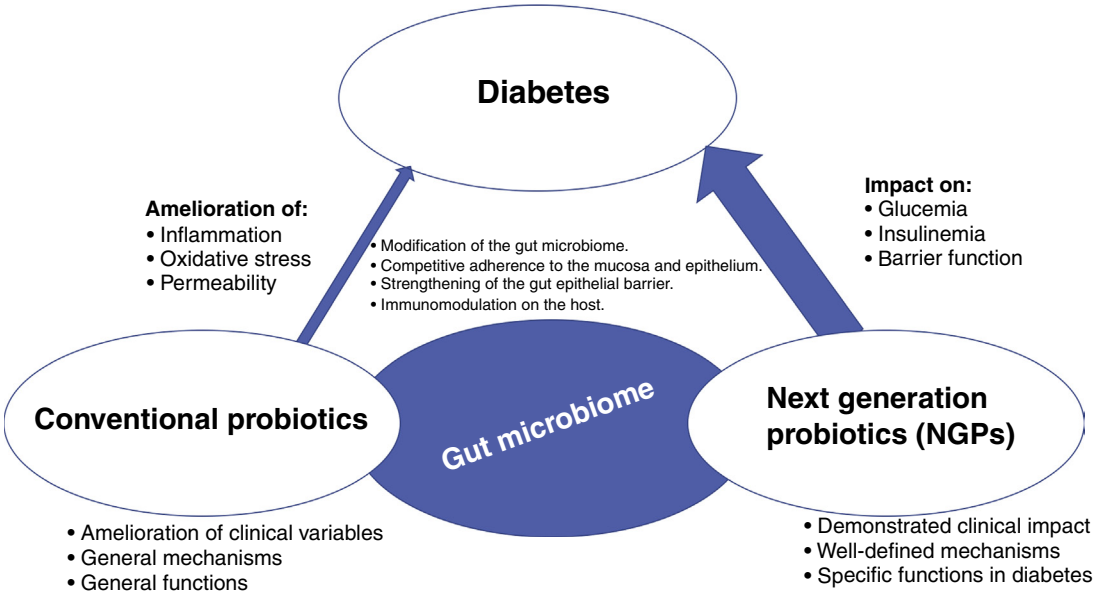


FIG. 20.1 Use of probiotics in the management of diabetes. The use of probiotics in diabetes conditions has been tried in multiple trials. Conventional probiotics, meaning those probiotics traditionally used such as those from Bifidobacterium and Lactobacillus species, have demonstrated a limited action over the diabetes-related variables, being their results more general. However, the irruption of the Next Generation Probiotics (NGPs), belonging to the knowledge achieved by the gut microbiome science, has increased the expectative on the use of these products targeted through diabetes. These NGPs, to be designated by this term, must demonstrate particular clinical impacts over a particular disease, through well-defined mechanisms and with specific functions on, in this case, diabetes. *No permission required*.

impacting the target diseases (López-Moreno et al., 2021). Without focusing only on diabetes, some of the most promising candidates are *Prevotella copri*, *Christensenella minuta*, *Parabacteroides goldsteinii*, *Akkermansia muciniphila*, *Bacteroides thetaiotaomicron*, *Faecalibacterium prausnitzii*, and *Bacteroides fragilis*. These bacteria participate in different processes such as the control of insulin resistance and obesity, the reduction of inflammation, or even show anticancer properties (Chang et al., 2019). However, the development of this new kind of probiotics for their use and commercialization is a difficult process because they receive a treatment of drug as Live Biotherapeutic Products (LBP). In general terms, the conditions that must be probed are among others: the understandings of its effectiveness on a particular disease, safety, characteristics about physiology, genomics, and metabolomics, and the potential virulence factors are mandatory. Moreover, the interaction between NPG and the host in order to maintain intestinal integrity and host homeostasis must also be addressed (Lin et al., 2019). Besides, the safety assessment for the potential to carry and spread antibiotic resistance genes is nowadays mandatory (Saarela, 2019), as the antibiotic resistance problem is increasingly growing.

Thus, in this manner, there is a growing number of research teams working on the development of the previously cited NGPs or deciphering others. Indeed, as a matter of

conclusion, it is more and more frequent to find this kind of sentence as in the work of Le Roy about *Dysosmobacter welbionis* J115T stating "*D. welbionis* J115T directly and beneficially influences host metabolism and is a strong candidate for the development of next-generation beneficial bacteria targeting obesity and associated metabolic diseases" (Le Roy et al., 2021). As the number of novel strains grows, besides the knowledge about their mechanisms within the metabolism of the host, we will be spectators of a turning point in the probiotics field. And the trend in the isolation of new strains is now emerging with new reports every month.

Thus, the human gut microbiome is a promising target for managing type 2 diabetes (T2D). However, the inconsistent usage of strains and formulas, the heterogeneity of the target population, and various qualities and validities across the studies might be the reasons for the controversial results of probiotic intervention (Suez, Zmora, Segal, & Elinav, 2019). We need more randomized clinical trials to be able to determine the most beneficial bacteria, the optimal dosage, and the treatment period. Interestingly, it is very possible that the first NGP totally commercially available will be to treat this disease, and especially T2D with respect to obesity. Indeed, the most advanced example is *A. muciniphila*. Its knowledge will serve for the more rapid development of NGPs. However, the use of inanimate parts of the bacterium, such as the Amuc_1100 previously commented, may overtake the use of live bacteria.

Another interesting field is the development of functional foods, in which the introduction of these probiotics into food products such as dairy products for the management of diabetes could be a good candidate to improve the daily life of the patients. In fact, as the field of NGPs is developing, the introduction or reinforcement of prebiotic substances in food to grow a targeted known probiotic is something that it is being successfully tried. In this way, in a collaborative study, we have recently reported that an alcohol-free beer formulated with isomaltulose and a resistant dextrin was able to modulate the gut microbiome in subjects with T2D, and in particular, the acetate producer *Parabacteroides* (Mateo-Gallego et al., 2021). Following this approach, it seems that synbiotic products could have better anti-diabetic properties than conventional dairy fermented foods (Zepeda-Hernández, Garcia-Amezquita, Requena, & García-Cayuela, 2021), so it is imperative to search for good combinations of probiotics with their preferred prebiotics.

20.10 Conclusions

Therefore, the current knowledge about the use of probiotics in diabetes makes us to think that conventional probiotics are good adjuvants to diabetes medication. These probiotic substances help to ameliorate the main diabetes variables, such as glucose, HbA1c, or insulin levels, as well as parameters related to inflammation or permeability. In fact, most of the ameliorative effects have been reported to be connected with the gut microbiome, an endocrine organ that has been reported as an important part of the homeostasis of the host. And in this manner, gut microbiome knowledge has been the starting point to the development of the next-generation probiotics, which are the future of the management of diabetes. We are looking forward to seeing the development of this field in the next years.

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CHAPTER

21

Probiotic for dyslipidemia prevention and treatment

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21.1 Introduction

Dyslipidemias cover an extensive spectrum of lipid abnormalities characterized by an increase in total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and triglyceride (TG) concentrations, and a decrease in high-density lipoprotein cholesterol (HDL-C) concentrations, either occurring individually or in association. Dyslipidemia is recognized as a relevant risk factor for the development of atherosclerosis, cardiovascular disease, and premature disability and mortality.

The intestinal microbiota has been the subject of much discussion, having been found to be responsible for circulating cholesterol level as well as weakening of composition and function of gut dysbiosis. Gut dysbiosis is characterized by low bacterial diversity, increased gut pathobionts and enhanced intestinal permeability, conditions characteristic of dyslipidemia (Le Roy et al., 2019; Neto et al., 2018).

A role of the gut microbiota in the regulation of host lipid metabolism, particularly cholesterol, has been proposed (Ghazalpour, Cespedes, Bennett, & Allayee, 2016). One mechanism relies on the effect of fermentation-derived propionate on the activity of the enzyme 3-hydroxy-3-methyl glutaryl-coenzyme A synthetase in the liver, leading to an inhibition of cholesterol synthesis (Lin, Vonk, Slooff, Kuipers, & Smit, 1995). Furthermore, several gut bacteria, particularly lactobacilli, possess bile acid hydrolase activities (Begley, Hill, & Gahan, 2006). Bile acid hydrolase activity impacts the enterohepatic cycle of bile acids, leading to increased fecal loss of bile salts and secondary reduction of serum cholesterol due to stimulation of bile acid synthesis in the liver (Jones, Martoni, & Prakash, 2012). Other modifications of bile acids by gut microbiota lead to the production of secondary bile acids that can activate receptors in the intestinal tract and other tissues (e.g., FXR, PXR and TGR5), resulting in important regulatory effects on whole-body metabolism and inflammation (de Aguiar Vallim, Tarling, & Edwards, 2013).

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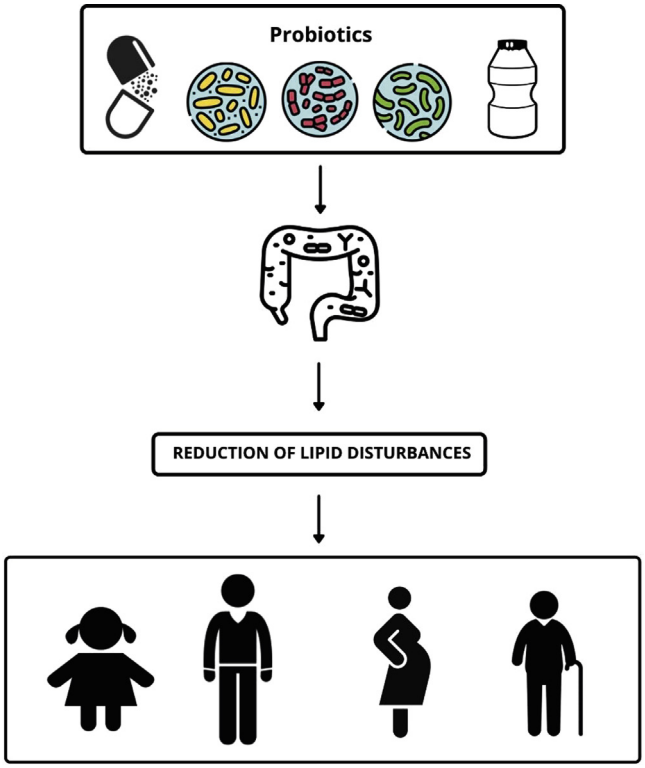


FIG. 21.1 Probiotics reduction lipid disturbances in different stages of human life. *No permission required.*

These findings reinforce modulation of the gut microbiota as part of a potential strategy to prevent or treat dyslipidemias. Probiotic-based therapy, conceptualized as the use of live non-pathogenic microorganisms capable of conferring health benefits to the host when administered in adequate amounts (Hill et al., 2014), has been studied as a promising innovative therapeutic option in the prevention and treatment of dyslipidemias at different stages in human life (Fig. 21.1). In this chapter, the main clinical findings related to probiotic-based therapy under dyslipidemias conditions will be discussed.

21.2 Evidence of probiotic therapy on dyslipidemias in children and adolescents

Dyslipidemia in childhood can be of genetic origin, also called primary dyslipidemia or familial dyslipidemia (Maliachova & Stabouli, 2018), or can be developed related to secondary factors, such as obesity, metabolic syndrome, type 2 diabetes, renal or liver diseases, autoimmune disorders or provoked by pharmacological treatment (Lazarte & Hegele, 2020). Obesity is the most common secondary factor driving the expression of dyslipidemia in youth. In this case, excessive consumption of ultra-processed food rich in saturated fatty acid,

sugar, sodium and diets poor in fiber, associated with sedentary life activities such as watching television, using computer and handheld electronics, playing video games, and time on cell phones have all been described as important risk factors for the development of dyslipidemias associated to obesity in early life (Lazarte & Hegele, 2020).

In this development phase, the adoption of an active lifestyle with daily physical exercise and nutritional counseling is advised. Encouragement of the daily consumption at least 5 servings of fruits and vegetables, increased fiber intake and limitation of ultra-processed foods, fast foods and saturated fatty acids should be implemented with children and their parents or caretakers as a primary strategy in the treatment of dyslipidemia (Corkins et al., 2016).

Current research has suggested that gut dysbiosis, a state characterized by impairment of gut microbiota diversity and increased intestinal permeability, can cause low-grade chronic inflammation, oxidative stress, obesity, and cardiometabolic disorder in the pediatric population (Yang & Ni, 2019). For them, gut microbiota modulation by probiotic administration has emerged as a safe and innovative strategy to improve gut microbiota composition and lipid disturbances (Pederiva et al., 2021).

In 70 healthy-looking children and adolescents (aged 6–18 years), daily administration of one symbiotic capsule (comprising: *Lactobacillus (L.) casei*, *L. rhamnosus*, *Streptococcus (S.) thermophilus*, *Bifidobacterium(B.) breve*, *L. acidophilus*, *B. longum* and *L. bulgaricus* at 2.0×10^8 CFU, fructo-oligosaccharides, vitamin E, vitamin A and vitamin C, for 8-week) reduced significantly cardiometabolic risk factors, such as total cholesterol, LDL-c and triglycerides among children and adolescents (Safavi, Farajian, Kelishadi, Mirlohi, & Hashemipour, 2013).

In children with primary dyslipidemia, daily administration of probiotic formulation containing *B. animalis* subsp. *lactis* MB 2409 (DSM 23733), *B. bifidum* MB 109 (DSM 23731), and *B. longum* subsp. *longum* BL04 (DSM 23233) in lyophilized form (at the dosage of 1×10^9 CFU, for 4-weeks) exerted a mildly improvement in the lipid profile when compared with placebo. Despite this, it is noteworthy that both probiotic and placebo groups had improvement in lipid profile at the end of each treatment period (Guardamagna et al., 2014).

In addition, a pilot study carried with obese children who were given a *L. casei* strain Shirota (LcS)-containing beverage (at 4×10^{10} CFU, for 6 months) combined with diet therapy and physical exercise demonstrated that the protocol intervention contributed to weight loss and improvement of lipid profile (Nagata, Chiba, Wang, & Yamashiro, 2017).

Although the above studies have found benefits from probiotic therapy in the lipid profile of children and adolescents, a randomized, placebo-controlled trial demonstrated that administration of probiotic VSL#3 , for 16 weeks, significantly increased total adiposity and did not improve gut microbiota composition, liver fat content, gut hormones, glucose or insulin fasting variables for obese Hispanic adolescents (Jones et al., 2018). Taken together, current evidence is still insufficient to assess the beneficial effects of probiotic therapy in reducing dyslipidemias in children and adolescents. This indicates that further studies are needed.

21.3 Evidence of probiotic therapy on dyslipidemia in pregnant women

During the gestational period, changes in lipid and lipoprotein metabolism are involved in the development of physiological hyperlipidemia (de Brito Alves et al., 2019). Gestational dyslipidemia is a clinical condition related to the increased synthesis of lipoproteins and

lipolysis in the adipose tissue, which serves as an energy substrate for fetal growth. This process promotes increases in serum levels of cholesterol, TG and LDL-c and a decrease of HDL-c. Expected elevations for triglyceride and cholesterol levels during a normal gestational period usually do not exceed 332 mg/dL and 337 mg/dL, respectively. Elevations over the 95th percentile values, however, can be observed during pregnancy. Patients with levels over these expected adaptation levels can be divided into 2 groups: (1) supraphysiologic hyperlipoproteinemia during pregnancy; and (2) extreme hyperlipoproteinemia limited to the gestational period (triglyceride level >1000 mg/dL) (Basaran, 2009).

Maternal dyslipidemia should be treated with diet and exercise intervention, as well as with glycemic control when appropriate. Bile acid sequestrates, such as colesevelam or mipomersen, may be used for the treatment of familial hypercholesterolemia during pregnancy; severe hypertriglyceridemia should be treated with omega-3 fatty acids, parenteral nutrition, plasmapheresis, and other lipid-lowering agents in the last trimester of pregnancy (de Brito Alves et al., 2019; Wild, Weedin, & Gill, 2015).

It is suggested that the impaired gut microbiota composition is a key mediator in the development of dyslipidemia during pregnancy (de Brito Alves et al., 2019), therefore the use of probiotics has been suggested as an important resource for the prevention or treatment of this condition.

In a randomized, single-blinded, controlled clinical trial, the supplementation with 200 g/day of probiotic yoghurt containing *S. thermophiles*, *L. bulgaricus*, *L. acidophilus* LA-5 and *B. animalis* BB12 (1×10^7 CFU, for 9 weeks) among 70 pregnant women in the third trimester of gestation, resulted in a significant reduction in total cholesterol (TC), LDL-c, and HDL-c levels as well as serum TG concentrations (Asemi et al., 2012).

In another study, 256 pregnant women were allocated into three groups: those who received dietary counseling with probiotics (capsule containing *L. rhamnosus* GG and *B. lactis*); those who received placebo dietary counseling; and those who received the placebo without counseling (control group). These women, who were followed from the first trimester of pregnancy to 12 months postpartum, exhibited similar lipid serum levels during pregnancy. However, those who received maternal dietary counseling showed higher reductions of postpartum TC and LDL-c serum levels in comparison to pregnant control (Hoppu, Isolauri, Koskinen, & Laitinen, 2014).

Recently, a double-blind, randomized, placebo-controlled trial carried with 432 pregnant women in Auckland and Wellington, New Zealand, demonstrated that a probiotic-based therapy in early pregnancy, using specifically-designed tablets containing 6×10^9 CFU of *L. rhamnosus* HN001. Although this did not have a significant effect on fasting lipid in pregnant women with or without gestational diabetes mellitus, daily administration of *L. rhamnosus* HN001 decreased plasma conjugated bile acids and fasting glucose, suggesting a role in the improvement of glucose metabolism in pregnant (Chen et al., 2021).

A randomized, double-blind, placebo-controlled trial was carried out with 87 women with gestational diabetes allocated in following groups: 1) receiving vitamin D (50,000 IU/every 2 weeks) plus a probiotic (8×10^9 CFU/day); 2) group receiving only probiotic (8×10^9 CFU/day); and 3) placebo group. This study found that vitamin D and probiotic co-supplementation, for 6 weeks promoted a significant reduction in TG ($p < 0.001$), VLDL-C ($p < 0.001$) and HDL-C/TC ratio ($p < 0.001$), as well as an increased serum level of HDL-C ($p = 0.008$) and total antioxidant capacity ($p < 0.001$) in comparison to the pregnant women who had received

the placebo treatment. Additionally, the study demonstrated that that vitamin D and probiotic co-supplementation decreased TG ($p = 0.02$) and VLDL-C ($p = 0.02$) and increased total antioxidant capacity ($p = 0.006$) when compared to the results from pregnant women receiving only the probiotic (Jamilian, Amirani, & Asemi, 2019). These findings suggest that co-supplementation strategy with probiotics and vitamin D can be safe and effective against lipid disturbances in women with gestational diabetes.

Sixty pregnant women with gestational diabetes mellitus, primigravida and aged 18 to 40 years received probiotic capsules ($n = 30$) or a placebo capsule ($n = 30$). The women in the probiotic group took daily capsules containing (2×10^9 CFU/g) of *L. acidophilus*, *L. casei* and *B. bifidum*, for 6 weeks. After the intervention, the study revealed that probiotic supplementation decreased serum TG ($p = 0.03$) and VLDL-cholesterol ($p = 0.03$) concentrations. However, there was no effect on the other lipid markers (Karamali et al., 2016). In another similar study, participants who consumed probiotic capsules containing *L. acidophilus*, *L. casei*, *B. bifidum* and *L. fermentum* (2×10^9 CFU/g each) for 6 weeks showed a reduction in serum levels of TG ($p = 0.02$), VLDL-C ($p = 0.02$) and TC/HDL-C ratio ($p = 0.006$). In addition, there was a significant increase in HDL-C levels ($p = 0.03$) (Babadi et al., 2019), highlighting the potential of this probiotic composition to improve lipid profile.

21.4 Evidence of probiotic therapy on prevention and treatment of dyslipidemias

Probiotic therapy has been proposed as a safe strategy for the prevention and treatment of dyslipidemias (Neto et al., 2018). This section discusses the main clinical studies regarding the effects of probiotic therapy in the prevention and treatment of dyslipidemias.

A parallel, double-blind, placebo-controlled, randomized study with 46 volunteers was conducted with the administration of *L. plantarum* ECGC 13110402 (a dairy isolate, at a concentration of 2×10^9 CFU, twice daily in capsular format) for 12 weeks. Although *Lactobacillus* relative abundance was not increased, the treatment with *L. plantarum* ECGC 13110402 lowered serum levels of LDL-C ($p = 0.03$) in subjects with normal total cholesterol. Additionally, TC ($p = 0.045$) was reduced in volunteers with high total cholesterol serum levels, but without a hypocholesterolemic effect in subjects with mildly elevated total cholesterol (Costabile et al., 2017). Despite the reduced sample size represents the main limitation of the study, the findings suggest a preventive and therapeutic effect on dyslipidemia condition promoted by the *L. Plantarum* ECGC administration.

In moderately hypercholesterolemic men, daily administration of an isoflavone-supplemented soy product fermented with *Enterococcus faecium* CRL183 and *L. helveticus* 416 (at 10^9 CFU, for 6-weeks), improved lipid profiles and reduced the oxidation of LDL particles (Cavallini et al., 2016). This suggests that co-supplements in food could potentialize the effects of probiotic therapy in dyslipidemia conditions and that further studies are needed. A similar study with thirty-six subjects with mild hypercholesterolemia showed that a dietary combination of soy with a probiotic or prebiotic food significantly reduced total and LDL-cholesterol (Larkin, Astheimer, & Price, 2009).

A randomized, double-blind, placebo-controlled, parallel-group trial, carried out with 33 subjects displaying LDL-c in the 130 to 200 mg/dL range demonstrated that daily

administration of a nutraceutical combination containing *B. longum* BB536 at 10^9 CFU, red yeast rice extract (10 mg monacolin K), 16 mg niacin and 20 mg coenzyme Q10, for 12 weeks, reduced significantly the LDL-c (a drop of approximately 26%), total cholesterol and apolipoprotein B (about – 17%) and non-HDL-C (– 24%) when compared to subjects of the placebo group. This suggests that nutraceutical formulation (Lactoflorene Colesterolo, 1 sachet/d), besides being well-tolerated by participants, was considered safe and effective in terms of significant improvement of the atherogenic lipid profile (Ruscica et al., 2019).

A single-arm, open-label pilot study performed with 11 hypercholesterolemic adults demonstrated that daily administration of the probiotic *Saccharomyces boulardii*, at 5.6×10^{10} CFU for 8-week, reduced remnant lipoprotein particles, but did not improve serum levels of cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides (Ryan, Hanes, Schafer, Mikolai, & Zwickey, 2015). In fact, the reduced simple size of the study makes it difficult to clarify the hypocholesterolemic effects promoted by *Saccharomyces boulardii* treatment. Preclinical findings verified that administration of *Saccharomyces boulardii*, a non-bacterial microorganism classified as a probiotic agent, modified gut microbiota composition and exerted an anti-hypocholesterolemic effect on mice and hamsters (Albuquerque et al., 2019; Briand, Sulpice, Giammarinaro, & Roux, 2019). Certainly, further clinical studies need to be conducted to confirm or refute the effects of *Saccharomyces boulardii* on the condition of dyslipidemia.

Although a broad body of clinical studies has demonstrated effectiveness in probiotic therapy to reduce dyslipidemias, some did not find effects of probiotic administration on the lipid profile of hypercholesterolemic subjects. For example, administration of capsules of *L. fermentum*, twice a day and for a period of 10 weeks, did not lead to additional reduction in cholesterol and triglyceride serum levels in subjects with elevated serum cholesterol when compared to a placebo group (Simons, Amansec, & Conway, 2006). Similarly, a double-blind placebo-controlled study carried out with 80 volunteers displaying hypercholesterolemia did not find any serum lipid benefit from the administration of freeze-dried *L. acidophilus* at a daily dose of 3×10^{10} CFU, for 6 weeks (Simons et al., 2006).

According to Fuentes, Lajo, Carrion, and Cune (2013), the effectiveness of each strain can be related to baseline cholesterol values, as well as the duration of the intervention. The authors investigated the effects of a mix of three strains of *L. plantarum* (CECT 7527, CECT 7528 and CECT 7529) on their efficiency to lower cholesterol in hypercholesterolemic patients ($n = 60$) aged 18 to 65 years. A decreased of TC levels was observed in the probiotic group when compared with the placebo group. The lipid outcomes were also analyzed based on TC values at baseline. This study showed that *L. plantarum* had a better effect on patients with higher initial cholesterol levels. In the participants with high baseline cholesterol levels (2510–3000 mg/L), the *L. plantarum* treatment showed a reduction in serum levels of TC (17.4%), LDL-C (17.6%) and oxidized LDL-C (15.6%) ($p < 0.05$) after 12 weeks of consumption when compared with subjects in the placebo group. In the subjects with low baseline cholesterol levels (2000–2500 mg/L), there was a significant reduction only in serum levels of TC (9.4%) ($p < 0.05$).

Other randomized clinical trials demonstrated that consumption of a probiotic containing strains of *L. curvatus* (HY 7601) and *L. plantarum* (KY 1032) was effective for a 20% reduction in serum levels of TG ($p = 0.001$) and 25% increase ($p = 0.001$) in apolipoprotein A-V (Apo A-V). In this study, over a 12-week testing period, the probiotic group ($n = 46$) consumed 2

g of a powder containing 5×10^9 CFU of *L. curvatus* and *L. plantarum* each day, whereas the placebo group (n = 46) consumed the same product without probiotics. In part, the function of Apo A-V in accelerating plasma TG hydrolysis might be to enhance lipase lipoprotein activity, as, in the probiotic group, the changes in TG were negatively correlated with Apo A-V. This indicates that high levels of Apo A-V might contribute to the reduction of serum TG (10.1016/j.numecd.2015.05.002).

In another study performed with 200 healthy adult men and women for 12 weeks, consumption of fragmented *L. amylovorus* (CP1563) significantly improved TG ($p < 0.001$), TC ($p < 0.001$) and LDL-Cholesterol ($p < 0.001$) serum levels. In this case, subjects of one group (n = 100) received 500 mL/day of the test beverage and volunteers in the second group consumed 500 mL of a placebo beverage per day. The beneficial effects of increased HDL-C levels occurred mainly in men. Moreover, the study showed that a long period of intake is needed to potentialize health-promoting benefits, including antidyslipidemic effects. (Nakamura et al., 2016).

Finally, the increase in HDL-C levels ($p = 0.02$) after probiotic plus symbiotic consumption, when compared to the control, demonstrated in a study with 120 adult men and women, indicated that the use of probiotics in association with symbiotics can also be an important strategy to combat metabolic syndrome-associated diseases, such as dyslipidemia. Findings and new studies could have a significant impact on clinical practice and public health as a simple and effective adjunctive approach for prevention or management (Kassaian, Feizi, Aminorroaya, & Amini, 2019).

A meta-analysis including 11 randomized clinical trials showed that L. acidophilus reduced TC and LDL-C levels to a greater extent than other bacterial species. Further, a probiotic intervention duration of >4 weeks could be more effective in improving these parameters (Shimizu et al., 2015). Another meta-analysis including 15 randomized clinical trials demonstrated that probiotic supplementation was effective in reducing TC, LDL-C, BMI, WC, and inflammatory markers. Additionally, the study concluded that the consumption of probiotics was more effective when delivered by fermented milk or yogurt, consumed for at least 8 weeks, involving multiple strains. Further, the study concluded that L. acidophilus was the most effective species to reduce LDL-C levels (Sun & Buys, 2015).

More recently, a meta-analysis carried through 13 studies, involving a total of 818 participants in 8 countries. This study revealed that glycemic, lipids, blood pressure and inflammation indicators are significantly improved by probiotic supplementation, particularly in the subjects who are ≤55, baseline BMI <30 kg/m^2, who took the supplement for more than 8 weeks, and received multiple species probiotic (Liang et al., 2021).

21.5 Evidence of probiotic therapy on elderly

In this case, 156 men and women over 55 received daily administration of yoghurt and probiotic capsules that provided a minimum *L. acidophilus* and *B. animalis* dose of 3×10^9 CFU. The intervention, when compared to the control, did not change concentrations of TC, LDL-C, HDL-C and TG ($p > 0.05$). However, this lack of effect can be a result of a relatively good baseline of serum lipid profile (Ivey et al., 2015).

A randomized, double-blind, controlled trial carried out with 60 patients, demonstrated that administration for 6 weeks of a probiotic containing 7 viable strains of *Lactobacillus*,

Bifidobacterium and *Streptococcus*, increased HDL-c levels ($p = 0.002$) but did not improve TG, CT levels, and anthropometric parameters (Razmpoosh et al., 2019).

Cosupplementation can be an interesting way to improve serum lipid parameters. An investigation was conducted to evaluate the effects of probiotic and selenium cosupplementation on indicators of mental health and metabolic profiles in diabetic people with coronary heart disease. It was possible to identify that the subjects who had been receiving 200 μg/day selenium plus 2×10^9 CFU/day of each probiotic (*L. acidophilus*, *L. reuteri*, *L. fermentum*, and *B. bifidum*) for 12 weeks, as they had reduced levels of TG ($p = 0.003$), VLDL-C ($p = 0.003$), and TC ($p = 0.02$) when compared to the control. However, there was no significant improvement in HDL-C levels (Raygan, Ostadmohammadi, & Asemi, 2019).

Another randomized placebo-controlled study carried in type 2 diabetic patients with coronary heart disease, aged between 48 and 85 years, demonstrated that co-supplementation of vitamin D (50,000 IU every 2 weeks) plus probiotic (8×10^9 CFU/g of *Lactocare zisttakhmir* ($n = 30$) for 12 weeks significantly increased HDL-C serum levels ($p = 0.004$) (Raygan, Ostadmohammadi, Bahmani, & Asemi, 2018).

Overall, probiotics have great potential to reduce the risk of lipid disorders in elderly people, with the improvement in HLD-C levels being the most evident result. Furthermore, specific strains should be considered to target the treatment.

21.6 Conclusion

Nutrition, food security and a healthy lifestyle should represent the first choice for the prevention and treatment of dyslipidemia. The lack of gut microbiota analyses, appropriate dosages, intervention time, safety tests and small sample sizes in the clinical studies available represent the main limitation found for more extensive clinical application of probiotic therapy in patients suffering a dyslipidemia condition. In addition, an international culture bank containing well-cataloged anti-dyslipidemic probiotic strains is essential for the development of anti-dyslipidemic products for worldwide study and use.

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CHAPTER

22

Probiotics for urinary tract disease prevention and treatment

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22.1 Urinary tract infections and related microorganisms

Urinary tract infections (UTIs) are among the most common bacterial infections worldwide, with prevalence ranging from 12.9% in the United States, 19.6% in Europe, and up to 24% in developing countries. It is generally characterized by the presence of microorganisms in urine, in quantities greater than 10^5 colony-forming units in each milliliter (CFU/mL) of urine. They can usually cause a wide spectrum of clinical conditions, varying from asymptomatic and benign infections up to situations of high morbidity or even progressing to death (Flores-Mireles, Walker, Caparon, & Hultgren, 2015; Medina & Castillo-Pino, 2019; Wagenlehner et al., 2020).

Anatomically, the urinary tract is divided into two parts. The upper urinary tract includes the kidneys, renal pelvis, and ureters; and the lower urinary tract includes the bladder and urethra. Infections that affect the upper urinary tract are called pyelonephritis. In the lower tract, they are called cystitis if they affect the bladder and/or urethritis when they affect the urethra (Lee & Neild, 2007; Hickling, Sun, & Wu, 2015).

According to the guidelines of the European Association of Urology (Bonkat et al., 2020), UTIs are commonly classified as complicated, uncomplicated, recurrent, catheter-associated, or urosepsis. The uncomplicated cases are related to non-pregnant women and/or people without anatomical and/or functional alterations. In contrast, cases considered complicated occur in individuals with a greater predisposition to develop UTIs, such as pregnant women, patients with anatomical and/or functional alterations, as well as with comorbidities, which are high-risk factors for urosepsis, characterized as systemic infections secondary to UTIs with multiple dysfunctions and the possibility to progress to death.

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Some patients develop UTIs more frequently than the rest of the population, with at least three episodes in one year or two episodes in six months, being classified as recurrent. A considerable part of the cases occurs in individuals using a catheter or who did it in the last 48 hours (Bonkat et al., 2020). Lower tract infections may be asymptomatic or present varied symptoms; the most common being dysuria, pollakiuria, nocturia, urinary incontinence, macrohematuria, suprapubic pain, and cloudy urine. Fever, chills, flank or costovertebral angle tenderness, and nausea are more associated with upper tract infections (Kline & Lewis, 2016; Schmiemann, Kniehl, Gebhardt, Matejczyk, & Hummers-Pradier, 2010).

The indicatives of renal or urinary tract diseases are red blood cells (RBC) counts greater than 5000/mL and of leukocytes greater than 10,000/mL in urinary sediment. The diagnosis is confirmed through the qualitative and quantitative identification of the pathogen, with counts above 10^5 CFU/mL being considered positive. Laboratory detection of the microorganism must be associated with the manifestation of symptoms in the patient, as high microbial counts are not always associated with infections and vice versa, avoiding, for example, unnecessary treatment and interventions (Schmiemann et al., 2010; Addis, 1926; Kass, 1957; Foxman, 2010; Morand et al., 2019).

The urine has been considered a naturally sterile fluid for many years. However, culture techniques associated with metagenomics have shown the existence of a urinary microbiota (urobiome), which, for the most part, is not detected by usual culture methods, but which may play a fundamental role in the pathogenesis of urinary infections (Morand et al., 2019; Dubourg et al., 2020).

The composition of the urinary microbiota is directly related to the intestinal microbiota and, to a lesser extent, to other anatomical structures, such as the skin and vagina. The most prevalent bacterial phyla in the urobiome are Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes. Most of the species identified, under normal conditions, are considered commensals of the urinary tract. However, among those most frequently associated with urinary infections were found *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Enterococcus faecalis* (Morand et al., 2019). Fungi have also been identified in studies related to the urinary microbiota, mostly species belonging to the genus *Candida* (Dubourg et al., 2020).

UTIs can be caused by the proliferation of a single bacterial or fungal species considered uropathogenic or by more than one species. Cases of co-infection or polymicrobial infection are more associated with complicated infections in groups considered more susceptible to UTIs (Kline & Lewis, 2016). Although most UTI-causing microorganisms come from the intestine, the vagina plays a fundamental role in the pathogenesis of these infections. Infection of the bladder and, eventually, of the kidneys, is preceded by the colonization of the vaginal introitus region, and later, of the urethra by uropathogens (Stapleton, 2016; Klein & Hultgren, 2020).

Studies have shown that the vaginal microbiota can harbor uropathogenic bacteria. Among them, uropathogenic *E. coli* (UPEC), *Staphylococcus saprophyticus*, *S. aureus*, and *Staphylococcus epidermidis*, *Streptococcus agalactiae* (group B *Streptococcus*), *Gardnerella vaginalis*, and bacteria of the genus *Ureaplasma*. The prevalence of these bacteria is almost always related to low lactobacilli counts (Stapleton, 2016; Brannon et al., 2020; Lewis & Gilbert, 2020).

The UPEC is the most isolated species in both uncomplicated and complicated infections, being followed by *K. pneumoniae*, also found in both infections. *S. saprophyticus* has been more

associated with uncomplicated infections, while *Enterococcus* spp. and *Candida* spp. are more prevalent in complicated infections (Medina & Castillo-Pino, 2019).

Most of UPEC comes from the intestinal microbiota. The same *E. coli* serotypes have already been detected in stool samples, periurethral region, and urine of healthy women (Schlager, Hendley, Bell, & Whittam, 2002). It is also known that UPEC presence in the female periurethral region for long periods is often linked to a higher prevalence of bacteriuria, pyuria, and UTI symptoms (Czaja et al., 2009).

However, UPEC strains are also capable of adhering, invading, and colonizing not only the vaginal epithelium but also bladder epithelial cells and other urinary tissues (urothelium), which can serve as reservoirs for further infections. It corroborates the evidence of the existence of a close relationship between intestinal, urinary, and genital microbiota in the pathogenesis of UTIs (Mulvey, Schilling, & Hultgren, 2001; Brannon et al., 2020).

The most prevalent Gram-positive bacteria in uncomplicated infections in women are *S. saprophyticus*, *E. faecalis*, and *S. agalactiae* (Kline & Lewis, 2016). The first is the most isolated Gram-positive bacterium from uncomplicated infections in young non-pregnant women, being found mostly in community samples and more rarely causing hospital infections. In men without complicating conditions, it has been associated with urethritis and prostatitis, while in the elderly or institutionalized patients it can lead to serious conditions (Kline & Lewis, 2016; Lewis & Gilbert, 2020).

Infections with non-saprophytic Gram-positive cocci have been more closely related to complicated infections. *S. aureus* is the main responsible for cases of UTIs among pregnant women or in patients using urinary catheters. *S. agalactiae* is closely related to systemic infections due to UTIs and urosepsis in the elderly, pregnant women, diabetic, and/or immunocompromised patients. In pregnant women, screening and antibiotic prophylaxis for this bacterium are recommended, due to the increased risk of chorioamnionitis and neonatal sepsis (Kline & Lewis, 2016).

The most isolated species of the genus *Enterococcus* from UTIs are *E. faecalis* and *Enterococcus faecium*. They are not often found in community infections but are considered the third cause of hospital-acquired UTIs, especially in cases of the use of catheters (Kline & Lewis, 2016).

Although more recent research shows a wide repertoire of bacteria and fungi that are part of the human urobiome, the role of these microorganisms, as commensals and/or pathogens of the urinary tract, is still widely discussed (Morand et al., 2019; Ackerman & Chai, 2019). However, it is already known that the organisms most associated as uropathogens express several virulence factors for the effective establishment of the infection (Flores-Mireles et al., 2015).

Factors related to cell adhesion capacity are essential for urothelium colonization, including cell appendages such as pili and flagella found in UPEC and *P. mirabilis*; and different adhesins that bind to cell receptors. Obtaining nutrients and iron is the responsibility of toxins, enzymes (such as proteases and hemolysins), and siderophores. Uropathogens also have effective structures and mechanisms for evading the immune response, such as the presence of a capsule in strains of *K. pneumoniae*, *P. mirabilis*, and *P. aeruginosa*. The ability to form biofilms, already described in most uropathogenic species, has been also considered an important form of protection from antibiotics, immune system response, and stress conditions (Flores-Mireles et al., 2015).

The frequency and severity of urinary infections are related not only to the characteristics of the pathogen, as the species, high microbial counts, and expression of virulence factors,

but also to the characteristics of the infected individual. In patients, both anatomical and functional changes that modify the normal urinary flow can lead to an increased risk to develop UTIs (Hickling et al., 2015).

The most relevant factors for uncomplicated infections are individual and family history of UTIs, sexual activity, new partners, postmenopausal age, vaginal dysbiosis, and recent antibiotic use. In addition to these previous factors, anatomical urogenital abnormalities, vesicoureteral reflux, diabetes mellitus, pregnancy, catheterization, urolithiasis, immunosuppression, and history of pyelonephritis are more associated with complicated infections (Klein & Hultgren, 2020).

Men and women have anatomical differences in the urinary tract, thus resulting in distinct susceptibility to these infections, with the latter being disproportionately more susceptible. The male urethra, with a size ranging from 13 to 20 cm in length, is considerably larger than the female one, which varies from 3.8 cm to 5.1 cm in length. The genitals, despite not being part of the urinary tract, can act as an entry point for pathogenic microorganisms. The vagina in particular, besides its proximity to the anus, may harbor species considered to be uropathogenic (Hickling et al., 2015; Stapleton, 2016; Lewis & Gilbert, 2020).

The prevalence of UTIs in women increases proportionally with age, however, reaching its peak between 14 and 24 years. The frequency of sexual activity and number of partners tend to be associated with a greater predisposition in this group (Medina & Castillo-Pino, 2019; Klein & Hultgren, 2020). Pregnancy is a conditioning factor for urinary infection. The use of catheters and previous history of UTIs are also associated with greater susceptibility in pregnant women. The most frequently isolated bacterial species from pregnant women are *E. coli*, coagulase-negative *Staphylococcus*, *S. aureus*, and *K. pneumoniae* (Alemu et al., 2012).

The development of infections in postmenopausal women may be related to housing (institutional or not), health status, use of antibiotics, catheters, diabetes, as well as the decrease in vaginal lactobacilli counts. The use of catheters in the elderly is associated with an increase in the number of isolated species, the most frequent being those belonging to the genera *Proteus*, *Klebsiella*, *Serratia,* and *Pseudomonas*. When elderly people have diabetes mellitus, the infections caused by *Klebsiella*, *Enterobacter,* and *Candida* are more common (Medina & Castillo-Pino, 2019).

Diabetic patients have a high susceptibility to UTIs, both from bacterial and fungal etiologies. *E. coli*, as well as in other groups, is also more isolated in these patients, followed by coagulase-negative *Staphylococcus*, *Enterococcus* spp. and *S. aureus*. The genus *Candida* is the most prevalent and *C. albicans* the most isolated species (Alemu et al., 2012). Catheter-associated infections are usually caused by biofilm-forming bacteria and are therefore more resistant to antibiotic treatments. These infections had a higher risk of progressing to more severe cases, such as acute pyelonephritis, bacteremia, urosepsis, and death (Kline & Lewis, 2016).

The main antibiotics used for the treatment of UTIs are sulfonamides, fluoroquinolones, and, among β-lactams, ampicillin. However, the treatment of these infections has become a major concern due to the significant increase in antibiotic resistance rates, coupled with the high rates of recurrent infections. Another problem associated with the use of conventional treatments in patients with UTIs is that they often are in broad-spectrum, causing alterations in the healthy microbiota of the patient, making them more susceptible to new infections (Flores-Mireles et al., 2015).

Thus, new therapeutic approaches have been sought to prevent and control these infections, to reduce the high rates of antimicrobial resistance and cases of recurrent infections. Among these alternatives is the use of probiotic microorganisms.

22.2 Probiotics in the treatment and prevention of bacterial diseases of the urinary tract

The urinary tract is the main anatomical site for bacterial infections in adults. These infections can be caused by several pathogens, but the most common bacteria are UPEC, *E. coli*, *P. mirabilis*, *E. faecalis*, *K. pneumoniae*, and *S. saprophyticus*. Although they are most often treated as easily managed health problems with the use of antibiotics, the high recurrence rates are noteworthy, because they result in increased costs for the health system and the possibility of complications. Furthermore, inadequate clinical behavior and self-medication promote the emergence of strains resistant to antimicrobials used in their treatment, signalizing the need for the development of alternative approaches to the use of antibiotics (McLellan & Hunstad, 2016).

Most uropathogens originate from the intestinal environment. Through the perineum, they reach the urethra and possibly the bladder. This process takes place despite the mechanisms that the urinary tract has to prevent it, such as the chemical composition of the urine, the secretion of antimicrobial peptides by the urothelium, the unidirectional flow of urine, and complete emptying of the bladder. An important step towards the establishment of the infection is the transcription of genes that code for virulence factors, such as pili and adhesins, that allow tissue binding and invasion. The presence of pathogens triggers the innate immune response characterized by the activation of the complement, secretion of antimicrobial peptides and pro-inflammatory cytokines, and recruitment of phagocytic cells. The action of the immune system can lead to the elimination of bacteria, but the inflammatory process results in tissue damage and characteristic symptoms of cystitis (Spencer, Schwaderer, Becknell, Watson, & Hains, 2014; Becknell, Schwaderer, Hains, & Spencer, 2015).

Some bacteria can evade the immune system, either by invading host cells or by the ability to make morphological changes, rendering them resistant to the action of phagocytes. The process of colonization and ascension to the kidneys is aided by the production of toxins and the ability to form biofilms. The resulting tissue damage causes the release of nutrients that stimulate the multiplication of pathogens and facilitate their arrival to the kidneys (Flores-Mireles et al., 2015). In addition to factors related to the ability of bacteria of colonizing and invading, studies have shown an association between cases of recurrent acute pyelonephritis and single nucleotide polymorphisms in genes involved in the innate immune response, highlighting the need for deeper knowledge about the pathophysiology of UTIs, mainly because inadequate treatment or the lack of it can result in more serious conditions, such as bacteremia if the pathogens manage to cross the tubular barrier into the kidneys (Millner & Becknell, 2019; Table 22.1).

Conventional treatment of bacterial UTIs should be performed by evaluating patterns of antibiotic sensitivity and resistance, through the culture of urine samples. Commonly used antibiotics include amoxicillin-clavulanate, ciprofloxacin, cephalosporin, nitrofurantoin, trimethoprim-sulfamethoxazole, among others; however, the quantity of substances available for the treatment of urinary infections has decreased due to the emergence of multiresistant microorganisms, mainly in the hospital environment (Aragón et al., 2018). *E. coli* strains resistant to antibiotics are associated with outbreaks of UTIs in several countries. Some of them, in Africa, have already registered 100% resistance of this microorganism to amoxicillin in urine sample isolates (Vagios, Hesham, & Mitchell, 2020).

The resident microbiota is known for its fundamental role in the defense against pathogens, for its ability to synthesize antagonist substances, for competing for adhesion sites, or even for stimulating the maturation and effector functions of the immune system. In this sense, the

TABLE 22.1 Main virulence factors associated with the most frequent urinary tract pathogens.

Main UTI agents	Virulence factors	References
Gram-negative		
Escherichia coli	- Adhesion and biofilm formation: type 1 pili; pili associated with pyelonephritis; pili of Group C type-1; antigen 43- Tissue damage and dissemination: alpha-hemolysin toxin; cytotoxic necrotizing factor toxin 1; acquisition of iron - Immune Evasion: capsular antigens	Flores-Mireles et al. (2015)
Klebsiella pneumoniae	- Adhesion and biofilm formation: type 1 pili; type 3 pili- Tissue damage and dissemination: acquisition of iron - Immune evasion: capsule	Flores-Mireles et al. (2015)
Proteus mirabilis	- Adhesion and biofilm formation: mannose-resistant pili; mediated by autotransporter; trimeric auto agglutinin autotransporter - Tissue damage and dissemination: hemolysins; proteus toxic agglutinin; acquisition of iron; urease - Immune Evasion: capsule; metalloprotease	Flores-Mireles et al. (2015)
Gram-positive		
Staphylococcus saprophyticus	- Adhesion and biofilm formation: secreted hemagglutinins; hemagglutinins adhered to the cell wall - Tissue damage and dissemination: urease; D-serine deaminase - Immune evasion: capsule	Flores-Mireles et al. (2015), Kline and Lewis (2016)
Enterococcus faecalis	- Adhesion and biofilm formation: pili associated with endocarditis and biofilm; enterococcal surface protein; collagen adhesin - Dissemination: sortase A; methionine sulfoxide reductase A and B - Immune evasion: enterococcal polysaccharide antigen	Flores-Mireles et al. (2015), Kline and Lewis (2016)
Streptococcus agalactiae	- Adherence: capsule rich in sialic acid - Immune evasion: capsule rich in sialic acid; hyaluronidase secretion - Invasion and dissemination: pore-forming toxins	Kline and Lewis (2016), Siddhardha, Dyavaiah, & Syed (2020)

manipulation of this microbiota should be a promising alternative in the prevention and treatment of urinary infections without the use of antibiotics. The relationship between the imbalance in the normal microbial population and the development of infections is easily seen in the study of the vaginal ecosystem. This environment is dominated by *Lactobacillus* species that prevent the adhesion and proliferation of pathogenic bacteria. However, intrinsic factors, such as entering menopause, or extrinsic factors, such as the use of spermicides, intimate soaps, and antibiotics, or even sexual intercourse can cause a decrease in the population of lactic acid bacteria, resulting in an environment conducive to the development of infectious diseases (Flores-Mireles et al., 2015; Sihra, Goodman, Zakri, Sahai, & Malde, 2018; Aragón et al., 2018).

The recognition of the importance of the regulatory role of the commensal microbiota has motivated studies and the rational use of probiotics for the prevention and treatment of urinary tract infections. Most in vitro investigations seek to investigate the role of *Lactobacillus* spp. in the inhibition of pathogens through the production of antimicrobial substances, inhibition of adhesion to epithelial cells, and modulation of virulence factors (Vagios et al., 2020).

The antimicrobial activity of probiotics against uropathogens has been accessed by multiple approaches. Most of the studies focus on evaluating the inhibitory capacity of lactic acid bacteria isolated from fermented dairy products and the microbiota of individuals considered healthy. In these studies, the antimicrobial activity is tested by the agar diffusion method against reference strains or clinical isolates of urinary tract pathogens, mainly including enterobacteria, such as *E. coli*, *K. pneumoniae*, *P. mirabilis*, and other bacterial, including *P. aeruginosa*, *E. faecalis*, and *S. saprophyticus*. The species *Lactobacillus acidophilus*, *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*, and *Lacticaseibacillus rhamnosus* stand out for their broad spectrum of inhibition and their inhibitory capacity, in terms of the diameter of the inhibition zone, comparable to those obtained with the use of antimicrobials (Chapman, Gibson, Todd, & Rowland, 2013; Manzoor, Ul-Haq, Baig, Qazi, & Seratlic, 2016; Shim, Lee, & Lee, 2016). The inhibition exerted by *Lactobacillus* species is mainly due to the release of lactic acid resulting from the metabolism of carbohydrates, which leads to a decrease in pH, making the environment hostile to most pathogens. The antagonistic activity of lactic acid seems to act synergistically with H_2O_2, also released by several *Lactobacillus* species in an aerobic environment (Chapman et al., 2013). Despite numerous evidence of their probiotic potential, other genera of lactic acid bacteria such as *Lactococcus* and *Weissella* are studied for their capacity to produce bacteriocin-like substances with action against uropathogens (Ayeni et al., 2009).

Studies have shown the inhibitory effect of probiotics against bacteria that cause infections in children. Strains of lactic acid bacteria of the species *Levilactobacillus brevis*, *E. faecium*, and *Pediococcus acidilactici*, isolated from the feces of healthy children, were able to inhibit the growth of bacteria isolated from the urine samples of children hospitalized with UTIs. All potential probiotics were able to strongly inhibit the growth of *E. coli*, *S. saprophyticus*, *Citrobacter freundii*, *Proteus vulgaris*, *Enterobacter cloacae*, *P. aeruginosa*, and *Bacillus anthracis* (Lim, Lee, & Kim, 2009). The probiotic strain *E. coli* Nissle 1917 was able to antagonize the growth of uropathogenic bacteria isolated from the urine of children, such as *E. coli*, *Pseudomonas*, *Enterococcus*, *Klebsiella*, *Staphylococcus*, *Serratia*, and *Enterobacter*. These pathogens were found to possess virulence genes associated with kidney and bladder adhesion and immune system evasion. The ability to inhibit probiotic bacteria was attributed to the production of bacteriocins (Storm, Koff, Horvath, Li, & Justice, 2011).

Lactobacillus strains can inhibit even multiresistant bacteria. *L. acidophilus* ATCC3456, *Lacticaseibacillus casei* ATCC 39392, and *L. rhamnosus* ATCC 7469 were able to inhibit the growth of clinical isolates of uropathogenic *E. coli* strains resistant to cephalexin, ceftriaxone, ampicillin, cefotaxime, trimethoprim-sulfamethoxazole, tetracycline, nalidixic acid, ciprofloxacin, gentamicin, doxycycline, erythromycin, penicillin, and vancomycin. The largest halos of inhibition were observed when the *L. casei* supernatant was used (Naderi et al., 2014). Probiotics were able to inhibit the growth of extended-spectrum beta-lactamase-producing *E. coli* isolated from patients with UTIs. The supernatants of the strains *Lactobacillus gasseri* HLAB 414, *L. rhamnosus* HY7801, *L. acidophilus* SNUL, *L. plantarum* AK8-4, *Lacticaseibacillus paracasei* CH15-2, and *L. acidophilus* Antibio 300 showed inhibitory activity against the pathogen exhibiting halos that ranged between 10 and 20 mm in diameter. Antagonism against *P. vulgaris* and *E. faecalis* was also observed (Shim et al., 2016).

In addition to lactic acid bacteria, other microorganisms have shown antimicrobial activity against urinary tract bacterial pathogens. The bacterium *Staphylococcus succinus* AAS2, isolated from the fermented food Ngari, typical of northeastern India, inhibited the growth of the

uropathogens *E. coli*, *P. mirabilis*, *P. aeruginosa*, *S. aureus*, *K. pneumoniae*, and coagulase-negative *Staphylococcus*; although the inhibitory activity was greater against Gram-positive bacteria, indicating the possibility of the participation of bacteriocins in this action (Khusro, Aarti, Salem, Buendía Rodríguez, & Rivas-Cáceres, 2018). The bacterium of the actinomycetes group *Nocardia brasiliensis* PTCC 1422 could inhibit the growth of bacteria isolated from urine samples of patients who presented UTIs. Clinical isolates showed resistance to numerous tested antibiotics. The culture supernatant of the potentially probiotic tested strain could inhibit the growth of *E. coli*, *K. pneumoniae*, and *Serratia marcescens*. The antagonism was attributed to 17 secondary metabolites identified by gas chromatography (Jalali et al., 2016).

Among the antimicrobial substances produced by probiotic microorganisms, bacteriocins have received special attention due to their therapeutic potential against pathogenic bacteria, including those that present multi-resistance to antibiotics. Bacteriocins are a family of thermostable, ribosomal synthesized peptides or small proteins that generally exhibit strong and specific antimicrobial activity against other bacteria related to the producing strain. Bacteriocins produced by probiotics isolated from the vaginal environment have been identified and their action against uropathogens has been determined, such as the bioactive peptide DT24 produced by *L. brevis* DT24, which presents bacteriostatic action against the uropathogenic *E. coli* (Trivedi, Jena, Patel, & Seshadri, 2013). The bacteriocin produced by *L. fermentum* KT998657 was able to interfere with the biofilm formation by *P. aeruginosa*. The antibiofilm action was potentiated when the bacteriocin was tested together with the exopolysaccharide produced by the probiotic, showing the importance of bacterial cell products in reducing cell-cell and cell-surface interactions by pathogens (Sharma, Harjai, & Shukla, 2018).

The antagonism tests performed in vitro are critical in screening for microorganisms with the potential to prevent or treat infections caused by bacteria. Studies point to substances with vast antimicrobial bactericidal or bacteriostatic potential, and that interfere with biofilm formation. The diversity of compounds produced indicates a broad spectrum of action, including against pathogens resistant to many antibiotics. However, the lack of standardization of the studies, with the use of different strains and concentrations in each assay, makes the comparison of results practically impossible. Confirmation of the inhibitory action and its use as a therapeutic or prophylactic agent in patients with urinary infection depends on in vivo trials and controlled clinical studies.

Probiotics can act by preventing the binding of pathogens to receptors on the urothelium and vaginal epithelium, thus averting colonization and invasion. They can act preventively occupying sites that would eventually be taken by pathogenic bacteria, competing for available receptors, or turning off uropathogens that are already attached to the epithelium (Andreu, Stapleton, Fennell, Hillier, & Stamm, 1995; Osset, Bartolomé, García, & Andreu, 2001). In this sense, bladder epithelial cell lines have been frequently used in studies that seek to assess the non-adherent capacity of probiotics. *L. plantarum*, *L. fermentum*, *L. acidophilus*, *Ligilactobacillus salivarius*, and *L. rhamnosus* have stood out for their ability to inhibit the adhesion of bacterial pathogens in vitro (de Llano et al., 2017; Chapman, Gibson, & Rowland, 2014).

The ability to form biofilm is identified as a significant marker in the pathogenicity of uropathogenic bacteria due to its importance in the adhesion process. The strains *L. acidophilus* NCIMB 30184 (PXN 35), *L. fermentum* NCIMB 30226 (PXN 44), *L. plantarum* NCIMB 30187 (PXN 47), and *L. rhamnosus* NCIMB 30188 (PXN 54) can inhibit the formation of biofilm by *E. faecalis* NCTC 00775 and *E. coli* NCTC 9001, and also compete with these microorganisms

for binding sites in the bladder epithelium. Except for *L. rhamnosus*, cell-free supernatants of all other microorganisms tested alone could significantly inhibit biofilm formation by *E. faecalis*. The same result was observed for the mixture of *L. acidophilus*, *L. fermentum* and *L. rhamnosus*. In the assay with *E. coli*, all *Lactobacillus* mixtures resulted in significant inhibition of the biofilm, the same result was only achieved by the *L. rhamnosus* strain tested separately. The pH of the supernatants was associated with the interference effect on biofilm formation, as the inhibitory effect was not observed when the supernatant was neutralized. This highlights the role of organic acids produced by the microbiota in establishing an unfavorable environment for pathogens. All strains of *Lactobacillus* tested alone, or in combination, were able to significantly inhibit the binding of pathogens to human bladder cells (Chapman et al., 2014).

The antibiofilm activity was also observed in a study using the supernatant of the strains *Bacillus subtilis* KATRINA1933 and *Bacillus amyloliquefaciens* B-1895. Both bacteria induced a reduction of up to 80% in the biofilm formed by *P. mirabilis* isolates from patients with UTIs. Biofilm reduction was associated with the production of antimicrobial substances and the ability of probiotic bacteria to coaggregate with the pathogen (Algburi et al., 2020). Lactic bacteria isolated from kefir identified as *L. paracasei* GM12, *L. paracasei* MGH13, *L. rhamnosus* MG6, and *L. rhamnosus* MN125 could reduce the biofilm formed by uropathogenic *E. coli*. The isolates also showed antimicrobial activity not associated with the production of organic acids (Ghane, Babaeekhou, & Ketabi, 2020).

Lactobacillus strains can not only prevent adhesion but also inhibit pathogen invasion. The commercial strains *Lactobacillus johnsonii* NCC533 and NCC2917, *L. rhamnosus* NCC4007, and *L. acidophilus* NCC2463 were evaluated for their ability to prevent the binding and invasion of *E. coli* CFT073 and UT189 to the human bladder cell lineage UM-UC-3 (ATCC CRL-1749). The tested pathogens were isolated from patients with acute pyelonephritis and cystitis, respectively. NCC533, NCC2917, and NCC4007 prevented binding to UTI189. All probiotic strains tested prevented the invasion of UTI189 into bladder cells. The two strains of *L. johnsonii* were able to inhibit the binding of CFT03, but only NCC533 inhibited the invasion of the cells by the pathogen (Delley et al., 2015).

The strains *L. paracasei* PM2, *L. salivarius* PM78, *L. plantarum* PM206 and 229, *Lactobacillus crispatus* RY2, and *Pediococcus pentosaceus* PM68 and 201 also exhibited capacity to inhibit the adhesion and invasion of *Proteus mirabilis* BCRC 10725 to human uroepithelium SV-HUC cells -1. The strains of *L. paracasei*, *L. salivarius* and *L. crispatus* stood out for the ability, in exclusion and competition assays, to significantly inhibit bacterial adhesion and invasion (Lai, Lin, Hsieh, & Tsai, 2020).

In an in vivo study using mice, the *Lactobacillus murinus* LbO2 strain isolated from the vaginal tract of a healthy female, decreased the *P. mirabilis* count in the kidney and bladder of animals previously challenged in a prophylactic and therapeutic approach. This effect was associated with the ability of the lactic bacteria to interfere with the binding of the pathogen to the epithelial tissue. *Proteus mirabilis* is a bacterial pathogen that often causes urinary tract infections that result in complications, and many strains are already resistant to antibiotics such as ciprofloxacin, ceftazidime, imipenem, or aminoglycosides (Fraga, Scavone, & Zunino, 2005).

Probiotic microorganisms from different origins may have non-adhesive properties. *Lactobacillus salivarius* UCM572, isolated from the vaginal exudate of a healthy woman; *L. plantarum* CLC17, milk isolate with activity against uropathogens; and *L. acidophilus* 01, a

commercial strain indicated for UTIs, were evaluated for their capacity of interfering with the binding of uropathogens *E. coli* ATCC 53503, *E. coli* 10791, *E. faecalis* 04-1, *E. faecalis* 08-1, and *Staphylococcus epidermidis* 08-3 to the human bladder cell lineage T84. When tested for their ability to prevent adhesion, compete for binding sites, or turn off pathogens already attached to *L. salivarius* cells, those strains showed promise for presenting significant results against all pathogenic bacteria except for *E. coli* ATCC 5350, while *L. acidophilus* showed the best results in assays with Gram-positive bacteria (de Llano et al., 2017).

The antiadhesive activity of probiotics, specifically from the genus *Lactobacillus*, depends on the strain tested and the pathogen. The ability to adhere to cells of the urinary tract mucosa is a complex process that involves cell receptors from epithelial cells and the immune system, in addition to the interaction between pathogens and local microbiota. The varied results found in the diversity of available studies make it difficult to establish a standardization of prevention or treatment based on in vitro findings.

Parallel to the antiadhesive and antimicrobial activity, some studies have shown the ability of probiotics to interfere in the production of virulence factors. In this sense, metabolites produced by lactic acid bacteria have been identified as inhibitors of genes that code for virulence factors, such as those involved in pathogen adhesion and invasion (Vagios et al., 2020). The culture supernatant of *L. rhamnosus* GR-1 and *Limosilactobacillus reuteri* RC-14 bacteria could inhibit the expression of type 1 and P fimbriae by uropathogenic *E. coli*, which are critical factors in the process of adhesion to the urogenital tract. *L. rhamnosus* GR-1 also stimulated the inflammatory response in human urothelium T24 cells challenged with heat-inactivated *E. coli*. The presence of the probiotic significantly potentiated the immune response through the NF-KB route, increased the production of TNF and the TLR-4 receptor, involved in the recognition of the bacterial wall lipopolysaccharides (Karlsson, Scherbak, Reid, & Jass, 2012; Cadieux, Burton, Devillard, & Reid, 2009).

The great potential of probiotics as an alternative to the use of antibiotics highlights the need for a continuous search for new strains and for standardization that allows their rational use and of their products, therapeutically or prophylactically, in UTIs caused by bacteria, especially taking into account the emergence of pathogens with multi-resistance. In addition to antimicrobial substances, in vitro studies show promising activities of probiotics interfering with the expression of virulence factors and modulation of the immune system. Studies in this area should move towards the development of formulations and protocols involving them in conjunction with antibiotic therapy, defined by controlled clinical studies.

22.3 Clinical trials applied to the use of probiotics in bacterial urinary infections

The use of probiotic strains in the context of urinary tract infections appears in two main circumstances: prevention and treatment. In the latter, they appear as a treatment step, focused on the reestablishment of the resident microbiota (Akgül & Karakan, 2018). The clinical trials conducted for this type of evaluation present many variations regarding the strains used, the concentration of the formulations, the duration of administration, and the populations analyzed, limiting the results obtained (Sihra et al., 2018; Vagios et al., 2020; Hanson, Vandevusse, Jermé, Abad, & Safdar, 2016; Table 22.2).

TABLE 22.2 Evidence on the use of probiotics in bacterial urinary infections.

Reference	Sample	Type of intervention	Analyses	Results
Reid et al. (2003)	64 women, with no clinical history of UTI in the previous 12 months; average age of 35 years	Oral capsules of *Lacticaseibacillus rhamnosus* GR-1 and *Limosilactobacillus fermentum* RC-14 once a day, daily, for 60 days	Collection of vaginal swabs for total lactobacilli, yeast and coliform cultivation, Gram stain, and Nugent score assignment	Change from a bacterial vaginosis profile to one predominantly composed of lactobacilli
Morelli et al. (2004)	10 women, with no clinical history of UTI in the previous 12 months	Oral administration of lyophilized preparation of *Lacticaseibacillus rhamnosus* GR-1 and *Limosilactobacillus fermentum* RC-14 once a day, daily, for 14 days	Fecal and vaginal samples were collected on days 0, 7, and 14; and submitted to molecular identification of lactobacilli	Probiotic strains were identified in fecal samples and vaginal swabs. And, compared to the collected sample, there was an increase in the lactobacilli colonization
Lee et al. (2007)	120 children with persistent primary vesicoureteral reflux after 1 year of antibiotic prophylaxis	Intake of *Lactobacillus acidophilus* ATCC 4356 formulation (1 × 108 CFU/g) twice a day or trimethoprim-sulfamethoxazole (2/10 mg/kg) once a day	UTI recurrence rate and identification of the microorganisms involved in these infections	There was no statistical difference in the incidence of UTI between groups. The three main bacteria involved in these infections, in both groups, were: *E. coli*, *K. pneumoniae*, and *Enterobacter cloacae*
Stapleton et al. (2011)	100 women (18-40 years) with a clinical history of episodes of UTI treated with antibiotics in the last 12 months	Vaginal suppositories containing *Lactobacillus crispatus* CTV-05 (108 CFU/mL, Lactin-V) once a day and then once a week for 10 weeks	Number of urinary infection episodes and identification of *L. crispatus* in vaginal swabs	Reduction in the number of UTI episodes and high level of colonization by *L. crispatus* in the vaginal microenvironment
Beerepoot et al. (2012)	252 postmenopausal women with a history of urinary infections	Oral capsules of *Lacticaseibacillus rhamnosus* GR-1 and *Limosilactobacillus fermentum* RC-14 twice a day or trimethoprim-sulfamethoxazole (480 mg) once a day for 12 months	Number of UTI episodes and bacterial resistance development in *Escherichia coli*	Both types of prophylaxis reduced the occurrence of UTIs over 12 months. Bacterial resistance was only reported in the group that took antibiotic prophylaxis
Mezzasalma et al. (2017)	60 premenopausal women	14-day oral administration of *L. acidophilus* and *Limosilactobacillus reuteri* or *Lactiplantibacillus plantarum*, *Lacticaseibacillus rhamnosus* and *Bifidobacterium animalis* subsp. *lactis* or placebo	Analysis of the presence of probiotic strains in vaginal swabs	The probiotic strains of the formulations were identified in the vaginal swabs after oral administration
Olsen et al. (2018)	34 pregnant women (36th week)	Daily oral dose of *Lacticaseibacillus rhamnosus* GR-1 and *Limosilactobacillus fermentum* RC-14 for 3 weeks or until the baby is born	Vaginal swabs collected after 21 days and tested for the presence of Group B Streptococcal bacteria (GBS).	There was no difference in GBS colonization rates between the two. The probiotic increased rates of vaginal commensals

The diversity of strains used in clinical trials also makes comparisons and the establishment of more specific recommendations difficult because the properties of a certain strain cannot be attributed to another one, even if they are of the same species (Mezzasalma et al., 2017). Despite these considerations, well-characterized probiotic strains, such as *L. rhamnosus* GR-1 and *L. fermentum* RC-14, have shown significant results in the studies in which they were involved (Sihra et al., 2018; Reid et al., 2003; Morelli, Zonenenschain, Del Piano, & Cognein, 2004; Beerepoot et al., 2012).

In a clinical trial conducted with 64 women considered healthy (mean age of 35 years), the daily intake of capsules of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 restored the characteristic vaginal microbiota; from bacterial vaginosis to a microbiota composed predominantly of lactobacilli; in 37% of the women who were in the group that received the capsules (Reid et al., 2003). Similar results were obtained when analyzing the vaginal microbiota of 10 healthy women, without clinical history of recent urinary infections, who used capsules of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 for 14 days (Morelli et al., 2004).

When comparing the effect of traditional prophylaxis (trimethoprim-sulfamethoxazole, 480 mg) and alternative prophylaxis (capsules containing *L. rhamnosus* GR-1 and *L. fermentum* RC-14) on the occurrence of urinary infections in 252 postmenopausal women with episodes of recurrent infections, both types of prophylaxis were found to be effective in decreasing the recurrence of infections. However, traditional prophylaxis increased *E. coli* resistance from 20 to 40% to 80 to 95%. While in the group that used prophylaxis, no cases of bacterial resistance were reported (Beerepoot et al., 2012).

Unlike the clinical trials reported above, oral administration of *L. rhamnosus* GR-1 and *L. fermentum* RC-14 to 21 pregnant women (36 weeks) did not result in decreased vaginal colonization by Group B streptococcal bacteria after 21 days. But, after this period, there was an increase in the rate of commensals in the vaginal microenvironment (Olsen, Williamson, Traynor, & Georgiou, 2018).

Differences between oral and vaginal routes of administration have not significantly influenced the ability of probiotic strains to colonize and re-establish the vaginal microbiota (Sihra et al., 2018; Morelli et al., 2004). Vaginal administration is efficient in restoring the microbiota and maintaining it healthy even after treatment is discontinued. Additionally, it has the main advantage of conferring the provision of local and targeted action (Homayouni et al., 2014; Buggio, Somigliana, Borghi, & Vercellini, 2019; Cribby, Taylor, & Reid, 2008). The use of a vaginal suppository containing *L. crispatus* CTV-05 significantly reduced the recurrence of urinary infections and increased the level of colonization with *L. crispatus* CTV-05 in the vaginal microenvironment in phase 2 clinical screening with 100 premenopausal women with a history of recurrent infections (Stapleton et al., 2011).

When taken orally, the probiotic strain must withstand all adverse conditions of the gastrointestinal tract before reaching the urogenital one. Well-characterized probiotic strains administered orally can ascend to the urogenital tract, even in small numbers, and promote the reestablishment of the vaginal microbiota (Sihra et al., 2018; Morelli et al., 2004; Homayouni et al., 2014; Buggio et al., 2019). A recent clinical trial conducted in 60 premenopausal women reported the presence of *L. reuteri*, *L. rhamnosus*, *L. acidophilus*, *L. plantarum* and *Bifidobacterium animalis* subsp. *lactis* in vaginal swabs, after oral administration of a formulation containing these bacteria (Mezzasalma et al., 2017). In addition to the vaginal microbiota, oral administration can also contribute to the restoration of the intestinal microbiota during

the course, which would reduce the transfer of uropathogens from the rectum to the vagina. However, there is still no correlation between the level of fecal recovery of orally administered strains and the rate of vaginal colonization (Sihra et al., 2018; Morelli et al., 2004; Cribby et al., 2008).

Another aspect that must be observed in clinical trials is the homogeneity of the studied population. The composition of the microbiome can be influenced by ethnicity, factors related to behavior, hormonal levels, sociodemographic and socioeconomic factors. The microbiome evaluation of 1268 healthy African American women and 416 women of European ancestry demonstrated the predominance of three microbiome profiles in African American women: *G. vaginalis*, vaginosis-associated bacteria-1, and *Lactobacillus inner*. The latter is known for its inefficiency in preventing the growth and colonization of other species, making these women more likely to have dysbiosis in the vaginal environment and, consequently, more prone to colonization by uropathogens (Fettweis et al., 2014; Witkin et al., 2013).

In a similar study conducted recently in Brazil, it was found that about 36.5% of the population analyzed (609 women of childbearing age) had a microbiome composed predominantly of *Lactobacillus inner*. When these results were observed by region, it was possible to notice slight differences in the microbiome profiles, with a predominance of depletion of *Lactobacillus* in the North, Northeast, Midwest, and Southeast regions and a profile characterized by *L. crispatus* and *L. gasseri* in the South region (Marconi et al., 2020). Differences in the composition of the vaginal microbiome influence the development of urinary infections and indicate the most suitable probiotic strain and route of administration, influencing the impact and safety of the outcome.

There are not many clinical trials evaluating the effectiveness of preventing and treating UTIs in men and people with urinary tract disorders, such as the neurogenic bladder. In men, urinary infections are considered complicated UTIs, due to prostate involvement and neurological implications. Generally, the studies conducted with these groups mentioned have a very small population, which makes the reliability of the obtained results difficult (Wagenlehner, Weidner, Pilatz, & Naber, 2014; Lee, Shim, Cho, & Lee, 2007; Toh, Boswell-Ruys, Lee, Simpson, & Clezy, 2017).

The applicability of probiotic strains in preventing urinary tract infections is promising. However, for the development of clinical practice, clinical trials need to exhibit a standardized administration scheme and more homogeneous population characteristics in terms of age, ethnicity, population size, among other variables. In this way, it would be possible to establish specific medical recommendations, taking into account both the probiotic strain and its properties and the target group of the treatment.

22.4 Probiotic in the treatment and prevention of fungal diseases of the urinary tract

Urinary infections of fungal etiology are not common in immunocompetent patients. They are more common in diabetic patients and/or in those with some structural abnormality of the urinary tract (Sheerin, 2011).

According to numerous studies, the yeast of the genus *Candida* is the most prevalent fungus associated with urinary tract infections (UTIs) (Behzadi, Behzadi, & Ranjbar, 2015;

TABLE 22.3 Virulence factors of *Candida* sp. and their role in the pathogenesis.

Virulence factor	Function	References
Phenotypic change	*C. albicans* (and some strains of *C. dubliniensis*) can change form from yeast to pseudohyphae (germ tube), increasing adhesion and invasiveness to host tissues	Staniszewska (2020), Mayer et al. (2013)
Aspartyl proteases	Enzymes that degrade structural proteins associated with immune defenses, such as collagen, keratin, mucin, antibodies, complement, and cytokines	Fisher et al. (2011) Mushi et al. (2019)
Phospholipases	Enzymes that hydrolyze phospholipids, major components of mammalian cell membranes	Fisher et al. (2011), Mushi et al. (2019)
Hemolysins	Enzymes that induce the breakdown of red blood cells, releasing hemoglobin, a rich source of iron	Fisher et al. (2011), Mushi et al. (2019)
Biofilm production	Mediated by nonspecific mechanisms, such as hydrophobicity, and specific ones, such as adhesins. Allows yeast to adhere to host epithelium as well as inert surfaces	Staniszewska (2020), Mayer et al. (2013)
Tigmotropism and galvanotropism	The germ tubes of *C. albicans* respond to electric fields (galvanotropism) and changes in the substrate topography (thymotropism), reorienting their growth axis according to local calcium concentrations	Fisher et al. (2011) Mayer et al. (2013)

Helbig et al., 2013; Odabasi & Mert, 2020). The species that stands out is *Candida albicans*, an opportunistic pathogen strongly associated with nosocomial urinary tract infections. Other relevant species include *Candida glabrata*, *Candida tropicalis*, and *Candida parapsilosis* (Alfouzan & Dhar, 2017).

Candiduria is a condition rarely present in healthy individuals. However, it is a common finding in hospitalized patients, particularly those in intensive care units, who often have diabetes, long-term urinary catheters, and are exposed to antimicrobials for long periods. Other factors that may be associated with candiduria and *Candida* urinary tract infections include older age, female gender, bladder dysfunction, urinary stasis, nephrolithiasis, and kidney transplantation (Sobel, Fisher, Kauffman, & Newman, 2011).

The *Candida* species express numerous virulence factors that facilitate colonization and spread in host tissues. Among these virulence factors are phenotypic change, secretion of hydrolytic enzymes (aspartyl proteases, phospholipases, and hemolysins), biofilm production, galvanotropism, and thigmotropism (Fisher, Kavanagh, Sobel, Kauffman, & Newman, 2011). The role of each virulence factor in the pathogenesis of yeasts of the genus *Candida* is shown in Table 22.3.

22.4.1 Probiotic bacteria with anti-*Candida* potential

Studies evaluating the probiotic potential of bacteria against the *Candida* species employ mainly lactobacilli and, to a lesser extent, bifidobacteria and *Streptococcus strains* (Matsubara, Bandara, Mayer, & Samaranayake, 2016; Table 22.4).

The antimicrobial activity of bacteria with probiotic potential is a well-known property. In vitro and in vivo studies demonstrate the antimicrobial activity of lactobacilli and

TABLE 22.4 Probiotic bacteria with anti-*Candida* potential and their mechanisms of action.

Probiotic bacteria	Inhibited microorganisms	Mechanism(s) of action
Limosilactobacillus reuteri	*C. albicans; C. dubliniensis; C. glabrata; C. tropicalis*	Production of organic acids and H2O2 (Jørgensena et al., 2017)
Lacticaseibacillus rhamnosus	*C. albicans*	Filament reduction (Rossoni et al., 2018)
		Production of biosurfactants (Santos et al., 2019)
		Inhibition of biofilm formation (Santos et al., 2019)
		Inhibition of the expression of hydrolytic enzymes (Dube et al., 2020; Oliveira et al., 2016)
Lacticaseibacillus paracasei	*C. albicans*	Reduction to filament (de Barros et al., 2018; Wang et al., 2017; Rossoni et al., 2018)
		Production of biosurfactants (Santos et al., 2019)
		Inhibition of biofilm formation (Santos et al., 2019)
Limosilactobacillus fermentum	*C. albicans*	Filament reduction (Rossoni et al., 2018)
Lactobacillus crispatus	*C. albicans*	Filament reduction (Wang et al., 2017)
Lactobacillus gasseri	*C. albicans*	Filament reduction (Wang et al., 2017)
		Production of biosurfactants (Morais et al., 2017)
		Inhibition of biofilm formation (Morais et al., 2017; Matsuda et al., 2018)
Lactobacillus jensenii	*C. albicans*	Filament reduction (Wang et al., 2017)
		Production of biosurfactants (Morais et al., 2017)
		Inhibition of biofilm formation (Matsuda et al., 2018; Morais et al., 2017)
Lactobacillus acidophilus	*C. albicans*	Filament reduction (Wang et al., 2017)
		Biosurfactant production (Santos et al., 2019)
		Inhibition of biofilm formation (Santos et al., 2019)
Lactobacillus pentosus	*C. albicans; C. krusei; C. tropicalis*	Filament reduction (Wang et al., 2017)
		Inhibition of biofilm formation (Aarti et al., 2018)
Bifidobacterium longum	*C. albicans, C. krusei, C. glabrata, C. parapsilosis; C. tropicalis*	Production of organic acids (Inturri et al., 2019)
Bifidobacterium adolescentis	*C. albicans*	Inhibition of biofilm formation (Lakhtin et al., 2010)
Bifidobacterium bifidum	*C. albicans*	Inhibition of biofilm formation (Lakhtin et al., 2010)
Bifidobacterium gallinarum	*C. albicans*	Inhibition of biofilm formation (Lakhtin et al., 2010)
Streptococcus thermophilus	*C. albicans*	Filament reduction (Azad et al., 2021)
		Inhibition of biofilm formation (Azad et al., 2021)
Streptococcus salivarus	*C. albicans*	Filament reduction (James et al., 2016)
		Inhibition of biofilm formation (James et al., 2016)
		Inhibition of the expression of hydrolytic enzymes (James et al., 2016)

bifidobacteria against Gram-positive, Gram-negative bacterial pathogens and fungi, including *Candida* sp. (Coman et al., 2014; Prabhurajeshwar & Chandrakanth, 2017; Niţoi, Matei, & Cornea, 2018). One of the main mechanisms responsible for *Candida* inhibition by lactobacilli is the production of secondary metabolites, such as hydrogen peroxide, lactic acid, and bacteriocin-like compounds (Wang et al., 2017). The production of hydrogen peroxide by lactobacilli is a relevant factor in inhibiting *C. albicans*, but not other species, such as *Candida krusei* and *C. parapsilosis*. This is due to the presence of an oxidase that is part of an alternative oxidative pathway in non-*albicans Candida* strains (Jørgensena, Kragelunda, Jensenb, Kellera, & Twetmana, 2017; Costa-de-Oliveira et al., 2012).

Other mechanisms associated with the control of *Candida* sp. by lactobacilli with probiotic potential include (1) inhibition of germ tube formation; (2) reduced adhesion/inhibition of biofilm formation and (3) decreased/inhibited enzymatic activity.

The transition from yeast to hyphae in *C. albicans* can be triggered by several environmental signals in vitro, including neutral pH, presence of serum or N-acetyl glucosamine, elevated concentration of carbon dioxide, and a temperature of 37°C (Wang et al., 2017). This morphological change increases invasiveness to host tissues and plays such an important role in the pathogenesis of *C. albicans* that genetically modified strains are avirulent so that they can only grow as yeasts (Staniszewska, 2020; Mayer, Wilson, & Hube, 2013; Sudbery, 2001).

After incubation of clinical isolates of *L. rhamnosus*, *L. paracasei*, and *L. fermentum* with *C. albicans* in an experimental model of *Galleria mellonella*, a reduction of almost 60% of yeast filamentation was observed when compared to the control group (Rossoni et al., 2018). Culture supernatants of vaginal isolates of *L. crispatus*, *L. gasseri*, and *Lactobacillus jensenii* were able to inhibit, in vitro, the growth and hyphae formation of *C. albicans*, in addition, *L. crispatus* could inhibit the expression of the following related genes hyphal formation: hyphal wall protein 1 gene (HWP1), agglutinin-like protein gene 3 (ALS3), and extent of cell elongation gene 1 (ECE1) (Wang et al., 2017). Similar results were obtained after incubation of an oral isolate of *L. paracasei* with *C. albicans* in an experimental model of *Caenorhabditis elegans*. The reduction in hyphal formation was associated with down-regulation of the UME6 and TEC1 genes, transcription factors that regulate this process (de Barros et al., 2018).

The adhesion of *C. albicans* to the host epithelium is the first stage of the infection, being mediated by nonspecific mechanisms, such as hydrophobicity and biosurfactants; and specific ones, such as adhesins (Staniszewska, 2020; Mayer et al., 2013).

Lactobacilli with probiotic potential can produce biosurfactants reducing the adhesion of *C. albicans* to the host epithelium. Vaginal isolates of *L. jensenii* and *L. gasseri* can reduce, in vitro, the biofilm formation of *C. albicans*, *C. tropicalis* and *C. parapsilosis* between 25% and 35% through the production of biosurfactants (Morais et al., 2017). Similar results were obtained by using standard strains of *L. rhamnosus* and *L. acidophilus*, and a vaginal strain of *L. paracasei*. Biosurfactant molecules produced by these lactobacilli were able to decrease the adhesion of *C. albicans* and interrupt biofilm formation in vitro (Santos et al., 2019). The culture supernatant of the *Lactobacillus pentosus* LAP1 strain, isolated from the oral cavity, was able to reduce, in vitro, the biofilm formation of *C. albicans*, *C. krusei*, and *Candida tropicalis* due to the secretion of bioactive components that inhibited filamentation (Aarti et al., 2018). Similarly, the culture supernatant from *L. gasseri* and *L. crispatus* could suppress biofilm formation of *C. albicans*, as well as fungus adhesion to HeLa cells. The mechanism involved in the inhibition was the

downregulation of the following genes: HWP1 and ECE1 (hypha-specific genes), ALS3 (adhesion-related gene), BCR1 (biofilm-related gene), and EFG1, TEC1, and CPH1 (hypha-related transcription factors) (Matsuda, Cho, Sugita, Ogishima, & Takeda, 2018).

The main hydrolytic enzymes produced by *Candida* sp. are aspartyl proteases, phospholipases, and hemolysins. The first ones are capable of degrading structural proteins associated with immune defenses, such as collagen, keratin, mucin, antibodies, complement, and cytokines. Phospholipases are enzymes that hydrolyze phospholipids, which are the main components of mammalian cell membranes. Hemolysins induce the breakdown of red blood cells, releasing hemoglobin, a rich source of iron (Fisher et al., 2011; Mushi, Bader, Bii, Groß, & Mshana, 2019).

Some strains of lactobacilli are capable of decreasing or suppressing the expression of hydrolytic enzymes by *Candida*. Inhibitory and sub-inhibitory concentrations of the culture supernatant of *L. rhamnosus* were able to significantly inhibit the production of proteinases and phospholipases by *C. albicans* in vitro, in addition to changing the antifungal sensitivity profile, reversing the resistance to some drugs (Dube, Khan, Marimani, & Ahmad, 2020). By incubating the entire *L. rhamnosus* cell with *C. albicans* for 48 h, similar results are achieved, with a decrease in the expression of hydrolytic enzymes (proteases, phospholipases and hemolysins) and a change in the antifungal sensitivity profile (Oliveira, Santos, Silva, Jorge, & Leão, 2016). Strains of bifidobacteria and streptococci, although less used than lactobacilli in the treatment and prevention of fungal diseases of the urinary tract, also exhibit desirable probiotic characteristics, with potential anti-*Candida* activity.

Culture supernatants of *Bifidobacterium longum* associated with *L. rhamnosus* showed inhibitory activity against *C. albicans*, *C. krusei*, *C. glabrata*, *C. parapsilosis*, and *C. tropicalis*. The inhibitory process is associated with the release of metabolites with antimicrobial potential, such as organic acids (Inturri, Trovato, Volti, Oliveri, & Blandino, 2019). Lectins produced by *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, and *Bifidobacterium gallinarum* were able to destroy the biofilm formed by *C. albicans* in vitro (Lakhtin et al., 2010). The oral administration of probiotics containing *B. longum* and *B. bifidum* could reduce candiduria in pediatric patients, who use broad-spectrum antibiotics, as well as reduce colonization of the gastrointestinal tract by *C. albicans* (Kumar, Bansal, Chakrabarti, & Singhi, 2013).

A standard strain of *Streptococcus thermophilus* was able to inhibit the growth, transition, and biofilm formation of *C. albicans* in vitro and in vivo (Azad et al., 2021). Both the intact cell and the supernatant of the *Streptococcus salivarus* culture could inhibit growth and biofilm formation by *C. albicans*. This is related to the suppression of genes involved in the transition from yeast to hypha, and in the production of hydrolytic enzymes, such as ALS3 (adhesin/invasin), EFG1 (hyphae-specific gene activator), SAP5 (secreted protease), and HWP1 (hyphal wall protein critical to biofilm formation; James, MacDonald, Chanyi, Cadieux, & Burton, 2016).

22.4.2 Probiotic yeasts with anti-*Candida* potential

The only yeast available for probiotic use in humans is the *Saccharomyces boulardii*. Its probiotic potential against *Candida* sp. is well documented. *S. boulardii* can inhibit the growth, adhesion, and filamentation of *C. albicans*, *C. krusei*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, and *C. auris* in vitro, *ex vivo* and in vivo. The inhibition of these microorganisms is strongly associated with the release of small bioactive molecules and, in *C. albicans*, with the negative modulation of

TABLE 22.5 Probiotic yeasts with anti-*Candida* potential and their mechanisms of action.

Probiotic yeast	Inhibited microorganisms	Mechanism(s) of action
Saccharomyces boulardii	*C. albicans, C. krusei, C. tropicalis, C. parapsilosis, C. glabrata; C. auris*	Filament reduction (Kunyeit et al., 2020; Murzyn et al., 2010a, 2010b)
		Inhibition of biofilm formation (Fettweis et al., 2014; Marconi et al., 2020; Witkin et al., 2013)
Saccharomyces cerevisiae	*C. albicans, C. krusei, C. tropicalis, C. parapsilosis, C. glabrata*	Filament reduction (Kunyeit et al., 2019, 2020)
		Inhibition of biofilm formation (Kunyeit et al., 2019, 2020)
Issatchenkia ocidentalis	*C. albicans, C. tropicalis, C. krusei, C. parapsilosis and C. auris*	Filament reduction (Kunyeit et al., 2019, 2020)
		Inhibition of biofilm formation (Kunyeit et al., 2019, 2020)

genes involved in the transition from yeast to hypha (Niţoi et al., 2018; Kunyeit, Anu-Appaiah, & Rao, 2020; Murzyn et al., 2010; Murzyn, Krasowska, Stefanowicz, Dziadkowiec, & Łukaszewicz, 2010). Although *S. boulardii* is the only yeast available for probiotic use in humans, other yeasts have inhibitory properties on *Candida* sp., such as *Saccharomyces cerevisiae* and *Issatchenkia westernis* (Kunyeit et al., 2020; Table 22.5).

S. cerevisiae can inhibit adhesion, colonization, biofilm formation, and filamentation of *C. albicans* and non-*albicans*, in vitro and in vivo, including in animal models. Inhibition of host cell adhesion is mediated by probiotic-pathogen coaggregation and inhibition of biofilm formation, and the filamentation occurs through the secretion of bioactive molecules capable of negatively modulating the expression of genes associated with virulence (Kunyeit, Kurrey, Anu-Appaiah, & Rao, 2019; Kunyeit et al., 2020).

In a very similar way, *I. occidentalis* can inhibit adhesion, colonization, biofilm formation, and filamentation of *C. albicans*, *C. tropicalis*, *C. krusei*, *C. parapsilosis*, and *C. auris*, despite its mechanism of action is not fully elucidated, being associated with the release of bioactive metabolites (Kunyeit et al., 2019, 2020).

22.5 Conclusions

The UTI has been defined as a clinical situation in which microorganisms are identified in the urine in quantities greater than 105 colony-forming units per milliliter. Such infection can occur in sites of the upper and lower urinary tract, presenting itself as an asymptomatic infection or with mild, moderate, and even severe symptoms and death. Molecular biology findings bring a new paradigm in the definition of UTI, since the urinary tract, previously considered a sterile body niche, has an associated microbial community (microbiome). Identification and comparison studies of the microbiome in populations of healthy individuals and patients with uncomplicated, complicated, and recurrent UTIs aim to understand the role of these resident microorganisms and the pathogenesis of these infections. Among the microorganisms most commonly associated with UTIs are the bacteria *E. coli*, *S. aureus*, *P. aeruginosa*, *C. trachomatis*, *N. gonorrhoeae*, *K. pneumoniae*, *P. mirabilis*, *E. faecalis*, and the fungus of the genus *Candida*.

Greater susceptibility to UTIs seems to be associated with factors such as gender, as women are more susceptible than men; exposure to invasive procedures, such as the use of catheters; comorbidities that can compromise the immune system; and hormonal factors that impact homeostasis of the urinary tract, like pregnancy and menopause. Studies in the vaginal ecosystem, describing the relationship between an imbalance in the normal microbial population and the development of infections, have demonstrated the fundamental role of the microbiota in defending against pathogens. Such defenses involve mechanisms of competition for adhesion sites, synthesis of antagonist substances, and production of substances that inhibit biofilm formation by bacteria associated with vaginosis, and also activation and modulation of immune mechanisms in the host. In this context, probiotics (live bacteria that can be administered in association with the traditional treatment, with antimicrobials) have emerged, and mimic the supposed defenses promoted by the normal microbiome.

The genus *Lactobacillus* constitutes the main group of microorganisms with desirable probiotic properties in the control of UTIs associated with bacteria and also *Candida*. However, recent studies using *Staphylococcus succinus*, *Nocardia brasiliensis*, and *Escherichia coli* strains have also demonstrated the capacity of these species to act as probiotics in bacterial UTIs. The genus *Bifidobacterium*, mainly the species *Bifidobacterium longum* and yeasts of the species *Saccharomyces cerevisiae* and *Issatchenkia westernis*, have shown a relevant role as a probiotic in UTIs associated with *Candida*. Although the pharmaceutical market already sells the yeast *Saccharomyces boulardii* as a probiotic for human use, little is known about the pathophysiological mechanisms involved in UTIs. Further studies are needed to elucidate such mechanisms and to indicate, more precisely and effectively, the treatment with probiotics for this clinical situation.

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CHAPTER

23

The role of probiotics in people with cancer

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23.1 Introduction

In 2018, 17 million new cases of cancer were diagnosed worldwide and it is estimated that cancer incidence rates will increase by 62% by 2040 (CRUK, 2018). The most common cancers diagnosed worldwide are lung, breast, bowel or prostate cancers and these make up to 40% of new diagnoses yearly (CRUK, 2018).

People diagnosed with cancers may receive a range of therapies, including surgery, chemotherapy, radiotherapy, and immunotherapies. Because of this, they can also develop complications from treatments received, which may also require intervention. Management can involve preventive strategies (e.g., the delivery of co-trimoxazole in immunosuppressed patients diagnosed with ALL to prevent *Pneumocystis jiroveci*), interventional strategies (e.g., the use of platelets for a patient who has developed thrombocytopenia as a direct consequence of bone marrow suppression caused by chemotherapy), or treatment for long-term effects (for example, psychological therapy in a young adult who received cancer treatment as a child).

Curative strategies and supportive care implemented have resulted in improved survival (Steliarova-Foucher et al., 2017). Further improvements may come from focusing on why people with cancer die, understanding which deaths are due to a failure of the anti-cancer therapy, and which are from the toxicities of therapy. Cancers and cancer interventions are known to affect the gastro-intestinal microbiome, cause dysbiosis and significant gastrointestinal symptoms (known as mucositis), and result in gastrointestinal associated infections. Therefore, the use of probiotics has been investigated as a tool for cancer prevention and used as a supportive intervention for patients diagnosed with cancer.

23.2 The role of probiotics in cancer prevention and therapy

Probiotics, according to the World Health Organization and United Nations Food and Agriculture Organization (FAO), are defined as "live micro-organisms, which, when administered

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in adequate amounts, confer a health benefit on the host" (Schlundt, 2012). The most common strains used in probiotics belong to the genera *Lactococcus* and *Bifidobacterium* (Holzapfel, Haberer, Geisen, Bjorkroth, & Schillinger, 2001). Health benefits attributed to probiotics include improved gastrointestinal flora, reduction in serum cholesterol, reduced incidence of irritable bowel diarrheas, and prevention of cancer (Nagpal et al., 2012).

Interest of the use of probiotics for cancer prevention and cancer therapy has gained interest in recent decades. The association between a diet with *Lactobacillus* and reduced incidence of colon cancer was first demonstrated in rats in in the 1980s (Goldin & Gorbach, 1980). It is believed that probiotic bacteria can contribute to the regulation of homeostasis by modulation of the pH of bile acids, which can impact the colon epithelium (Bernstein, Bernstein, Payne, Dvorakova, & Garewal, 2005), bacterial enzymes involved in tumor genesis (Kim & Jin, 2001) and production of short-chain fatty acids, which is known to act as signaling molecules for the immune system and cell cycle (Commane, Hughes, Shortt, & Rowland, 2005).

The use of probiotics in conjunction with conventional cancer therapy has also been investigated. Research has demonstrated how certain cancer therapies can cause dysbiosis, which may impact the efficacy of chemotherapy and immunotherapy (Routy et al., 2018). The state of the microbiome before, during and following a cancer diagnosis can be linked to the development of toxicity (i.e., infection) and response to interventions. Dysbiosis may affect the health of an individual at different stages of a cancer patient's journey, that is, before diagnosis, during treatment, and end of treatment.

Investigations have also proposed how the gut microbiome can affect drug metabolism, efficacy and toxicity, as well as how an individual responds to chemotherapy in people with cancer (Pouncey, Scott, Alexander, Marchesi, & Kinross, 2018). During treatment for cancer the gut microbiome can be affected by a range of factors. Examples include includes diet, surgical intervention, supportive care interventions, that is, antibiotics and chemotherapy. Research by Panebianco et al. (Panebianco, Andriulli, & Pazienza, 2018) proposed that in mice bacterial translocation and T-helper 17 cell activation increases the efficacy of cyclophosphamide in a "healthy" gut microbiota when compared to mice raised in a sterile environment. Mice with a reduction of Gram-positive bacteria due to vancomycin (an antibiotic) were found to have a reduced therapeutic response to cyclophosphamide when compared to the control group. *Lactobacillus johnsonian*, *Lactobacillus murinus*, and *Enterococcus hirae* were found to stimulate an Th1 and Th17 immune response in the spleen and mesenteric lymph nodes thereby improving the efficacy of cyclophosphamide. However, the authors were unable to demonstrate this response in mice that received vancomycin (Viaud et al., 2013).

23.3 Gastrointestinal symptoms and infection

Gastrointestinal symptoms can occur in cancer patients receiving treatments. Examples include the development of diarrhea, ulceration and inflammation of the gastrointestinal system (also known as mucositis), and gastrointestinal associated bacteremia. Chemotherapy and radiotherapy-induced diarrhea is typically reported as an adverse event and is associated with fluorouracil, capecitabine and irinotecan-based chemotherapy cancer regimes. It is estimated that up to 45% of all chemotherapy patients experience severe diarrhea (Arbuckle,

Huber, & Zacker, 2000). Radiotherapy is believed to potentially alter bacterial flora and affect the intestinal motility and vascular permeability of mucosal cells (Salminen, Elomaa, Minkkinen, Vapaatalo, & Salminen, 1988). Chemotherapy is thought to alter the composition of intestinal flora and therefore affect the metabolism of intestinal enzymes vital for gut integrity. Changes to the gut flora may impact the gut defense barrier, immune function, and absorption of vital nutrients (Guarner & Malagelada, 2003). Radiotherapy or chemotherapy-induced diarrhea may interrupt or even stop treatment, impair the quality of life and prolong hospital stay of patients with cancer, potentially increasing health economic burdens too (Dranitsaris, Maroun, & Shah, 2005).

23.4 Mucositis

Mucositis is the inflammation and ulceration of the gastrointestinal mucosal lining, which can occur in people receiving cancer therapies. It may be caused by radiotherapy or cytotoxic agents that affect DNA synthesis (particularly S-phase specific agents), including cytarabine, methotrexate, and fluorouracil (Rubenstein et al., 2004).

Mucositis can occur in any part of gastrointestinal system from the mouth to the anus. Symptoms can range from mild erythema of the gastrointestinal lining to widespread ulceration. Development of mucositis can therefore result in pain, nausea, malabsorption, malnutrition, diarrhea, and increased risk of local and systemic infections (Blijlevens et al., 2008).

The Sonis hypothesis proposes 5 stages of the pathogenesis of mucositis:

1. Radiotherapy or cytotoxic exposure to the mucosal lining resulting in DNA damage and release of free radicals;
2. Activation of transcription factors including NF-kb, which results in the upregulation of pro-inflammatory cytokines causing mucosal destruction;
3. Signal amplification which may exacerbate or prolong mucosal injury;
4. Ulceration; and
5. Healing and gradual restoration of the flora (Kwon, 2016).

The relationship between mucositis and febrile neutropenia (a frequent complication for cancer patients) is recognized (Blijlevens et al., 2008). Patients with mucositis are believed to be most vulnerable to bacterial translocation from the gastrointestinal tract during stage 4 of the Sonis hypothesis, following damage to the epithelial lining of the mucosa, and inflammatory amplification (van Vliet, Harmsen, de Bont, & Tissing, 2010).

A study of adult patients diagnosed with multiple myeloma or non-Hodgkin's lymphoma demonstrated that there was a higher incidence of fever with severe mucositis when compared to those with less severe or no mucositis (68% vs. 47%, difference 21%, $p = 0.004$) (Blijlevens et al., 2008). Currently, the management of mucositis involves supportive care strategies. This may include the use of analgesia, loperamide to reduce diarrhea, and delivery of nutrition using both enteral and parental routes. There is no widespread preventative or therapeutic intervention for mucositis and febrile mucositis. However, studies have therefore explored the role of probiotics as a preventative and therapeutic intervention for people with cancer who experience mucositis.

23.5 Evidence

Several systematic reviews that have investigated the role of probiotics in cancer patients receiving chemotherapy, radiotherapy and surgery have been undertaken. A systematic search was undertaken for this chapter in 2021. Twenty systematic reviews were identified. However, 2 were excluded following full text screening because the reports were literature reviews. Included studies are summarized in Table 23.1.

The first published systematic review and meta-analysis investigating the efficacy and safety of the use of probiotics in people with cancer by Redman et al. were published in 2014 (Redman, Ward, & Phillips, 2014). It proposed that probiotics may reduce the severity and frequency of diarrhea in patients with cancer following the review of 11 randomized-controlled trials (RCTs). A meta-analysis of 4 RCTs found that participants receiving probiotics, when compared to the control group, showed a significant reduction in frequency of CTC grade ≥2 diarrhea (odds ratio [OR] 0.32, 95% confidence interval [CI] 0.13–0.79), and possibly grade ≥3 diarrhea (OR 0.72, 95% CI 0.41–1.25). It suggested that in the probiotic groups soft/semi-solid stools may occur more commonly (OR 0.46, 95% CI 0.04–5.64) and reduce the need for anti-diarrheal medicine (OR 0.63, 95% CI 0.13–0.79). Between-study heterogeneity of results was noted. This was attributed to the different treatments, strain, doses and duration of probiotics alongside comorbidities, cancers, and interventions delivered.

An update was then undertaken in 2018 (Hassan, Rompola, Glaser, Kinsey, & Phillips, 2018). It Identified 10 new RCTs and 8 further studies reporting adverse effects (AEs) of probiotics in people with cancer, giving a total of 21 studies for efficacy analysis and 25 studies for safety analysis. There was marked heterogeneity of the strain, dose and duration of probiotic used and age, cancers, and anti-cancer therapies under study. It was not possible to undertake subgroup analysis to explore between-study heterogeneity further. The updated systematic review undertaken for this thesis concluded there is still insufficient evidence to determine that probiotics are effective and safe in people with cancer. A meta-analysis demonstrated that probiotics might reduce the incidence of diarrhea, duration of pyrexia and may reduce incidence of septicemia and central line infection. However, these results should be interpreted cautiously because of the heterogeneous nature of included studies and the lack of studies with a clear low risk of bias.

Systematic reviews identified in Table 23.1 were undertaken for a range of cancer patients. This included patients diagnosed with abdominal and pelvic cancers, and patients who received surgery, radiotherapy or chemotherapy. One systematic review by Wardill, Van Sebille, Ciorba, and Bowen (2018) stated that probiotics may be beneficial for certain outcomes. However, several studies reported significant heterogeneity and concerns about study biases. Therefore, conclusions regarding the use of probiotics in cancer patients cannot be made with confidence.

23.6 Safety of probiotics

The use of probiotics in immunosuppressed patients causing probiotic-associated infections is one of the most concerning adverse outcomes that have prevented the recommendation of routine use of probiotics. Many national based guidelines do not recommend routine use

TABLE 23.1 Characteristics and findings of included studies.

Author	Paper	Included studies	Findings	Conclusion	Miscellaneous
Redman et al. (2014)	The efficacy and safety of probiotics in people with cancer: a systematic review	Eleven studies (N = 1557 participants) were included for assessing efficacy. Seventeen studies (N = 1530) were included in the safety analysis	Results show that probiotics may reduce the severity and frequency of diarrhea in patients with cancer and may reduce the requirement for anti-diarrheal medication, but more studies are needed to assess the true effect. For example, comparing probiotic use to control 25 groups on effect on Common Toxicity Criteria ≥2 grade diarrhea, odds ratio (OR) = 0.32 (95% confidence interval [CI] of 0.13–0.79; p = 0.01). Five case reports showed probiotic-related bacteremia/fungemia/positive blood cultures.	Probiotics may be a rare cause of sepsis. Further evidence needs to be collated to determine whether probiotics provide a significant overall benefit for people with cancer.	
Wang et al. (2016)	The efficacy and safety of probiotics for prevention of chemoradiotherapy-induced diarrhea in people with abdominal and pelvic cancer: a systematic review and meta-analysis.	Nine randomized and placebo-controlled studies (N = 1265 participants) were included for assessing efficacy Eleven studies, including 1612 people (873 consuming probiotics and 739 not consuming probiotics), were used for the analysis of safety of probiotics.	Probiotic groups were compared with control groups with respect to the incidence of diarrhea, OR = 0.47 (95% confidence interval 0.28–0.76; p = 0.002). Of the 11 studies, seven studies had no adverse events (AEs) caused by probiotics, whereas four studies reported varying degrees of AEs in their treatment.	Probiotics may have a beneficial effect in prevention of chemoradiotherapy-induced diarrhea generally, especially for Grade ⩾2 diarrhea. Probiotics may rarely cause AEs.	
de Andrade Calaça, Bezerra, Albuquerque, Porto, and Cavalcanti (2017)	Probiotics as a preventive strategy for surgical infection in colorectal cancer patients: a systematic review and meta-analysis of randomized trials.	Seven peer-reviewed articles with randomized controlled trials were identified.	Effect of probiotics to minimize complications of infection reported an OR 0.5388 (95% CI, 0.4058–0.7154, p < 0.0001)	Probiotics are suggested to prevent surgical inflammation of CRC, while the combination of microorganisms administered is beneficial to the treatment and surgical recovery.	Quality of reported studies not included in systematic review

(*Continued*)

TABLE 23.1 (Cont'd)

Author	Paper	Included studies	Findings	Conclusion	Miscellaneous
Hassan et al. (2018)	Systematic review and meta-analysis investigating the efficacy and safety of probiotics in people with cancer	Twenty-one studies (N = 2982 participants) were included for assessment of efficacy. Twenty-five studies (*N* = 2242) were included in the safety analysis.	Probiotics may reduce the incidence of diarrhea in patients with cancer [odds ratio (OR) = 0.52, 95% confidence interval (CI) 0.34–0.78, 95% prediction interval (PI) 0.3–0.92, I-sq 36.9%, 5 studies] and the duration of pyrexia [standardized mean difference 0.39 days, 95% CI 0.35–0.43, I-sq 0.01%, 5 studies]. Five case reports showed probiotic-related bacteremia/fungemia/positive blood cultures.	There remain insufficient studies to assess the true effect of probiotics in people with cancer. Meta-analysis suggests probiotics may be beneficial but further studies are still required. Improved reporting of outcomes and adverse events in clinical trials are required to improve accuracy and confidence of conclusions drawn in future updates.	
Wei et al. (2018)	Probiotics for the prevention or treatment of chemotherapy- or radiotherapy-related diarrhea in people with cancer	12 studies involving 1554 participant identified	For occurrence of any diarrhea, risk ratios (RRs) ranged from 0.35 (95% confidence interval (CI) 0.26 to 0.47) to 1.0 (95% CI 0.94 to 1.06) (three studies; low-certainty evidence). For occurrence of grade 2 or higher diarrhea, the pooled RR was 0.75 (95% CI 0.55 to 1.03; four studies; 420 participants; low-certainty evidence), and for grade 3 or higher diarrhea, RRs ranged from 0.11 (95% CI 0.06 to 0.23) to 1.24 (95% CI 0.74 to 2.08) (three studies; low-certainty evidence). For probiotic users, time to rescue medication was 36 hours longer in one study (95% CI 34.7 to 37.3), but another study reported no difference (moderate-certainty evidence). For the need for rescue medication, the pooled RR was 0.50 (95% CI 0.15 to 1.66; three studies; 194 participants; very low-certainty evidence).	This review presents limited low- or very low-certainty evidence supporting the effects of probiotics for prevention and treatment of diarrhea related to radiotherapy (with or without chemotherapy) or chemotherapy alone, need for rescue medication, or occurrence of adverse events. All studies were underpowered and heterogeneous. Severe side effects were absent from all studies.	

Author	Paper	Included studies	Findings	Conclusion	Miscellaneous
Wardill et al. (2018)	Prophylactic probiotics for cancer therapy-induced diarrhea: a meta-analysis.	Seven trials with a total of 1091 patients were included in this meta-analysis.	Compared with placebo, prophylactic probiotics did not prevent or reduce the overall incidence of diarrhea or severe CTCAE Grade at least 3 diarrhea [relative risk (RR) = 0.81, 95% confidence interval (95% CI) = 0.60-1.09, Z = 1.41, p = 0.16; RR = 0.54, 95% CI = 0.25–1.16, Z = 1.58, p = 0.11], nor did it influence the use of rescue medication (RR = 0.93, 95% CI = 0.53–1.65, Z = 0.24, p = 0.81).	Current evidence does not support widespread implementation of probiotics for diarrhea secondary to cytotoxic therapy and the tyrosine kinase inhibitor, dacomitinib. Research efforts should be diverted to pair specific forms of gastrointestinal toxicity and their unique microbial phenotype to develop the ideal microbial protectant.	
Ouyang et al. (2019)	Probiotics for preventing postoperative infection in colorectal cancer patients: a systematic review and meta-analysis	Thirteen identified studies Data were extracted from 1186 participants,	Probiotics administration contributed to the reduction of overall infection rate after colorectal surgery, with a pooled odds ratio (OR) of 0.51 (95% CI: 0.38–0.68, p =0.00). Meanwhile, the incidence of incision infection (pooled OR = 0.59, 95% CI 0.39–0.88, p =0.01) and pneumonia (pooled OR = 0.56, 95% CI 0.32–0.98, p = 0.04) as well as the first flatus time (SMDs = −0.70, 95% CI −1.13 to −0.27, p =0.002) were also reduced by probiotics.	Probiotics have potential efficacy on preventing postoperative infection and related complications in cancer patients undergoing colorectal surgery.	

(*Continued*)

TABLE 23.1 (Cont'd)

Author	Paper	Included studies	Findings	Conclusion	Miscellaneous
Qiu et al. (2019)	The significance of probiotics in preventing radiotherapy-induced diarrhea in patients with cervical cancer: A systematic review and meta-analysis,	Nine randomized, placebo-controlled studies (N = 1508 participants) were included for assessing the efficacy of probiotics. Eight studies, included 1410 participants (726 consuming probiotics, 657 consuming placebo, 27 lost to follow-up), were used for the analysis of safety of probiotics.	Compared with placebo groups, participants in probiotic groups experienced much lower incidence of RID with RR of 0.61 (95% CI 0.46-0.81; p = 0.0007). In addition, significant results were also observed in CTC grade ≥2 and grade ≥3 RID, with the pooled RRs of 0.52 (95% CI 0.30-0.98; p = 0.02) and 0.32 (95% CI 0.12-0.82; p = 0.02) respectively. Of the 8 studies, 4 studies had no AEs caused by probiotics, while another 4 studies reported varying degrees of AEs during their treatment.	Probiotics may have a beneficial effect in prevention of RID generally, especially for Grade ≥2 or 3 diarrhea. Probiotics may be safe and rarely cause severe AEs during treatment.	
Devaraj et al. (2019)	The Effects of Probiotic Supplementation on the Incidence of Diarrhea in Cancer Patients Receiving Radiation Therapy: A Systematic Review with Meta-Analysis and Trial Sequential Analysis of Randomized Controlled Trials.	There were eight trials, and a total of 1116 participants were included in the primary analysis.	Compared with placebo, probiotics were associated with a lower risk of radiation induced diarrhea (RID) [risk ratio (RR) = 0.62, 95% CI = 0.46, 0.83]. A requisite heterogeneity-adjusted trial sequential analysis indicated conclusive evidence for this beneficial effect. No statistically significant reduction in RID (RR = 0.52, 95% CI = 0.14, 1.91) was observed on subgroup analysis in patients receiving both radiation therapy and chemotherapy. However, those patients receiving only radiation therapy (RT) demonstrated significant benefit (RR = 0.61, 95% CI = 0.48, 0.78). There was a significant difference in the antidiarrheal medication use (RR = 0.54, 95% CI = 0.35, 0.84) observed with the use of probiotics. However, no significant difference was observed for the incidence of soft and watery stool.	The use of probiotics is beneficial in preventing RID in patients receiving RT.	

Author	Paper	Included studies	Findings	Conclusion	Miscellaneous
Chen et al., (2020)	Probiotics Used for Postoperative Infections in Patients Undergoing Colorectal Cancer Surgery.	6 studies involving a total of 803 patients	For the incidence of total postoperative infection (odd ratios (OR) 0.31, 95% confidence interval (CI) 0.15–0.64, $I^2 = 0\%$), surgical site infection (OR 0.62, 95% CI 0.39–0.99, $I^2 = 0\%$), surgical site infection (OR 0.62, 95% CI 0.39-0.99, $I^2 = 0\%$), surgical site infection (OR 0.62, 95% CI 0.39–0.99, $I^2 = 0\%$), surgical site infection (OR 0.62, 95% CI 0.39–0.99, $I^2 = 0\%$), surgical site infection (OR 0.62, 95% CI 0.39–0.99, $I^2 = 0\%$), surgical site infection (OR 0.62, 95% CI 0.39–0.99).	Probiotics is beneficial to prevent postoperative infections (including total postoperative infection, surgical site infection, pneumonia, urinary tract infection, and septicemia) in patients with colorectal cancer.	
Kamaluddin et al. (2020)	Probiotic inhibits oral carcinogenesis: A systematic review and meta-analysis.	Only five articles were included in the qualitative synthesis. Two out of the five papers were further analyzed for quantitative synthesis in meta-analysis.	Most of the included studies were found to be of "moderate quality". The qualitative synthesis found four probiotics that exhibited potential therapeutic effects in oral carcinogenesis, including *Acetobacter syzygii*, AJ2, *Lactobacillus plantarum*, and *Lactobacillus salivarius* REN. Among them, the application of *L. salivarius* REN resulted in a 95 % lower risk for developing oral cancer ($p < 0.05$).	It is known that probiotics have the potential to inhibit oral carcinogenesis, thus supporting the hypothesis of the study. The ability of *L. salivarius* REN to inhibit the development of oral cancer suggested that this bacterium can be a potential inhibitory agent against oral carcinogenesis.	Participant number for each trial was not specified
Lin et al.(2020)	The efficacy and safety of probiotics for prevention of chemoradiotherapy-induced diarrhea in people with abdominal and pelvic cancer: A systematic review and meta-analysis based on 23 randomized studies	Twenty-three randomized, placebo-controlled studies (N = 2570 participants) were included in the efficacy assessment.	The incidence of all diarrhea (risk ratio [RR] 0.16; 95% confidence interval [CI] 0.51-0.73), grade 3 diarrhea (RR 0.36; 95% CI 0.18–0.72), and grade ≥2 diarrhea (RR 0.65; 95% CI 0.54–0.78), but not that of grade ≤2 diarrhea (RR 1.07; 95% CI 0.95–1.21), was significantly reduced in the probiotics compared to the placebo groups. No significant increase in the incidence of AEs was found in the probiotics group, although four studies reported a variety of AEs.	Probiotics prevented chemoradiotherapy-induced diarrhea, particularly high-grade diarrhea. Probiotics rarely cause AEs.	

(*Continued*)

TABLE 23.1 (Cont'd)

Author	Paper	Included studies	Findings	Conclusion	Miscellaneous
Dikeocha, Al-Kabsi, Eid, Hussin, and Alshawsh (2021)	Probiotics supplementation in patients with colorectal cancer: a systematic review of randomized controlled trials	A total of 23 RCTs were eligible for inclusion.	Probiotics supplementation in patients with CRC improved their quality of life, enhanced gut microbiota diversity, reduced postoperative infection complications, and inhibited pro-inflammatory cytokine production. The use of certain probiotics in patients with CRC also reduced the side effects of chemotherapy, improved the outcomes of surgery, shortened hospital stays, and decreased the risk of death. *Bifidobacterium* and *Lactobacillus* were the common probiotics used across all studies.	Probiotics have beneficial effects in patients with CRC regardless of the stage of cancer. However, larger clinical trials of selected or a cocktail of probiotics are needed to confirm the efficacy, dosage, and interactions with chemotherapeutics agents.	
Amitay, Carr, Gies, Laetsch, and Brenner (2020)	Probiotic/Synbiotic Treatment and Postoperative Complications in Colorectal Cancer Patients: Systematic Review and Meta-analysis of Randomized Controlled Trials,	Altogether, 16 randomized placebo-controlled probiotics/synbiotic clinical trials that included patients undergoing CRC surgery and investigated postoperative complications and QoL side effects were found. Meta-analyses using random-effects model were performed on data from 11 studies to calculate the effects of probiotics/synbiotics on common CRC resection postoperative side effects and complications.	Perioperative probiotics/synbiotics administration was associated with lower infection incidence (odds ratio [OR] = 0.34, $p < 0.001$), lower diarrheal incidence (OR = 0.38, $p < 0.001$), faster return to normal gut function (mean difference [MD] −0.66 days, $p < 0.001$), shorter postoperative antibiotics use (MD −0.64 days, $p < 0.001$), lower incidence of septicemia (OR = 0.31, $p < 0.001$), and shorter length of hospital stay (MD −0.41 days, $p = 0.110$).	The results support the hypothesis that short-term perioperative administration of probiotics/synbiotics, which are easy to administer, have few side-effects, and are low cost compared with alternatives, might help to alleviate gastrointestinal symptoms and postoperative complications among CRC patients.	Did not state total number of participants

Author	Paper	Included studies	Findings	Conclusion	Miscellaneous
Shu et al. (2020)	The effectiveness of probiotics in prevention and treatment of cancer therapy-induced oral mucositis: A systematic review and meta-analysis,	Five studies involving 435 patients were included in this study.	Pooled results showed a moderate heterogeneity ($p = 0.15$, $I^2 = 44\%$). The pooled RRs indicated that the use of probiotics decreased the risk of OM for grade ≥3 (RR = 0.66, 95%CI = 0.54–0.81, $p < 0.0001$) as well as all grades (RR = 0.83, 95% CI = 0.72–0.97, $p = 0.02$). There was no significant difference between probiotics and placebo for cancer therapy completion rate (RR = 1.14, 95%CI = 0.65–2.00, $p = 0.64$). The subgroup analysis indicated that the use of probiotics was not statistically significant for patients receiving chemo-radiotherapy (RR = 0.52, 95% CI = 0.26–1.04, $p = 0.07$).	Probiotics may reduce the incidence and mitigate the severity of cancer therapy-induced OM. Further trials with a randomized, double-blind and multicentric study design are needed to confirm this effect.	
Miarons et al. (2021)	The role of pro-, pre- and symbiotics in cancer: A systematic review. Journal of Clinical Pharmacy and Therapeutics.	22 articles with 2287 participants were included in the analysis.	The most studied bacteriotherapies were probiotics and symbiotics, in 10 and 7 studies, respectively. Both *Lactobacillus* and *Bifidobacterium* strains were used in 18 studies, while *Lactobacillus* and *Bifidobacterium* strains were individually used in 9 and 2 studies, respectively. Diarrhea incidence rates were 3.2–39.1% in intervention groups and 6.7–60.9% in control groups, while infection incidence rates were 11.1–22.7% in intervention groups and 17.3–28.7% in control groups.	Pro-, pre- and symbiotics may potentially be efficacious in reducing complications associated with chemotherapy, radiotherapy, and surgery in patients with cancer.	
Rodriguez-Arrastia, Martinez-Ortigosa, Rueda-Ruzafa, Folch Ayora, and Ropero-Padilla (2021)	Probiotic Supplements on Oncology Patients' Treatment-Related Side Effects: A Systematic Review of Randomized Controlled Trials	Twenty clinical trials published between 1988 and 2020 were included in this review 2508 participants	Seventeen studies (85%) revealed predominantly positive results when using probiotics to reduce the incidence of treatment-related side effects in oncology patients, while three studies (15%) reported no impact in their findings.	This study has shown that some probiotic strains (*L. acidophilus*, *L. casei*, *B. longum*, or *L. rhamnosus* among others) are a valid therapeutic strategy in some common treatment-related side effects in adult oncology patients, using both single or multiple strain combinations for at least 4 weeks of treatment.	

of probiotics in people with cancers. The Multinational Association of Supportive Care in Cancer (MASCC) 2014 issued guidelines on the management of mucositis secondary to cancer therapy. This guideline advises that probiotics containing *Lactobacillus* species should be used to prevent diarrhea in patients receiving chemotherapy and/or radiation therapy for a pelvic malignancy, but has not referenced the evidence for this recommendation.

In 2018, the European Society for Medical Oncology (EMSO) issued guidelines for the management of diarrhea in adult patients with cancer (Bossi et al., 2018) discussing the use of probiotics. The guideline discusses the conflicting evidence reviewed for the use of probiotics (grade II B) for the use of probiotics and mentions how probiotics may reduce the incidence of diarrhea, but that there is a higher risk in immunocompromised patients of developing a severe infection.

In the UK, neutropenic diet guidelines for hematology patients state that patients should avoid consuming foods or supplements with probiotic cultures. It states that lactobacilli and bifidobacteria do not have a more significant infection risk than strains found in the mouth, ileum and colon, and that reported infections are mostly limited to *Saccharomyces boulardii*, *Saccharomyces cerevisiae*, *Bacillus subtilis,* and *Lactobacillus rhamnosus* LGG. However, safety reviews (Redman et al., 2014) have reported that probiotics are safe, being a rare source of infection and no death have been attributed to their consumption.

The systematic review by Hassan et al. (2018) identified twenty-five studies (n = 2242) that included the safety analysis. It reported how it was unclear to determine how many individuals sustained adverse events as reported between studies. Some studies reported on individual events rather than people sustaining an adverse event, and it is unclear how this may overlap (for example, some studies reported on the incidence of septicemia, pneumonia, and urinary tract infection - making it challenging to identify the number of individuals or indeed if the same episode of illness was counted in two categories). An estimated 237 adverse effect events occurred in those consuming probiotics and 314 in those not consuming probiotics. Of the 8 studies identified during the updates, there were no deaths attributed to probiotics. In the initial review, 2 deaths were reported in probiotic groups, but this was not attributed to the intervention. There were 5 case reports identified during the initial review of associated probiotic infections. Some studies did not report on bacterial isolates from positive blood cultures identified (in both probiotic and control groups). Therefore, the systematic review could not determine if adverse events sustained were attributed to probiotics consumed due to the heterogeneity of malignancies and treatment regimens. As adverse events were also not clearly or uniformly defined in identified studies, it cannot be determined if all relevant data were appropriately identified, recorded or documented.

23.7 The use of probiotics in children diagnosed with cancer

A systematic review undertaken in 2018 (Hassan, Rompola, Glaser, Kinsey, & Phillips, 2018) reported that it was not possible to perform subgroup analysis on the use of probiotics in children due to the lack of available randomized-controlled trials available. During a systematic search undertaken for this chapter, only 1 randomized control trial, 1 randomized controlled feasibility study and 1 ongoing randomized-controlled trial was found when a systematic search was undertaken (Table 23.2). One Children's Oncology Group

TABLE 23.2 Characteristics and findings of identified pediatric studies.

Author	Paper	Included studies	Findings	Conclusion
Children's Oncology Group (trial ongoing)	Lactobacillus Plantarum in Preventing Acute Graft Versus Host Disease in Children Undergoing Donor Stem Cell Transplant	Estimated enrolment 500 participants Estimated completion date 30th October 2023		
Ladas et al. (2016)	The safety and feasibility of probiotics in children and adolescents undergoing hematopoietic cell transplantation.	A convenience sample of 30 children and adolescents undergoing HCT during the period of January 2010 to June 2014 was recruited to the study.	Administration of *LBP* was feasible with 97% (30/31, 95% confidence interval (CI) 83–100%) of children receiving at least 50% of the probiotic dose (median 97%; range 50–100%). We did not observe any case of *LBP* bacteremia (0% (0/30) with 95% CI 0–12%).	Our study provides preliminary evidence that administration of *LBP* is safe and feasible in children and adolescents undergoing HCT.
Hassan et al. (2021)	Mucositis reduction with probiotics in children with cancer: a randomised-controlled feasibility study	Ten patients were recruited	Between May and November 2019, 34 out of 39 eligible participants were approached. Ten patients were recruited (4 probiotics and 6 placebo) of which 2 participants withdrew. Seven participants partially completed the diary but only two participants completed 80% or more appeared to prefer giving informal verbal feedback when in direct contact with research and healthcare professionals.	The MaCROS study has demonstrated that undertaking an RCT may only be feasible if recruitment is improved. Modifications to the protocol will need to focus on recruitment strategies and adherence to data capture. More support for participants and their families to complete diaries is essential.
Ekert et al. (1980)	Prophylactic co-trimoxazole and lactobacilli preparation in neutropenic patients	A randomized study of intestinal decontamination was undertaken in 68 children with leukemia and solid tumors.	The diseases, severity of neutropenia, and incidence of infection at entry into study were comparable in the two groups. There was no significant difference in the incidence of infections developing during the phase of neutropenia.	Co-trimoxazole and lactobacilli preparation improved quality of life during a neutropenic episode and had the additional advantage of being relatively inexpensive.

randomized-controlled trial is also ongoing, and limitations identified in pediatric studies are mostly due to the concerns regarding probiotic associated infections.

23.8 Other considerations

Other aspects, which directly and indirectly impact the use of probiotics in people with cancer, should be also considered. However, evidence around these topics is limited and further research is required before conclusions can be made with confidence.

23.8.1 The microbioma and its impact on cancer therapy

Research has recently explored how the state of the microbiome before, during and following a cancer diagnosis can be linked to the development of toxicity (i.e., infection) and response to interventions (Alexander et al., 2017; Iida et al., 2013; Whisner & Athena Aktipis, 2019). This dysbiosis (the disruption of the microbiome) can impact affect the health of an individual and may vary at different stages of a cancer patient's journey, that is, before diagnosis, during treatment, and end of treatment. Overall, there is a lack of research to understand the role of dysbiosis.

As previously discussed, the gut microbiome may affect drug metabolism, efficacy and toxicity of how an individual responds to chemotherapy in people with cancer (Pouncey et al., 2018). The gut microbiome is impacted during cancer treatment by a range of factors. Examples include diet, surgical intervention, supportive care interventions, that is, antibiotics and chemotherapy.

The term pharmacomicrobiomics is used to describe how the microbiota can affect drug metabolism and toxicity. Pharmacomicrobiomics is believed to determine the toxicity versus the efficacy of chemotherapy in different individuals (Dasari et al., 2017). A "balanced" microbiome is believed to enhance therapeutic effects and reduce toxicity by manipulating how an individual responds to chemotherapy regimens and is based on an evaluation of the individual's microbiome (Pouncey et al., 2018).

It is believed the gut microbiota impacts an individual's response to chemotherapy through its relationship with the immune system (Alexander et al., 2017). The microbiota is thought to contribute to the metabolism of chemotherapy and production of toxic metabolites, therefore altering the microenvironment and indirectly impacting how an individual metabolizes chemotherapy. One study proposed the bacterial metabolism of bacterial vitamin B6, B9, and ribonucleotide can strengthen or weaken the effects of the chemotherapy drug 5-fluorouracil (5-FU) (Whisner & Athena Aktipis, 2019). A further study proposed disruption of bacterial deoxynucleotide pools can amplify 5-FU-induced autophagy and apoptosis (Chi et al., 2016).

The gut microbiota is impacted by multiple factors during chemotherapy. This includes diet, surgical intervention, supportive care interventions (i.e., antibiotics), and chemotherapy. A negative impact can result in dysbiosis, which can impair the symbiotic relationship of the microbiota and the individual, causing adverse side effects (i.e., diarrhea) and thereby weakening the efficacy of the chemotherapeutic intervention (Taur et al., 2014).

The microbiota can also influence the host's response to chemotherapy through modulation of the immune system. It is believed the interaction of the adaptive and innate immune

system and the gut microbiota can regulate immunomodulation (Taur et al., 2014). Chemotherapy is thought to cause bacterial translocation through damage to the gastrointestinal mucosal epithelium, which causes systemic infections and exposure to pathogens and leads to priming of the adaptive immune system, thereby impacting how an individual responds to chemotherapy (Erdman & Poutahidis, 2017; Iida et al., 2013).

It has also been proposed bacterial translocation and T-helper 17 cell activation may increase the efficacy of cyclophosphamide in mice with a "healthy" gut microbiota when compared to mice raised in a sterile environment (Panebianco et al., 2018). Mice with depletion of Gram-positive bacteria (i.e., commensal bacteria) due to vancomycin were found to have a reduced therapeutic response to cyclophosphamide when compared to the control group (Viaud et al., 2013). Intraluminal myeloid cell activation is thought to enhance the action of oxaliplatin (Daillere et al., 2016). However, the impact of the human gut microbiota on cyclophosphamide has not been investigated.

A study by Ziegler et al. (2019) evaluated the impacts of a prophylactic antibiotic (levofloxacin) to prevent febrile neutropenia when compared to broad-spectrum beta-lactam (BSBL) antibiotics on the gut microbiome in patients with hematological malignancies. In sixty patients, the gut microbiome of patients with BSBL exposure had significantly reduced diversity when compared to those without (median, interquartile range [IQR], 3.28 [1.73–3.71] vs. 3.73 [3.14–4.31]; $p = 0.01$). Patients receiving levofloxacin were found to have increased gut microbiota diversity when compared to those not receiving it (median IQR, 3.83 [3.32–4.36] vs. 3.32 [2.35–4.02]; $p = 0.03$). Levofloxacin exposure was also associated with a trend toward a lower risk of the dominance of non-Bacteroidetes genera compared to those without levofloxacin exposure (3 [14%] vs. 15 [38%]; $p = 0.051$). All this suggests that gut microbiome interacts with chemotherapy delivered and supportive care strategy deliveries could lessen toxicity experienced by a patient in current and future courses of chemotherapy. There is a need to investigate how the gut microbiota could be manipulated to reduce infections, and, therefore, potentially reduce mortality (Ziegler et al., 2019).

A further systematic review (Touchefeu et al., 2014) reported that patients receiving chemotherapy or radiation therapy demonstrated changes in intestinal microbiota, particularly, a decrease in *Bifidobacterium*, *Clostridium* species, *Faecalibacterium prausnitzii*, and increase in *Enterobacteriaceae* and *Bacteroides*, which may increase the risk of developing mucositis, and that probiotics significantly reduce the incidence of diarrhea. However, these conclusions were drawn from studies with pre-clinical models, and the methodology for how the systematic review was undertaken was not reported.

23.8.2 The use of antibiotics as a supportive care strategy

Antibiotics are frequently used as supportive care for cancer patients who are immunosuppressed and present with febrile neutropenia. One particular concern identified is whether the use of antibiotics would also impact the delivery and benefits of probiotics in people with cancers.

A study by Ziegler et al. (2019) evaluated the impact of a prophylactic antibiotic (levofloxacin) to prevent febrile neutropenia when compared to broad-spectrum beta-lactam (BSBL) antibiotics to treat episodes of fever on the gut microbiome in patients with hematological malignancies. In the study of sixty patients, it was found that the gut microbiome of

patients with BSBL exposure had significantly reduced diversity when compared to those without. This study proposes that the gut microbiome may interact with chemotherapy and supportive care interventions deliveries, potentially impacting the toxicity experienced by a patient in their current and future courses of chemotherapy (Ziegler et al., 2019). This research stands alongside the work suggesting probiotics delivered during a course of antibiotics may reduce antibiotic-associated diarrhea (Szajewska & Kolodziej, 2015), and emphasizes how we remains uncertain how antibiotics given during a course of probiotic can impact the efficacy of both.

23.9 Conclusion

Even though several systematic reviews and meta-analysis have been undertaken to explore the use of probiotics in people with cancer, the evidence for its use is still limited. It is not possible to conclude with certainty that probiotics are effective and safe in people with cancer. However, reviews have suggested that probiotics may reduce the incidence of diarrhea, duration of pyrexias, incidence of septicemia, and central line infection. Evidence also suggests that probiotics may be a rare source of infection, but that no deaths have been attributed to their consumption. There is limited evidence exploring the use of probiotics in children with cancer.

There is a lack of information exploring the impact of dysbiosis on the microbiome during the different stages of a cancer journey. This includes a distinct lack of research exploring the role of antibiotics, which is used frequently in patients with cancer and its impact on probiotic consumption. A better understanding of these factors and how they interact would help guide the dosing and delivery of probiotics and refine methodologies of future studies by improving the scientific understanding of this topic. Further research investigating the clinical impacts of probiotics on the microbiome and mucositis is required.

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CHAPTER

24

Probiotics for immune disease prevention and treatment

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24.1 Introduction

The intestinal microbiota is a set of archaea, bacteria, fungi, and yeasts that colonize the gastrointestinal tract, especially the colon (Dominguez-Bello, Godoy-Vitorino, Knight, & Blaser, 2019; Human Microbiome Project, 2012a; WGO, 2017). It has a varied composition, and the greater the microbial diversity, the healthier and more balanced the intestinal microbiota. In addition, a healthy microbiota produces and stimulates the release of various compounds, such as mucins, antimicrobial peptides, short-chain fatty acids, and cytokines, which can strengthen the intestinal barrier, aiding in the integrity of the immune system (Human Microbiome Project, 2012b).

Therefore, strengthening the immune system is key to developing and maintaining a healthy organism, which can both prevent and treat diseases (Levy, Kolodziejczyk, Thaiss, & Elinav, 2017). In this context, the study of the intestinal microbiota and agents capable of modulating it has increasingly gained prominence, as new technologies, such as DNA sequencing and the study of omics, allow understanding how the microbiota works in disease situations (Kelsen & Wu, 2012; Yurkovetskiy, Pickard, & Chervonsky, 2015).

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Among the modulating agents of the intestinal microbiota, probiotics stand out, which are live microorganisms that, when consumed in adequate amounts, provide benefits to the host's health (FAO/WHO, 2002). In addition, these can reinforce the immune response by developing a balance of intestinal microorganisms, characterizing a state of intestinal eubiosis (WGO, 2017). In order to do so, probiotics use immunological mechanisms, such as macrophage activation, production of immunoglobulin A (IgA), and increased production of anti-inflammatory cytokines, as well as non-immunological mechanisms, such as mucin production, stimulation of intestinal integrity, and competition for adhesion of pathogens (Azad, Sarker, & Wan, 2018; WGO, 2017).

Therefore, throughout this chapter, we discuss the construction of the immune system, the main cells involved in immunity, and how probiotics act in autoimmune diseases and strengthen immunity. We also discuss the intestinal dysbiosis present in autoimmune diseases, reporting how the modulation of the intestinal microbiota may be promising for treating and preventing these diseases.

24.2 Microbiota formation and immune system structuring

The type of childbirth, the form of breastfeeding, as well as the period of food introduction with the offer of fresh, processed, and ultra-processed foods may directly influence the construction of the intestinal microbiota. Children who underwent food introduction with consumption of fresh and minimally processed foods have been reported to have better metabolic programming, characterized by a higher diversity of microorganisms in the intestinal microbiota and, consequently, a lower risk of developing diseases in adulthood (Butel, Waligora-Dupriet, & Wydau-Dematteis, 2018; Milani et al., 2017).

It is noteworthy that several other factors also contribute to the formation of the intestinal microbiota, such as geographic location, number of siblings, presence of pets, use of medication, and recurrent hospitalizations (De Luca & Shoenfeld, 2019; Milani et al., 2017). In this way, the critical moments of microbiota formation (individual conception, breastfeeding, and food introduction period) allow for greater adhesion of microorganisms to the intestinal mucosa and stimulate the adaptive immunity of children (De Luca & Shoenfeld, 2019; Xiao et al., 2017).

The formation of an intact and diverse microbiota is essential for the prevention of several diseases in adulthood. Similarly, intestinal dysbiosis and microbial malformation in childhood is related to the occurrence of several diseases in adult life, as discussed in section 6 "Autoimmune diseases and probiotics," with intestinal modulation being a possibility for both prevention and treatment of these diseases.

24.3 Interaction between immune cells and probiotics

As previously reported, probiotics have different mechanisms of action to provide health benefits to the host. Among these, one may highlight the occupation of binding sites, which allows the blocking of bacterial pathogenic effects through the production of bactericidal substances, competition for nutrients with pathogens, and adherence to the intestinal

epithelium. Furthermore, probiotics promote intestinal epithelial cell survival, enhance barrier function, and stimulate protective intestinal epithelial cell responses for intestinal epithelial homeostasis. More importantly, modulating the immune system is one of the most plausible mechanisms underlying the beneficial effects of probiotics on human health as they allow for increased innate immunity and modulate pathogen-induced inflammation through signaling pathways regulated by toll-like receptors (TLRs) (Yan & Polk, 2011).

Some intestinal bacteria, such as lactic acid bacteria and bifidobacteria, are directly related to the stimulation of the immune response by increasing the production of antibodies, activation of macrophages, proliferation of T cells, and production of interferon and other cytokines. However, the immunomodulatory properties vary considerably depending on the strain (Van Overtvelt et al., 2010).

Next, we describe the role of the intestinal epithelium and the main immune cells involved in intestinal homeostasis and, consequently, in strengthening the immune system.

24.3.1 Gut-associated lymphoid tissue (GALT)

The intestinal epithelium is composed of a single layer of enterocytes or colonocytes, interspersed with specialized cells. In general, goblet cells produce mucins that form a dense inner mucus layer and a loose outer mucus layer. In contrast, Paneth cells enriched at the bases of small intestinal crypts produce antimicrobial peptides (AMP). In addition, a sampling of antigens across the intestinal barrier is mediated by microfold (M) and dendritic (D) cells extending their dendrites into the lumen. Thus, the intestine has a complex and differentiated immune system that allows it to tolerate a massive load of dietary antigens and commensal microorganisms (Jung, Hugot, & Barreau, 2010). In this sense, the secondary lymphoid tissue (TLS) is widely distributed in the various portions of the intestine, forming the so-called gut-associated lymphoid tissue (GALT) (Jung et al., 2010).

GALT represents the largest mass of lymphoid tissue in the human body, containing approximately 70% of all lymphocytes. Most antibodies produced by healthy individuals are of the IgA type, secreted by the intestinal epithelium (Magrone & Jirillo, 2013). Regarding their main functional characteristics, the different constituents of the intestinal immune compartment may be generally classified as inductive and effector sites. Inductive sites include the most organized structures, such as a) Peyer's patches, which are specialized lymphoid follicles in the wall of the small intestine that contain naïve B cells, follicular DCs, and T lymphocyte-rich areas (Spahn & Kucharzik, 2004); b) mesenteric lymph nodes, which function as a second line of defense for organized lymphoid tissues, filtering the contents of the mesenteric lymphatic vessels; c) isolated lymphoid follicles, which are groups of 100 to 200 B lymphocytes, located throughout the colon; and d) crypt patches, characterized by organized clusters of approximately 1000 cells located at the base of the small intestine crypts (Magrone & Jirillo, 2013).

However, the effector sites include lamina propria and intraepithelial lymphocytes (Hamada et al., 2002; Newberry & Lorenz, 2005). Diffuse lymphoid tissues are composed of IgA-producing plasma cells and mature T cells. Effector cells are dispersed throughout the intestinal mucosa, with most intraepithelial T lymphocytes having the CD8 phenotype (cytotoxic or suppressor), while lamina propria T lymphocytes have the CD4 (auxiliary) phenotype. Lamina propria also contains lineage B lymphocytes, memory B cells, and plasma cells, with 70 to 90% of these IgA producers (Bourlioux, Koletzko, Guarner, & Braesco, 2003).

24.3.2 Phagocytic cells

Known as the front line of the immune defense system, phagocytic cells (phagocytes, neutrophils, monocytes, macrophages, and NK cells) are responsible for the recognition of foreign molecules with subsequent formation of adaptive immunity by means of exposure of antigens through antigen-presenting cells (APC) (Azad et al., 2018).

Together with prebiotics, probiotics can modulate systemic immune responses, increasing the number and activity of the host's phagocytic cells. This action assumes great importance in the intestinal tract, which is the key organ for the development of general immunity in animal species. These lymphoid tissues (SLT and GALT) capture antigens available in the digestive tract, such as probiotics and microorganisms. These, in turn, stimulate B cells, precursors of IgA, and T cells, which contribute to the development of general and nonspecific immunity together with Peyer's patches. Furthermore, through immunological stimulation of the mucosa, IgA-type antibodies are produced, which reduce the number of pathogenic bacteria in the intestinal lumen. In addition, the immune stimulus produces macrophage activation, T cell proliferation, interferon production, among others, generating an increase in mucosal immunity and, consequently, in the host (Begum, Buyamayum, Lingaraju, & Dev, 2021).

24.3.3 Dendritic cells

Dendritic cells (DCs) are APCs that play a central role in immune responses to self and non-self-antigens. Contact with antigens or inflammatory stimuli may induce DC maturation, accompanied by functional and phenotypic changes (Jonkers, Penders, Masclee, & Pierik, 2012). In their mature state, DCs are primed to activate T cells towards a Th1/Th2 polarization (You, Dong, Mann, Knight, & Yaqoob, 2014). However, in the absence of inflammation, DCs remain immature, leading to the deletion of effector T cells or the generation of regulatory T cells (Treg) (Alagón et al., 2019).

In this context, it is noteworthy the role of naturally occurring CD4+ and CD25+ Treg cells in the control of normal immunological homeostasis (Ashraf & Shah, 2014; Sakaguchi, 2000). So far, regarding the role of APC in the development and/or peripheral expansion of Treg and the mechanisms by which this occurs, the notion that, by controlling DC maturation, the outcome of the immune response can be modulated remains (Ashraf & Shah, 2014). Furthermore, interactions with TLRs and DCs in the intestine are believed to be involved in this communication (You et al., 2014).

It has been reported that lactobacilli can differentially modulate DC maturation (Foligne et al., 2007; Ghavami et al., 2020; Jonkers et al., 2012; You et al., 2014). In fact, it has been shown that probiotics may have distinct immunomodulatory capacities for specific strains, both in vitro and in vivo (Alagón et al., 2019; Ghavami et al., 2020; You et al., 2014). This in vitro strain-specific immunomodulation capability appears to be closely associated with the in vivo anti-inflammatory potential of the strain (Ashraf & Shah, 2014; Foligne et al., 2007; You et al., 2014). The anti-inflammatory effects of lactobacilli observed both after oral and systemic administration suggest that mechanisms distant from the site of inflammation could involve populations of regulatory cells (Engevik et al., 2021; Foligne et al., 2007; Gardner, Mamotte, Jackaman, & Nelson, 2017).

Thus, the interaction of the probiotic microorganism with DCs is the factor that promotes the production of cytokines, major histocompatibility complex (MHC) molecules, for antigen presentation, and co-stimulatory molecules that polarize T cells into regulatory T cells and types 1 and 2 auxiliary cells (Ashraf & Shah, 2014). Moreover, probiotic bacteria can reach GALT by crossing special intestinal cells (transcytosis), called M cells, and interact directly with DCs, modulating the immune response (Lebeer, Vanderleyden, & De Keersmaecker, 2010; You et al., 2014).

24.3.4 Microfold cells (M)

M cells are present in Peyer's patches, located in the small intestine. They lack a brush border but play both endocytosis and transcytosis functions. These cells, consequently, can transport macromolecules and microorganisms between the intestinal lumen and the lymphoid system (Azad et al., 2018). In addition, it is noteworthy that Peyer's patches are inserted in a mucous layer, which means that the intestinal cells organize together in order to increase the protection against foreign substances. Thus, when translocation of bacteria to M cells occurs, they lead to the excretion of IgA in the basolateral epithelial domain with consequent binding to microorganisms and neutralization of their toxins (Neutra & Kraehenbuhl, 2003).

Therefore, because they can recognize what is outside the intestinal lumen, these cells activate the immune system, both innate and adaptive, in the presence of foreign substances that can damage the intestinal lumen, mobilizing macrophages and activating B cells. This leads to IgA stimulation, which aids in the homeostasis of the intestinal microbiota (Gerbe et al., 2011).

24.3.5 Paneth cells

Paneth cells produce AMP, such as defensins, lysozymes, and cathelicidins, which attack microbes that behave like lytic enzymes. In addition, the epithelium that is superior to Peyer's patches has specialized cells, called microfold cells or M cells (Neutra & Kraehenbuhl, 2003).

The connection of probiotic strains to intestinal epithelial cells, such as Paneth cells and enterocytes, is through the stimulation of the production of defensins and mucus, respectively, important substances in protecting mucosal surfaces against invasion by pathogens, becoming another mechanism of action on the host (Lebeer et al., 2010).

24.3.6 Goblet cells

Goblet cells are found on all mucosal surfaces, not restricted to the intestine. Thus, they are important in monitoring the extracellular environment, interacting with the microbiome and its products, and communicating with the underlying immunity, especially in relation to mucosal homeostasis, defense against mucosal infections, and inflammation. Furthermore, goblet cells have the important function of producing mucus. This compound, rich in glycoproteins and excellent viscosity, helps protect the gastrointestinal tract and acts as a source of immunological protection (Azad et al., 2018; Gerbe et al., 2011).

The mucus layer containing mucin (MUC) that coats the gastrointestinal epithelium is the front line of the innate defense. Mucins are probably the first molecules with which invading

pathogens interact on the cell surface and, consequently, may limit binding to other glycoproteins and neutralize the pathogen (Strugnell & Wijburg, 2010).

The large, highly glycosylated gel-forming mucins MUC2, and MUC5AC are the major components of mucus in the intestine and stomach, respectively. Small intestine mucus limits the number of bacteria that can reach the epithelium and Peyer's patches. In the large intestine, the inner mucus layer separates commensal bacteria from the host's epithelium, as the outer mucus layer is the natural habitat for commensal bacteria (McGuckin & Hasnain, 2017).

Intestinal goblet cells secrete not only MUC2 mucin but also a number of typical mucus components such as CLCA1, FCGBP, AGR2, ZG16, and TFF3. It has also been shown that goblet cells play a new role in the presentation of oral antigens to the immune system as they deliver luminal material from the small intestine to the DCs of tolerogenic CD103+ type lamina propria (Howe, Lickteig, Plunkett, Ryerse, & Konjufca, 2014; McDole et al., 2012). In addition to the gel-forming mucins, the transmembrane mucins MUC3, MUC12, and MUC17 form the enterocyte glycocalyx. Mucin MUC17 can slide from a surface to an intracellular vesicle location, suggesting that enterocytes are able to control and report an epithelial microbial challenge (Birchenough, Johansson, Gustafsson, Bergström, & Hansson, 2015).

There is communication of the epithelial cells with the immune system and in the opposite direction. An example of this is IL-10, which can affect and improve the properties of the inner mucus layer of the colon. Thus, mucus and epithelial cells from the gastrointestinal tract are the main guardians and controllers of bacterial interactions with the host's immune system (Kim & Khan, 2013).

Commensal bacteria and probiotics can modulate the normal intestinal microbiome by direct action and by signaling to the host, which in turn adjusts the immune response (Brestoff & Artis, 2013). Probiotics such as *Lactobacillus plantarum* were used to induce MUC2 and MUC3 mucins and inhibit the adherence of enteropathogenic *Escherichia coli*, indicating increased mucus layers glycocalyx coat the intestinal epithelium. Furthermore, the occupation of microbial binding sites by *Lactobacillus* spp. protect against pathogen invasion, which indicates that probiotics, in addition to stimulating production, also cause qualitative changes in mucins, preventing pathogen binding (Kim & Khan, 2013).

24.3.7 B and T lymphocytes

Cells of the adaptive immune system, B and T lymphocytes, generate immunological memory after contact with antigens presented by APCs (Azad et al., 2018), both lymphocytes being stimulated by the action of probiotics.

Regarding B lymphocytes, it is observed that probiotics stimulate macrophages to present antigens to B lymphocytes to stimulate the secretion of IgA, both locally and systemically (WGO, 2017). This humoral response mechanism aims to increase the production of an antibody whose main biological function is to protect against invading microorganisms such as viruses and bacteria, preventing the adherence of foreign microorganisms to epithelial cells. In addition, locally, IgA acts on its secretion site, the intestinal mucosa (Macpherson, Yilmaz, Limenitakis, & Ganal-Vonarburg, 2018), and mucus production, as discussed earlier, is also an intestinal protective mechanism stimulated by probiotics.

Regarding T lymphocytes, probiotics act by stimulating a balance between the maturation of Th1 and Th2 cells, which will result in a balance between the production of anti-inflammatory and pro-inflammatory cytokines (Azad et al., 2018). This mechanism is interesting to generate immunological tolerance, being also an immunological mechanism of action of probiotics.

In a framework of intestinal imbalance, with the presence of pathogenic bacteria, there is a stimulus to the production of inflammatory cytokines such as IL-17, IL-1, interferon-gamma (IFN-γ), and tumor necrosis factor-alpha (TNF-α). This generates chronic and systemic inflammation, which can lead to the development of various diseases. Hence, the action of probiotics in restoring intestinal eubiosis is essential for the functionality of the immune system and the consequent prevention and treatment of many diseases (Raheen, Liang, Zhang, & Cui, 2021).

24.4 Interaction between MAMP and probiotics

Intestinal epithelial cells are endowed with receptors that act as danger sensors, the TLRs, and nucleotide oligomerization domain-type (Nod) receptors. Both receptors recognize microbe-associated molecular patterns (MAMP), such as lipopolysaccharides (LPS), peptidoglycan, lipoteichoic acid, flagellin peptides, and formylate (Gwénaël, 2019; Sanders, Benson, Lebeer, Merenstein, & Klaenhammer, 2018). The binding of MAMP to specific receptors triggers a cascade of intracellular events that culminate in the activation of nuclear transcription factor-kappa B (NF-kB), MAP kinases, or caspases (Lebeer et al., 2010; Strober & Watanabe, 2011). Furthermore, these pathways induce the expression of pro-inflammatory cytokines, in which IL-1β, IL-6, IL-8, and TNF-α stand out as the main mediators (Sanders et al., 2018).

A high dose of MAMP is recognized during the administration of probiotics, which may interact with pattern recognition receptors (PRR), resulting in several immunomodulatory effects, as cited. TLRs are the best-documented PRR in the literature, detecting several probiotic MAMP (Teame et al., 2020). Furthermore, since the most common probiotics are gram-positive bacteria, TLR2 is a key PRR, as peptidoglycan and lipoteichoic acid are MAMP conserved among lactobacilli and bifidobacteria (Colagiorgi et al., 2015; Sanders et al., 2018).

Multiple other MAMP-PRR interactions between probiotic bacteria and host cells may also occur and impact final host responses. Within probiotic bacterial cells, unmethylated DNA containing cytosine-guanine is an important ligand for TLR9, which is expressed by many types of cells located in the intestine, including epithelial cells and classical immune cells. TLR9 signaling is also important for intestinal epithelial homeostasis (Kant, de Vos, Palva, & Satokari, 2014).

However, not all probiotic immunomodulatory molecules work through PRR. For example, some widely secreted enzymes seem to have an active impact on cytokine activity, such as the PrtP proteases associated with the conserved cell envelope (also called lactocepin), which have been shown to selectively degrade pro-inflammatory cytokines (Von Schillde et al., 2012). In addition, several bacterial metabolites, such as short-chain fatty acids (SCFA) and various amino acids (for instance, tryptophan-derived products), may directly impact the host's immune status (Corrêa-Oliveira, Fachi, Vieira, Sato, & Vinolo, 2016; Teame et al., 2020).

Thus, the immunomodulation induced by probiotics is the result of the interaction of conserved molecules commonly found in the cell wall of these microorganisms, the MAMP, with PRR, as well as commensal microorganisms inducing the signaling pathways of the immune system, and the type of immune response generated is directly dependent on the ingested probiotic strain and the cell type to which it binds (Oelschlaeger, 2010; Sanders et al., 2018). Thus, they can stimulate the release of specific cytokines, transmitting signals to adjacent cells, or exert antiviral or anti-inflammatory effects. In addition, the same recognition mechanism also regulates the homeostasis of the commensal microbiota in the mucosa (Gwénaël, 2019).

24.5 Probiotics and the modulation of inflammatory cytokines

The immunomodulatory effects of probiotics are mainly due to the induction of the release of cytokines, including interleukins, transforming growth factor, TNF, interferon, and immune cells released from chemokines, which further regulate the immune system (Azad et al., 2018; Foligné et al., 2010; Savan & Sakai, 2006).

The immunostimulant and immunoregulatory actions of probiotics are reported in the literature. Immunostimulatory probiotics are able to act against infections and cancer cells, inducing the release of IL-12, which stimulates NK cells and produces Th1 cells. By maintaining the balance between Th1 and Th2, these probiotics also work against allergies (Azad et al., 2018; Raheen et al., 2021). Contrary to this finding, immunoregulatory probiotics are attributed to Treg cells and IL-10 production to attenuate excessive inflammatory responses, inflammatory intestinal disease, and autoimmune disorders (Azad et al., 2018; Chiba et al., 2010).

Thus, the immunomodulatory effects of probiotics via cytokines are strain-specific, as indicated by the in vitro study by Haller et al. (2000) using Caco-2 cells in which they reported that *Lactobacillus sakei* are able to induce pro-inflammatory cytokines (IL-1β, TNFα, and IL-8) while *Lactobacillus johnsonii* induced anti-inflammatory cytokines (TGF-β) (Haller et al., 2000). Furthermore, a mixture of *Lactobacillus paracasei* and *Lactobacillus reuteri* in mice with IL-10 deficiency for *Helicobacter hepaticus* leads to reduced production of colitis and pro-inflammatory cytokines (Peña et al., 2005). Kourelis et al. (2010), in their study in Fisher-344 and BALB/c pure mice, demonstrated that *Lactobacillus acidophilus* NCFB 1748 and *Lactobacillus paracasei* subsp. *paracasei* DC412 induced specific immune markers and innate immune responses through polymorphonuclear cell recruitment and TNF-α production (Kourelis et al., 2010).

In this sense, the studies cited in this topic indicate that cytokines, as they are signaling substances, act in innate and adaptive immunity, transporting information between inflammatory cells, increasing the induction of effector cells and proteins that boost the effects of probiotics on immunomodulation. These effects shown in studies tend to be strain-specific; hence, combinations of different probiotic strains are among the possible alternatives to treat tissue damage associated with inflammation and reduce the risk of autoimmune diseases.

24.6 Autoimmune diseases and probiotics

After industrialization, there was a great increase in autoimmune, inflammatory, and metabolic diseases in the last two centuries, that is, in multifactorial diseases. However, due to

the relatively recent period, it is believed that genetic causes are not enough to explain this prevalence, which indicates that environmental and lifestyle changes (diet, especially the consumption of processed and ultra-processed foods, sleep pattern, hygiene, physical activity, exposure to antibiotics and xenobiotics, type of childbirth, family size, among others) are contributing to the outcome of these diseases (Kelsen & Wu, 2012; Levy et al., 2017).

Furthermore, it is observed that these environmental and lifestyle factors influence human cells and the microorganisms that inhabit the intestinal microbiota. In fact, microorganisms residing in the intestines evolved with human beings, developing important functions in the body metabolism, such as the production of SCFA and vitamins, aiding in the metabolization of non-digestible carbohydrates, and regulation of satiety (Collen, 2016; Pascale et al., 2018). As previously reported, in cases of intestinal dysbiosis, there may be a picture of immunological intolerance, where the organism may recognize something inherent to it as harmful, that is, the immune system can destroy the organism itself by mistake, which characterizes the occurrence of an autoimmune disease (US National Library of Medicine, 2021a).

In these cases, the recognition of MAMP and the production of SCFA are important ways of preventing and treating autoimmune diseases (Marietta, Mangalam, Taneja, & Murray, 2020), and it is noteworthy that probiotics act in both ways. Therefore, as discussed below, the modulation of the intestinal microbiota and the increase in intestinal integrity, that is, strengthening the immune system through probiotics, can prevent and treat several autoimmune diseases.

24.6.1 Crohn's disease

Crohn's disease is caused by a dysfunctional interaction between the microbiota and the immune system (Al-Judaibi, 2021). The disease is more common among older adults and is characterized by a transmural granulomatous inflammation, which occurs in a discontinuous pattern and tends to form fistulas (Mills, von Roon, Tekkis, & Orchard, 2011). The cause is unknown, but it may depend on interactions between genetic predisposition, environmental factors, and mucosal immunity (Jakubczyk, Leszczyńska, & Górska, 2020; Pavel et al., 2021). Furthermore, a study with mice shows that the urease enzyme and microbial activities may represent one of the main causes of this disease (Ribaldone et al., 2019).

Symptoms vary but generally include diarrhea, abdominal pain, weight loss, blood or mucus in feces, perineal pain, secretion, and irritation resulting from perianal fistulas (Mills et al., 2011). In addition, extra-intestinal manifestations of the disease, such as arthritis, uveitis, and rash, are also observed. Thus, intestinal changes related to Crohn's disease interfere with the commensal relationship between the immune system and the intestinal microbiome, leading to pathological symptoms, such as inflammation of the digestive tract (Jakubczyk et al., 2020; Lavoie et al., 2019).

The most used treatments for Crohn's disease are immunosuppressants, which are used to reduce the host's response, and antibiotics, which are used to suppress the microbiota, with a consequent decrease in the activation of the intestinal immune system. In this sense, probiotics may be used to manage the microbiome and, thus, they were considered a potential adjuvant therapy for Crohn's disease. There are evidences that probiotics benefit other gastrointestinal conditions, such as irritable bowel syndrome and ulcerative colitis. However, its effectiveness in Crohn's disease is not very clear, as most studies, in experimental models,

that evaluated the role of probiotics in this disease did not demonstrate any benefit or efficacy in maintaining the clinical remission of the disease (Bourreille et al., 2013; Coqueiro, Raizel, Bonvini, Tirapegui & Rogero, 2019; Limketkai, Akobeng, Gordon, & Adepoju, 2020; Müllner, Miheller, Herszényi, & Tulassay, 2014; Pavel et al., 2021).

In Crohn's disease, the increased mucosal permeability caused by disease progression may play a key role in causing and perpetuating intestinal inflammation. Therefore, the administration of probiotics in the early stages of Crohn's disease may reduce pathological damage and worsen symptoms by stabilizing the intestinal barrier (Fedorak et al., 2015).

In a meta-analysis that considered 27 randomized clinical trials, nine articles verified, under different conditions, the effectiveness of probiotics in Crohn's disease in humans. The authors showed that probiotics can generally significantly affect Crohn's disease, especially shortly after surgery, but in children with inflammatory bowel disease (IBD), mixed probiotics, using *Lactobacillus* and VSL#3, have a significant advantage (Gupta, Andrew, Kirschner, & Guandalini, 2000). Furthermore, the combination of probiotics *Saccharomyces boulardii*, *Lactobacillus*, and VSL#3 in Crohn's disease proved efficient (Ganji-Arjenaki & Rafieian-Kopaei, 2018). Thus, children with IBD who are at risk of developing Crohn's disease could be treated with probiotics to reduce intestinal permeability and counteract pathogenic bacteria (Ganji-Arjenaki & Rafieian-Kopaei, 2018; Pavel et al., 2021).

Therefore, the available data suggest that the concomitant use of multiple microorganisms in patients with IBD leads to better results in disease progression than the use of a single microorganism (Ganji-Arjenaki & Rafieian-Kopaei, 2018). The most effective have been the microorganisms of the *Bifidobacterium* and *S. boulardii* families. Still, in relation to determining the dose of probiotic to be offered, many studies use higher doses than those recommended (>109 colony-forming units). In contrast, other studies do not specify the dose used. In addition, two special categories of patients among these studies are children and elderly patients, and the data on the use of probiotics in IBD in these categories are much more limited. Therefore, more studies are needed to monitor the effective use of probiotics in IBD, specifically in Crohn's disease, as there is still not enough evidence to support the use of probiotics in daily clinical practice in Crohn's disease, and its use is only recommended in clinical studies (Bourreille et al., 2013; Coqueiro et al., 2019; Limketkai et al., 2020; Müllner et al., 2014; Pavel et al., 2021).

24.6.2 Celiac disease

Mediated by gluten consumption, celiac disease is an autoimmune disease that affects genetically predisposed individuals (Fasano & Catassi, 2012). In addition, environmental factors such as viral infections, quality and quantity of consumed wheat, and intestinal dysbiosis contribute to the development of this disease (Caio et al., 2019).

Gluten is a protein in wheat and other cereals, such as rye and barley (Caio et al., 2019). Also, there may be cross-contamination during grain processing or food preparation, which results in the presence of gluten in foods that do not originally contain it (Parsons et al., 2021). It is noteworthy that gluten is a difficult-to-digest protein, consisting of several non-digestible immunogenic peptides. The large amount consumed by the general population adds to that, resulting in intolerance to gluten (Caio et al., 2019).

The treatment of celiac disease is based on a diet that excludes gluten-containing foods. However, this diet must be carried out in the long term, and this practice is difficult,

involving social and psychological issues, risky for potentially consuming contaminated food, and high-cost, as gluten-free foods are more expensive than their traditional versions (Marasco et al., 2020; Serena, Kelly, & Fasano, 2018).

In addition, the characteristic inflammatory condition of celiac disease (Fasano & Catassi, 2012) stimulates the development of intestinal dysbiosis, thus forming a vicious cycle that involves intestinal inflammation, T cell recruitment, gluten intolerance, and increased intestinal permeability (Marasco et al., 2020; Serena et al., 2018). Therefore, it is believed that the use of probiotics as agents that modulate the intestinal microbiota is promising in celiac disease, acting both in prevention and in treatment (Marasco et al., 2020).

In a recent literature review, the authors highlight the main mechanisms of action used by probiotics in the management of celiac disease, namely: modulation of the composition of the intestinal microbiota, regulation of the immune response, competition for nutrients, decreased intestinal permeability, and protection from pathogens (Marasco et al., 2020). These mechanisms have been related to the ability of probiotics to improve intestinal homeostasis.

Also, it is observed that probiotics (*Lactobacillus fermentum*, *Lactobacillus casei*, and *Bifidobacterium lactis*) generate protection against the toxic effects of gliadin, one of the protein fractions of gluten (D'Arienzo et al., 2011; Lindfors et al., 2008; Marasco et al., 2020; Serena et al., 2018). This protection happens because probiotics help to control inflammation, modulate the intestinal microbiota, and develop GALT. Also, when added to yeast for bread production, probiotics help break down gluten peptides, all of which reduce the immunotoxicity caused by gluten (Marasco et al., 2020; Serena et al., 2018).

Therefore, the use of probiotics improves symptoms and quality of life for celiac patients (Marasco et al., 2020; Serena et al., 2018), and their use is interesting for this population.

24.6.3 Type 1 diabetes mellitus

Type 1 diabetes mellitus (DM1) is an autoimmune disease that leads to the destruction of insulin-producing pancreatic beta cells by the action of autoreactive CD8 + T lymphocytes. This disease has a high prevalence among children, although it is able to affect individuals of any age. Furthermore, once diagnosed, DM1 requires immediate therapy with insulin replacement (ADA, 2016).

The incidence of DM1 has increased in recent decades and has become a global public health issue, representing approximately 5% to 10% of all diabetes cases (Kahanovitz, Sluss, & Russell, 2017). The main factors driving the increase in the incidence of DM1 are the modern lifestyle and abrupt environmental changes, which have predominantly led to changes in food consumption. Consequently, there are alterations in the genome's epigenetic regulations and the composition of the intestinal microbiota, which may ultimately play a role in the pathogenesis of DM1 through immune dysfunction (Mishra et al., 2019).

The occurrence of pancreatic diseases, such as diabetes, may mean changes in the intestinal microbiota and immune function; after all, dysbiosis affects signaling pathways and remodels the functions of intestinal barriers, which directly implies the generation of insulin resistance. In general, these interactions between the immune system and the microbiota also vary according to age (Durazzo, Ferro, & Gruden, 2019). A recent review indicates that improvement in intestinal dysbiosis using specific probiotics is associated with a decline in the

autoimmune response and improvement in intestinal integrity (Mishra et al., 2019). Thus, the manipulation of the intestinal microbiome in children has shown positive results in reducing the risk of DM1, especially in lowering blood glucose levels and improving beta-cell function over time, but in children at high risk for DM1, using probiotics alone or in combination with other dietary supplements, clinical management is not yet well understood (Dayal & Rohilla, 2021; Kim, Lee, Im, & Lee, 2020; Mishra et al., 2019; Uusitalo et al., 2016).

Many probiotics are used for the prevention or adjuvant treatment of diabetes but their known mechanisms so far remain to be clarified. Among these mechanisms, it is already known in the literature that the administration of specific probiotic strains can increase SCFA production and, thus, balance intestinal cell homeostasis, activating free fatty acid 2 (FFAR2) and FFAR3 receptors, which contribute to the regulation of the immune system and in the pathogenesis of autoimmune diseases such as DM1 (Priyadarshini, Navarro, & Layden, 2018; Tan, McKenzie, Marino, Macia, & Mackay, 2017). Furthermore, SCFA-mediated activation of FFAR2/3 may also increase the production of a peptide-1 similar to glucagon (GLP-1) from intestinal L cells. GLP-1 is a hormone that stimulates insulin secretion from pancreatic β cells, thus lowering blood glucose (known as the "incretin effect") (Lee & Jun, 2014; Priyadarshini et al., 2018; Psichas et al., 2015). These facts demonstrate the potential of probiotics in the prevention or control of DM1 through maintaining or restoring homeostasis of the intestinal microbiota-immune system axis in an experimental model.

A study by Yadav et al. (2018), who worked with *Lactobacillus rhamnosus* MTCC:5957 and MTCC:5897, and *Lactobacillus fermentum* MTCC:5898 demonstrated improvements in glucose metabolism (fasting glucose, serum insulin, and glycated hemoglobin), oxidative stress, serum inflammation status (TNF-α and IL-6), and serum lipid profile in diabetic rats. In addition, it also significantly reduces the mRNA expression of genes related to gluconeogenesis, such as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase). Also, in another study, *L. acidophilus* showed antidiabetic characteristics by reducing glycated hemoglobin, fasting blood glucose level, and increasing GLP-1 concentrations in the serum of mice (Yan et al., 2020).

In a study with C57BL/6 mice, using specific probiotic strains of *Lactobacillus brevis*, it was shown to protect against DM1 induced by streptozotocin (STZ) and helped reducing blood glucose levels via gamma-aminobutyric acid (Abdelazez et al., 2018). Furthermore, the treatment of non-obese diabetic mice with probiotic strains belonging to the *Bifidobacteriaceae* and *Lactobacillaceae* families and the *Streptococcus thermophilus* genus improves DM1 through the positive modulation of the composition of the intestinal microbiota and reduction of intestinal inflammation, maintaining the immune homeostasis of the intestine and inhibiting the IL-1β expression. Furthermore, in STZ-induced C57BL/6 mice, administration of the probiotic strains *Lactobacillus kefiranofaciens* M and *L. kefiri* K has also been shown to stimulate the production of IL-10 in the pancreas (Wei, Chen, & Chen, 2015). The production of IL-10 in this context helps to contain the levels of cytokines associated with the T-helper 1 cell (TH) (IL-1β, IL-6, and IL-2) and proinflammatory cytokines (TNF-α) present in the pancreas. In addition, these strains were also responsible for an increase in GLP-1 production and regulation of insulin synthesis from pancreatic islet β cells (Wei et al., 2015).

In a non-obese diabetic mouse model, the probiotic strains belonging to *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus* spp., and *Bifidobacterium* spp. were used to activate the cytokine secretion pattern from pro-inflammatory to anti-inflammatory in GALT

(Calcinaro et al., 2005). This decreases the chances of occurrence of an islet-specific autoimmune condition, reducing insulitis and regulating the maintenance of B cell diversity, protecting autoimmune DM1 (Calcinaro et al., 2005). Likewise, high doses of butyrate in the diet have been shown to significantly reduce the incidence of DM1 in non-obese diabetic mice through the expansion of Treg (Mariño et al., 2017). Therefore, the application of such probiotic strains can be considered helpful in restoring intestinal cells and insulin-secreting cells, maintaining a healthy intestinal microbiota spectrum, thus helping to improve hyperglycemia and autoimmune DM1.

The effects of *L. casei* Shirota were also studied in two experimental models of insulin-dependent diabetes mellitus: alloxan-induced diabetes and non-obese diabetic mice. In both models, *L. casei* Shirota decreased the incidence of diabetes, slightly reduced plasma glucose levels, and prevented the destruction of beta cells and islets of Langerhans. The effects of *L. casei* Shirota in non-obese diabetic mice may be explained by the observed shift in the balance between Th1/Th2 to Th2. Furthermore, after treatment with *Lactobacillus casei* Shirota, the number of CD8+ T cells involved in beta cell destruction was lower. *L. casei* Shirota seemed to stimulate Th1 responses in this experimental model of diabetes. This suggests that the immunomodulatory effects of probiotics may not simply be explained by the distortion of the Th1/Th2 to Th1 balance. Supposedly, immunomodulation is regulated at another level (Ezendam & Loveren, 2006; Wei et al., 2015).

Different species of other *Lactobacilli* and yeast strains also act as antidiabetics, preventing apoptosis of pancreatic cells through upregulation of the PI3K/AKT pathway, increased production of protein similar to GATA 1, GLP1-induced insulin secretion by upregulation of the G protein-coupled receptor 43/41 (GPR43/41), proconvertase 1/3, and proglucagon activity in mice (Mishra et al., 2019).

Probiotics, therefore, reduce the risk of diabetes by regulating different cell signaling pathways and the expression of glucose metabolism hormones (Mishra et al., 2019). However, considering the health benefits of probiotics, there is a need to conduct high-quality clinical studies better to understand their effects on autoimmune diseases, including DM1.

24.6.4 Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic autoimmune disease that may lead to conditions of disability due to joint swelling, pain, stiffness, cartilage degradation, and osteoporosis (de Oliveira, Leite, Higuchi, Gonzaga, & Mariano, 2017). Genetic, epigenetic, and sex-specific factors, as well as smoking, air pollution, food, oral hygiene, periodontitis, increased fecal Prevotella, and imbalance in the gastrointestinal microbiota, are possible sources of onset or progression of rheumatoid arthritis, although the detailed mechanisms still need to be elucidated (Brusca, Abramson, & Scher, 2014; Paul et al., 2021).

RA manifests with increased fluid retention and migration of neuronal and inflammatory cells to joints, with increased secretion of pro-inflammatory cytokines (interleukins [IL]-1, IL-8, IL-12, IL-15, IL-17, IL-18, IL-29, and TNF-α) (Mateen, Zafar, Moin, Khan, & Zubair, 2016). RA is currently treated with some drugs such as non-steroidal anti-inflammatory, methotrexate (disease-modifying anti-rheumatic drug) used as a front-line drug for the treatment of RA, as well as hydroxychloroquine and chloroquine, which are antimalarial drugs and are also used for the treatment of RA. However, despite benefits such as pain and inflammation

reduction, all medications available in the clinical treatment of RA produce adverse reactions and may lead to severe toxicities (Paul et al., 2021). Thus, although still limited, there is growing interest in the possible use of probiotics in rheumatic diseases to improve quality of life, relieving symptoms, and as an alternative treatment (Vaghef-Mehrabany et al., 2014).

In this context, treatment with *L. casei* or *L. acidophilus* for 28 days supposedly prevented the development of arthritis in a preclinical model that reduced arthritic scores and pro-inflammatory cytokines such as IL-17, IL-1β, IL-6, and TNF-α, similar to indomethacin treatment (Amdekar, Singh, Kumar, Sharma, & Singh, 2013). Treatment with *Lactobacillus* spp. also increased the release of anti-inflammatory cytokines such as IL-4 and IL-10 in body fluids (Amdekar et al., 2013). The consumption of *L. acidophilus* and *L. casei* reduces the oxidative stress of animals with collagen-induced arthritis (Amdekar et al., 2013). Oral treatment with *L. casei* also induced anti-inflammatory effects in rats in a collagen-induced arthritis model by inhibiting the COX-2 enzyme by reducing pro-inflammatory cytokines (Amdekar et al., 2011).

In a placebo-controlled clinical trial, patients were randomized to receive *L. rhamnosus* GG (n = 8) or placebo (n = 13) for 12 months. Intervention with *L. rhamnosus* GG did not improve clinical symptoms or serum anti- and pro-inflammatory cytokines. However, ingestion of probiotics appeared to reduce the occurrence of swollen joints, although this effect was not significant (Hatakka et al., 2003). On the other hand, Zamani et al. (2016) reported that supplementation with probiotics resulted in better disease activity scores (observing 28 joints) in RA patients compared to placebo. A study by Chen et al. (2016) evaluated the intestinal microbiota profile in 40 RA patients and 32 healthy controls. A decrease in intestinal microbial diversity was observed in RA compared to controls, which correlated with disease duration and serum rheumatoid factor levels. Alipour et al. (2014) showed that an *L. casei* 01 supplementation decreased serum C-reactive protein levels, reduced sore and swollen joint counts, and improved overall health score. A significant difference was observed between the two groups regarding circulating levels of IL-10, IL-12, and TNF-α, in favor of the probiotic group.

In a meta-analysis, Mohammed et al. (2017) showed that the proinflammatory cytokine IL-6 was lower in volunteers with rheumatoid arthritis treated with probiotics compared to the controls treated with placebo. However, this study did not show an overall difference in clinical symptoms between the probiotic and placebo groups. Furthermore, in a meta-analysis, Rudbane et al. (2018) showed that among the included randomized clinical trials in which probiotics did not change inflammatory parameters (erythrocyte sedimentation rate), TNF-α, IL-1β, IL-6, IL-10, and IL-12) and indices of oxidative stress. The borderline significant reduction as a result of probiotic administration was determined only in C-reactive protein. Among the disease activity indices (disease activity score [DAS], sore joint count, and swollen joint count), DAS showed a significant improvement after treatment with probiotics. They also argue that the number of trials was too small to determine whether stress, dose, or duration-response effect was present. Thus, they suggest that probiotics seem to be less effective in RA; however, more studies are needed.

Another study by Liu, Zou, Zeng, Fang, and Wei (2013) aimed to investigate the human fecal lactobacilli community and its relationship with RA. By comparing quantitative polymerase chain reaction (PCR) in fecal samples from 15 RA patients and 15 healthy controls, the authors reported increased absolute numbers of *Lactobacillus salivarius*, *Lactobacillus iners*, and *Lactobacillus reminis* in untreated RA patients. Thus, they suggested a relationship potential between the lactobacilli community and RA development.

Clearly, more clinical trials are needed to confirm the effects of probiotics on immunity that have been observed in experimental models of arthritis. Indeed, evolving evidence suggests a relationship between altered intestinal microbiota and rheumatoid arthritis. However, more studies are needed to outline the microbiota profiles that may contribute to RA and the potential for treatment with adjuvant probiotics.

24.6.5 Infertility

Genital tract infections, both male and female, may lead to the development of several adverse effects, one of them being infertility, that is, the inability of a couple to become pregnant after a year of unprotected attempts (Motrich et al., 2018; Ravel, Moreno, & Simón, 2021; US National Library of Medicine, 2021b).

An inflammatory condition causes this infection-related infertility. Therefore, Bhandari, Rishi, and Prabha (2016) induced a vaginal inflammation with LPS and subsequently evaluated the use of the probiotic *L. plantarum* as an inflammatory modulator and consequent treatment for infertility. The researchers observed that higher levels of LPS (20 μg), that is, a more intense inflammation, resulted in infertility, while low levels of LPS (5 to 10 μg) were not enough to prevent pregnancy in animals. Also, the topical use of the probiotic *L. plantarum* effectively prevented the development of the inflammatory condition, allowing the pregnancy of 100% of the tested animals (Bhandari et al., 2016).

The promising results reinforce the importance of the vaginal microbiota for the development of pregnancy, as well as suggest that the use of probiotics may be used for the treatment of microorganisms that transmit sexual diseases related to infertility, such as *N. gonorrhoeae* and *C. trachomatis* (Bhandari et al., 2016). However, more studies on this topic are needed, especially in humans.

24.6.6 Myasthenia gravis

Myasthenia gravis is an autoimmune disease that attacks nervous signals and, consequently, voluntary muscles, causing a feeling of weakness, and difficulty in movement. This weakness worsens with the performance of physical activities, requiring the patient to remain at rest (US National Library of Medicine, 2021c).

In an animal model, offering the probiotics *Lactobacillus crispatus*, *L. rhamnosus*, and two strains of *Bifidobacterium animalis* subsp. *lactis* has been demonstrated to lessen the characteristic symptoms of myasthenia gravis, assessed through a decrease in the clinical score and an increase in body weight gain. Also, histologically, there was a reduction in myelin loss in the spinal cord and a reduction in astrocytosis, which means a reduction in the inflammatory process. Finally, the authors evaluated the immunomodulatory capacity of probiotics, observing DC maturation and increased expression of CCR7, TGF-β, IL-10, and IL-6 (Consonni et al., 2018). This indicates that the tested probiotics were able to modulate and generate immune tolerance in animals, leading to the control of myasthenia gravis.

A similar result was found by Rinaldi et al. (2019), who evaluated the same probiotics and animal model. In this case, the authors found a reduction in AChR antibodies in the group treated with probiotics, emphasizing that this antibody is responsible for developing neurological alterations that are characteristic of the disease. Also, in relation to the intestinal

microbiota composition, the animals that consumed probiotics had higher diversity and microbial balance (Pielou index), with bifidobacteria being responsible for restoring the microbial balance. The sick animals reduced the phyla *Tenericutes* and *Verrucomicrobia*, which was expected to indicate dysbiosis and intestinal inflammation (Rinaldi et al., 2019).

The obtained results are promising, indicating that probiotics can modulate the intestinal microbiota and the immune system, thus changing the outcome of the disease.

24.6.7 Sjogren's syndrome

One of the most common autoimmune diseases (Marietta et al., 2020; Tsigalou, Stavropoulou, & Bezirtzoglou, 2018), Sjogren syndrome, is characterized by the damage of exocrine glands and extra-epithelial tissues. It is a disease that is difficult to diagnose and treat, and its pathophysiology is not yet fully understood. Yet, its occurrence is related to genetic and environmental factors (Tsigalou et al., 2018).

In this syndrome, there is a lymphocytic infiltration in the salivary and lacrimal glands, which causes these organs to remain dry, denoting a classic symptom of this autoimmune disease (Kamal, Kandil, Eissa, Yousef, & Elsaadany, 2020; Meulen et al., 2016; Moon, Yoon, Choi, & Kim, 2020; Moutsopoulos, 1994; Tsigalou et al., 2018). Furthermore, due to this immune dysfunction, patients with Sjogren's syndrome are more likely to develop lymphomas (Meulen et al., 2016; Tsigalou et al., 2018) and oral candidiasis (Meulen et al., 2016).

The mechanism of action of Sjogren's syndrome involves the release of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-6, IL-10, IL-17, and TNF-α, generating chronic inflammation, which over-activates lymphocytes B, triggering the production of autoimmune antibodies that attack tear and saliva production (Meulen et al., 2016; Moon et al., 2020; Tsigalou et al., 2018). In addition, CD8 T cells destroy tear ducts (Meulen et al., 2016; Moon et al., 2020), resulting in the disease's ocular and salivary dryness characteristics.

As in other autoimmune diseases, recent studies show that there is an intense connection between microbiota and Sjogren's syndrome, noting that imbalances in the intestinal microbiota are associated not only with intestinal or systemic but also with ocular inflammation (de Paiva et al., 2016; Moon et al., 2020). In addition, in cases of Sjogren's syndrome, changes in microbial diversity and microbial profile are observed compared to a healthy pattern (Moon et al., 2020).

This suggests that the use of probiotics and, consequently, the intestinal balance could be a way to control this disease. In these patients, it is believed that probiotics may act on Treg, preventing the self-activation of T cells and restoring the balance between TCD8 and TCD4 cells, which would not result in autoimmune disease (Tsigalou et al., 2018). Also, a condition of intestinal dysbiosis may stimulate the production of autoantibodies, and the use of probiotics can correct this dysfunction (Moon et al., 2020).

Probiotics may also be used to treat other symptoms resulting from the syndrome, contributing to the well-being of patients with this disease. For example, in a study by Kamal et al. (2020), patients with Sjogren's syndrome treated with Lactobacillus acidophilus, Lactobacillus bulgaricus *S. thermophilus*, and *B. bifidum* for five weeks had a significant decrease in oral candidiasis. Furthermore, no patient treated with a mix of probiotics had erythema, unlike the placebo group, in which two patients were affected (Kamal et al., 2020). Given this potential, it is suggested that further studies evaluate the use of probiotics in the treatment of Sjogren's syndrome.

24.7 Conclusion

As discussed throughout this chapter, probiotics can both prevent and treat different diseases, and their use is interesting and necessary. However, one cannot lose sight of how these bacteria act, whether by immunological or non-immunological mechanisms and depending on the clinical context in which they are administered. For example, they may increase innate immunity and reduce inflammation induced by pathogens to promoting intestinal epithelial cell survival. Furthermore, we emphasize the importance of the intestinal epithelium and the main immune cells involved in intestinal homeostasis and, consequently, in strengthening the immune system under disease conditions.

Notably, the intestine has a complex and differentiated immune system that allows it to tolerate a massive load of dietary antigens and commensal microorganisms, such as SLT, which is widely distributed in various parts of the intestine forming GALT. We also highlight the TLRs and Nod-type receptors, the MAMP recognizer receptors, in addition to the metabolites arising from the process of metabolizing probiotics (SCFA, lipopolysaccharides, peptidoglycan fragments, lipoic and lipoteichoic acid, flagellin, and formylate peptides).

The probiotic doses used in treating diseases and their exact therapeutic effect still raise doubts based on the controversies of the results presented according to different specific strains. However, probiotics are known to manage the microbiome and, therefore, have been considered a potential therapeutic adjuvant for inflammatory intestinal diseases such as Crohn's and celiac diseases. In the latter, specifically, probiotics such as *L. fermentum*, *L. casei*, and *B. lactis* generate protection against the adverse effects of gliadin, one of the protein fractions of gluten. Furthermore, the therapeutic use of *L. rhamnosus* MTCC:5957 and MTCC:5897, in addition to *L. fermentum* MTCC:5898 in DM1 studies, has shown improvement in glucose metabolism changes caused by oxidative stress and in the status of serum inflammation and profile serum lipid in experimental studies.

Probiotics have been tested as prophylactics in some diseases or for use in therapeutic supplements for the human gastrointestinal tract, based on many in vivo studies that support their roles in this target organ, as well as in the complex tuning of the gastric microbiota or even its link between the intestine-brain axis. The current state of studies makes us think that we should not increasingly dissociate the action of probiotics from human eating habits, transcending their use beyond dairy products. A significant challenge for the food industry is associating probiotics with products that are easily accessible to the population, aiming to improve health. Therefore, health professionals must have a critical sense and responsibility when prescribing any supplement or probiotic. Moreover, it is necessary to disseminate scientific knowledge and create guidelines, so that professionals may be informed about the possible risks of products recognized only by their positive appeal.

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CHAPTER

25

Probiotics for liver disease prevention and treatment

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25.1 Introduction

Liver diseases (LD), such as nonalcoholic fatty liver disease (NAFLD), including non-alcoholic fatty liver (NAFL), non-alcoholic steatohepatitis (NASH), associated cirrhosis, and hepatocellular carcinoma, are considered one of the major causes of disease and death in the world (Wang, Fan, Zhang, Gao, & Wang, 2014). The growing increase in these pathologies, despite the development of vaccines and antiviral agents, is due to the increasing influence of modulating health factors such as a sedentary lifestyle and poor eating habits (Xiao et al., 2019).

It is known that the consumption of foods, especially those considered obesogenic, can cause dysbiosis, a condition in which changes in the composition of the microbiota and the function of the intestinal barrier are observed. This dysbiosis favors metabolic endotoxemia and low-grade inflammation related to pathologies such as LD (Acharya & Bajaj, 2017). The term "gut-liver axis" comes from the bidirectional relationship between gut and liver in terms of its anatomy and functionality with a complex metabolic, immunological, and endocrine interaction. This interaction ensures homeostasis but also interferes with the development of pathologies (Milosevic et al., 2018).

In summary, the interference of microbiota in the development of LD occurs when, under dysbiosis conditions, metabolites considered antigens (also known as pathogen-associated molecular patterns (PAMPs), produced by pathogenic microorganisms or from food, can cross the intestinal barrier and be initially recognized by dendritic cells or can activate the adaptive immune system. Even in low concentrations, these antigens, such as

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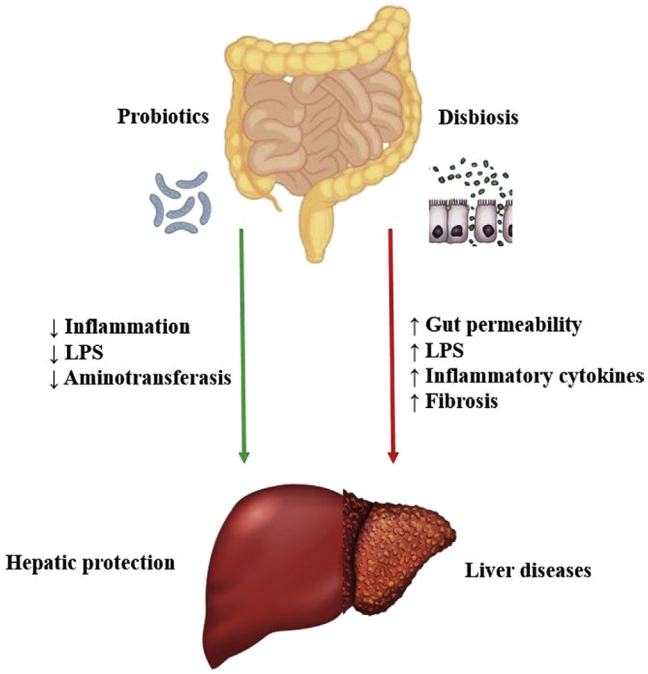


FIG. 25.1 Influence of the intestinal microbiota on the development and protection of liver diseases.

lipopolysaccharides (LPS), can lead to the production of inflammatory cytokines and chemokines that enters portal circulation. This inflammatory response occurs since LPS activates nuclear factor kappa B (NFKβ) through toll-like receptors (TLRs) and nod-like receptors (NLRs) (Yiu, Dorweiler, & Woo, 2017). In addition, LPS interacts with hepatocytes and Kupffer cells through hepatic TLR and NLRs resulting in the promotion and progression of fibrosis (Seki & Schnabl, 2012).

Given this interaction between microbiota and LD, the manipulation of the microbiota with the use of probiotics has been under investigation. The beneficial effects of probiotics are thought to include restoring intestinal homeostasis, strengthening intestinal barrier epithelium, regulating the immune system, and inflammation which leads to preventing gut-microbiota-related diseases. Most of these mechanisms are promoted by gene regulation, particularly in the intestine and liver (Plaza-Diaz, Gomez-Llorente, Fontana, & Gil, 2014). As shown in Fig. 25.1, probiotics improve intestinal microbiota (MI) composition, which ameliorates liver inflammation, decreases LPS concentrations, and improve aminotransferase concentrations (Mokhtari, Gibson, & Hekmatdoost, 2017; Wong et al., 2013). In this chapter, we discuss the main experimental and clinical findings using probiotics as a proposed treatment for various liver disorders.

25.2 Probiotics for treatment of NAFL

NAFL is an important cause of chronic liver dysfunction worldwide and refers to excessive accumulation of fat liver in the absence of a history of alcohol abuse or other secondary

causes (Zhang, Ji, Wang, & Li, 2018). Although studies on microbiota composition and development of NAFL are scarce, evidence has pointed to the role of dysbiosis in the development of this pathology, especially with regard to the immune response (Zheng, Liwinski, & Elinav, 2020).

Studies have been proposed to use probiotics as a NAFLD treatment strategy. In an animal model of diet-induced NAFLD, the researchers used MIYAIRI 588, a strain of *Clostridium butyricum*. This bacterium produces butyrate and is found in intestine of healthy animals and humans. The administration of 8.5 × 109 cfu/g for 8, 16, and 50 weeks produced a reduction of triglyceride deposits, improvement in insulin resistance, lower serum levels of endotoxins, and lower hepatic inflammatory indices. In addition, animals showed greater expression of proteins associated with lipolysis and junction proteins, reducing oxidative stress markers. Data suggest that MIYAIRI 588 prevented the progression of NAFLD (Endo, Niioka, Kobayashi, Tanaka, & Watanabe, 2013).

A double-blind randomized controlled pilot study with NAFLD patients was conducted using a treatment with *Lactobacillus bulgaricus* and *Streptococcus thermophilus* in a dose of 500 million units for 3 months. Compared to the placebo group, patients who received probiotics showed improvement in aminotransferases concentration (alanine aminotransferase – ALT and aspartate aminotransferase – AST) (Aller et al., 2011). An Iranian study also demonstrated the beneficial effects of probiotics on non-alcoholic fatty liver disease. In patients aged between 23 and 64 years, yogurt consumption containing *Lactobacillus acidophilus* La5 and *Bifidobacterium lactis* Bb12 in the amount of 300 mg/day for 8 weeks was investigated. The data point out a reduction in serum levels of LDL-cholesterol compared to the group that received conventional yogurt. In addition, probiotic consumption was effective to reduce aminotransferases (ALT and AST) levels, which improves liver functionality (Nabavi, Rafraf, Somi, Homayouni-Rad, & Asghari-Jafarabadi, 2014).

In 2018, a randomized clinical trial was conducted using an administration of "Symbiter" (concentrated biomass of 14 probiotic bacteria that includes Bifidobacterium, Lactobacillus, Lactococcus, Propionibacterium) or placebo for 8-week; 1 sachet (10 g) per day for diabetic patients with NAFLD. Probiotics use modulated intestinal microbiota, significantly decreasing liver fat, aminotransferase levels, and an inflammatory biomarker (TNF-α and IL-6) whereas restored IL-10 (Kobyliak et al., 2018).

More recently, patients with liver biopsy-proven NAFLD underwent ingestion for 1 year of probiotic capsules (Each capsule containing 112.5 billion live bacteria such as *Lactobacillus paracasei* DSM 24733, *Lactobacillus plantarum* DSM 24730, *Lactobacillus acidophilus* DSM 24735, and *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 24734, *Bifidobacterium longum* DSM 24736, *Bifidobacterium infantis* DSM 24737, *Bifidobacterium breve* DSM 24732, and *Streptococcus thermophilus* DSM 24731. The data showed an improvement in the histological imaging of the liver, in serum ALT levels, and in the profile of inflammatory cytokines (Duseja et al., 2019).

In a randomized, double-blind placebo-controlled study, a probiotic mix containing 109 cfu/1.4 g was used (*L. acidophilus* CBT LA1, *L. rhamnosus* CBT LR5, *L. paracasei* CBT LPC5, *P. pentosaceus* CBT SL4, *B. lactis* CBT BL3, and *B. breve* CBT BR3) for 12 weeks. The results showed that obese patients with NAFLD after using this formulation had reduced triglycerides serum levels and a lower percentage of intrahepatic fat (Ahn et al., 2019). Besides, probiotic use promotes a better impact on leptin levels and glycemic parameters in obese patients

using *L. casei*, *L. rhamnosus*, *L. acidophilus*, *B. longum*, and *B. breve* combined with lifestyle intervention (Behrouz, Jazayeri, Aryaeian, Zahedi, & Hosseini, 2017).

Although the mentioned beneficial aspects for NAFL treatment are associated to probiotics use by experimental and clinical studies, the mechanism of action is not fully understood (Yao et al., 2021).

25.3 Probiotics in the treatment of NASH

Non-alcoholic steatohepatitis is characterized by fatty acids deposit, inflammation, hepatocyte injury (known as ballooning), and eventual fibrosis (Chalasani et al., 2018). Velayudham et al. (2009) demonstrated that VSL#3 administration (mix with 8 probiotics, being *S. salivarius* subsp. *thermophilus*, *Bifidobacterium* (*B. breve, B. infantis, and B. longum*), *L. acidophilus*, *L. plantarum*, *L. casei*, and *L. delbrueckii* subsp. *bulgaricus* for 9 weeks modulated liver fibrosis, but no effect on reducing inflammation on NASH. Despite this, a study also conducted with animals and using the same probiotic mix revealed additional beneficial effects on NASH. Mencarelli et al. (2012) suggest that the administration of VSL#3, 6x a week for 3 months, reduced biomarkers of inflammation and liver fibrosis in mice.

In a more recent study, using an animal model with induction of NASH, the administration for only 10 days of a probiotic formulation containing *Bifidobacterium lactis*, *Lactobacillus bulgaricus*, and *Streptococcus thermophilus* associated with blueberry juice resulted in a reduction of apoptosis and inflammation in hepatocytes. In addition, there was an improvement in the antioxidant activity with a reduction in lipid peroxidation (Ren, Zhu, Zhu, & Cheng, 2017).

In humans, VSL#3 was used for 4 months in obese children who had a proven diagnosis of NASH. After supplementation, the children showed, in addition to improvement in BMI, less likelihood of steatohepatitis worsening (Alisi et al., 2014). Using a different probiotic, Malaguarnera et al. (2012) compared treatment with *Bifidobacterium longum* associated with fructooligosaccharides (FOS) with those patients who only underwent lifestyle modification (i.e., diet and exercise). After supplementation for 24 weeks, patients showed a reduction in TNF-α, C-reactive protein, AST, circulating endotoxins, steatosis, and NASH activity index. A Chinese study using treatment with a probiotic formula containing *Lactobacillus plantarum*, *Lactobacillus deslbrueckii*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus* and *Bifidobacterium bifidum* in patients diagnosed with NASH, supplementation for 6 months was able to reduce fat liver content and improve AST levels when compared to the placebo group (Wong et al., 2013).

In a recent review, Yao et al. (2021) summarize an update either on the efficacy of probiotics for the treatment of hepatic steatosis disease, illustrating results from clinical and experimental studies. Downregulation of lipogenesis, reduction of fat liver, and parameters associated with NASH diseases such as Body mass index (BMI), acid uric serum levels, and a NASH index were observed after administration of a symbiotic compound. Besides, probiotic use including six strains of lactobacillus and three strains of Bifidobacterium has been associated to reduce fat liver deposition through upregulation of SFCA receptor. It has been proposed that no conventional probiotics could promote decreased risk for metabolic disorders and obesity. These probiotics include *A. muciniphila*, *Bacteroides* spp, *F. prausnitzii*,

Roseburia (R.) intestinalis, *R. hominis*, *R. inulinivorans*, *R. faecis*, and *R. cecicola* and, although this subject needs more studies to elucidate the beneficial effects on NASH and NFL, we can consider these strains for treatment of hepatic inflammation, and fat liver deposit. An example of a new probiotic is *A. muciniphila* and their metabolites, propionate, and acetate, which interfere with lipid metabolism through epigenetic aspects and/or activating or silencing genes expression.

25.4 Probiotics in the treatment of cirrhosis

Cirrhosis is the final common consequence of liver damage resulting from various liver pathologies. Although its etiology is multifactorial in nature, the pathology has as common features necrosis of hepatocytes, in which liver parenchyma was replaced by fibrous tissue with nodules formation and consequent decreased liver functionality (Zhou, Zhang, & Qiao, 2014).

In an animal model of carbon tetrachloride-induced cirrhosis, 46 rats were randomized into 2 groups: group VSL#3 in drinking water (n = 22) and a group receiving only drinking water (n = 24). Daily doses, administered for approximately 3.5 weeks, were calculated according to the weight of the rats: 0.0128 × 109 bacteria per g of body weight. The results demonstrate that the administered VSL#3 decreased bacterial translocation, inflammatory state, as well as ascites formation. On the other hand, no improvement in liver damage was found (Sánchez et al., 2015). Using the same experimental model of cirrhosis induction, a recent study demonstrated that the administration of VSL#3 for 8 weeks reduced inflammatory cytokines and collagen fiber hyperplasia with a reduction in the degree of cirrhosis (Feng et al., 2021).

In humans, probiotic therapy with VSL#3 has also been used in cirrhosis (Dhiman et al., 2014; Gupta et al., 2013; Jayakumar et al., 2013). A more recent work using a formulation with 8 probiotics (*Streptococcus thermophilus* DSM 24731, *Bifidobacterium breve* (*B. breve*) DSM 24732, *B. longum* DSM 24736, *B. infantile* DSM 24737, *Lactobacillus paracasei* (*L. paracasei*) DSM 24733, *L. acidophilus* DSM 24735, *L. delbrueckii* subsp *bulgaricus* DSM 24734, and *L. plantarum* DSM 24730, in a total dose of 450 10^9 live bacteria, demonstrated improved cognitive function, decreased risk of falls and modulation of the systemic inflammatory response in patients with cirrhosis (Román et al., 2019).

Despite the promising effects of probiotics in the maintenance or improvement of liver function in patients with stable cirrhosis, especially the immune function, the effects, however, on the intestinal barrier and bacterial translocation still seem discreet (Horvath et al., 2016).

25.5 Probiotics in the treatment of hepatocellular carcinoma

Hepatocellular carcinoma (HCC) is the most common primary malignant liver tumor, usually developing in the LD context (Calderaro, Ziol, Paradis, & Zucman-Rossi, 2019). Among the major risk factors for developing HCC are smoking, alcohol abuse, chronic hepatitis B, C, and D virus infection, obesity, diabetes mellitus, mycotoxin liver damage, in addition to NAFL and NASH (Ghouri, Mian, & Rowe, 2017; Younes & Bugianesi, 2018).

Mice were used to investigate the effect of fermented milk containing *L. rhamnosus* GG administration and *Lactobacillus casei* strain Shirota and chlorophyll in animal-induced HCC. After 6 weeks of probiotic use, a reduction in the incidence of tumor and lower expression of genes associated with oncogenesis was found, suggesting a protective effect of the strains against hepatocarcinogenesis (Kumar et al., 2011). Later, also using an animal model of induced HCC, Li et al. (2016) demonstrated that supplementation with "Prohep" (composed formulation of *Lactobacillus rhamnosus* GG (LGG), viable *Escherichia coli* Nissle 1917 (EcN), and heat-inactivated VSL#3 (1:1:1)) for approximately 38 days was able to reduce liver tumor growth. The researchers suggest that this effect is associated with microbiota modulation, reducing the secretion of IL-10 molecule (anti-inflammatory), and suppressing the differentiation of Th-17, a T-cell subtype responsible for increasing tumor microenvironment.

Similarly, in a study with patients diagnosed with HCC, intestinal modulation with probiotic VSL#3 proved to be a promising therapeutic target as it improved dysbiosis and intestinal inflammation, and more importantly, reduced liver tumor multiplication and growth (Zhang et al., 2012).

Among proposed mechanisms, one review pointed out that probiotic use can attenuate the risk of hepatocarcinoma by modulating intestinal host microbiota. Thus, improvement of dysbiosis and the growth of beneficial bacteria to health result in preventing liver inflammation mediated by pathogen-associated molecular patterns (PAMPs). In addition, probiotics have antiviral activity against hepatitis B and C virus infections, as well as antioxidant, antiproliferative, antiangiogenic, and antimetastatic effects that can prevent the onset of hepatocellular carcinoma. In addition, they up-regulate the expression of tumor suppressor genes and down-regulate the expression of tumor-promoting genes (Thilakarathna, Rupasinghe, & Ridgway, 2021).

25.6 Probiotics in the treatment of hepatic encephalopathy

Characterized by changes in personality, consciousness, cognition, and motor function, hepatic encephalopathy (HE) is a significant complication of severe, acute, or chronic liver failure (Weissenborn, 2019). It is recognized that dysbiosis with increased intestinal permeability, bacterial translocation, and subsequent inflammatory response are among the factors implicating cognitive dysfunction in established LD (Clària et al., 2016; Felipo et al., 2012).

In a previous Indian study, researchers compared the results obtained between patients with decompensated cirrhosis on treatment with probiotics or without treatment. During a 3-month follow-up study, 8% of patients using probiotics developed HE compared to those without probiotic use (19%) (Mittal, Sharma, Sharma, & Sarin, 2011). A meta-analysis evaluated the efficacy of probiotics in relation to lactulose, which data demonstrates that both treatments are effective in the treatment of minimal HE (Shukla, Shukla, Mehboob, & Guha, 2011). Some mechanisms proposed include microbiota change by colonic acidification and fiber fermentation, immune system effect, decreased pathogenic bacteria adhesion, and attenuation of ammonia-producing bacteria, which leads to decreased ammonemia (Moran, López-Sánches, Milke-García, & Rodríguez-Leal, 2021).

In a more recent study, probiotic supplementation containing *Clostridium butyricum* and *Bifidobacterium infantis* strains was used in the treatment of minimal hepatic encephalopathy in patients with liver cirrhosis induced by the hepatitis B virus. 67 patients were evaluated

for 3 months, with 30 patients receiving the probiotic and 37 receiving placebo. At the end of the study, it was seen that patients who used the probiotic showed an improvement in the relationship between beneficial and pathogenic bacteria, intestinal barrier permeability, and cognitive function (Xia et al., 2018). Román et al. (2019), as previously mentioned, observed that the use of a formulation containing 8 probiotics improved the cognitive function of patients with cirrhosis and with proven associated cognitive dysfunction.

Previously, a Cochrane review analyzed 21 clinical trials, which included more than 1400 participants evaluating the use of probiotics in the treatment of HE. The findings point to beneficial effects of improving symptoms when compared to those with no therapy or placebo. However, most studies pointed out many biases, thus leading to the conclusion of the data being of low quality (Dalal, McGee, Riordan, & Webster, 2017).

Recently, Moran et al. (2021) analyzed clinical trials studies in order to approach current alternatives to the treatment of minimal HE. Beyond strategies including maintenance of nutritional status and reduction of sarcopenia, the utilization of different probiotics was performed. However, the absence of a standard protocol for diagnosis of HE and multiple therapeutic treatments observed in the literature cause difficulties to elucidate the real impact of these variables on health.

25.7 Conclusions

Evidence of changes in the intestinal microbiota may contribute to the pathogenesis of liver diseases. Modulation of the microbiota with the use of probiotics is a promising strategy to treat these diseases. Further experimental and clinical investigations are needed to evaluate for which strains, dosages, and duration of use are suitable for the prevention and treatment of liver diseases in their various forms. The current lack of these investigations prevents recommendations on the use of probiotics in clinical practice.

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Probiotics for Human Nutrition in Health and Disease

Presents the most updated information and potential healthcare applications and perceptions for using probiotcs for human nutrition

Probiotics for Human Nutrition in Health and Disease provides a comprehensive resource of information about the traditional and emerging health concepts, development, and application evolution of probiotics and their role in prevention and treatment of human metabolic disorders and illnesses. Key issues related to general aspects of probiotics, probiotics in human nutrition, and probiotics in human health promotion and disease treatment are described and discussed in three sections. This book provides the most significant knowledge, mechanistic bases, uses, clinical perceptions, case studies, and perspectives about probiotics for humans considering possibilities and limitations in the light of the appropriate available reference materials.

Written by highly qualified researchers and edited by a team of experts, each chapter summarizes the latest available information on probiotics in human health and presents and critically interprets the most significant evidence by applying the author's own practical experience from investigations with probiotics. The book's first section describes general features of probiotics, such as its relationship with prebiotics, its role in bioactive metabolite production, and its functions as a supplement. The second section describes probiotics specifically interacting with human nutrition, including pregnancy, lactation, in children, and in the elderly. The third and final section of the book assesses the role of probiotics in human health promotion and disease treatment, in such cases as periodontal health, gastrointestinal surgery, obesity and cardiovascular disease, and cancer prevention. This book is a valuable and comprehensive resource for researchers and practitioners involved in the field of human nutrition and will benefit academics, students, consultants, and industry professionals interested in the subject.

Key Features

- Presents traditional and emerging concepts, development, and application evolution of probiotics
- Provides key insights characterizing probiotics as promising innovative options for human nutrition
- Discusses how probiotics can be used in a perspective of health promotion and prevention and treatment of human metabolic disorders and illnesses

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