Functional Foods

Sources, Health Effects and Future Perspectives

Food Science and Technology

David L. Nelson
Editor

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FOOD SCIENCE AND TECHNOLOGY

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PREFACE

Food has significant societal, historical and religious roles, in addition to nutritional value. It represents cultures and customs, provides opportunities for sharing, giving and social interaction and offers nutrition, pleasure and satisfaction. Technically, all foods are functional, as they fulfill a basic human need and provide nutritive value. However, the term 'functional food' implies an additional physiological benefit beyond meeting basic nutritional needs. Functional food is not only a dietary product providing basic nutritional function of supplying nutrients but it is also known as a health-promoting and/or disease-preventing substance. Functional food has been recognised as a separate category in the food market and it is one of the world's most intensive areas of food product innovation. This book discusses the sources, health effects and future perspectives of functional foods.

Chapter 1 - New developments on edible films and coatings are gaining the interest topics of researchers in food science, consumers and the food industry. The term edible film refers to a biodegradable matrix performed by the use of natural biopolymers that could form a network. This network is applied onto the surface of foods products, spraying, dipping or used as the shell of microcapsules. Edible films have been introduced as oxygen and moisture barrier and also used as very good carriers for the delivery of several bioactive compounds such as vitamins, antioxidants, antimicrobials and probiotics in functional food systems. The most used matrices for this purpose are gelling polysaccharides (starch, cellulose, β-glucan, alginate, pectins, carrageenan, chitosan), proteins (whey, soy, gelatin, casein) and lipids. When used as carrier, the selection of the material is linked to their physicochemical properties and the interaction between the host compound and the media where it should express their activity. For example, for drugs delivery or probiotics treatment, the material should protect the active compound from acidic digestion (pH 1.2-1.5) but dissolves at around pH 7.5 in the intestine. However, some biopolymers present bioactivity by themselves, without the addition of any compound, e.g.,: chitosan and β-glucan. This review is aim to describe the alternatives used to perform functional systems by using biopolymers combined with functional compounds.

Chapter 2 - Double or water-in-oil-in-water ($W_1/O/W_2$) emulsions are complex systems consisting of an aqueous phase dispersed in a lipid phase, which in turn is dispersed in a continuous aqueous phase. Because these systems include water within the dispersed lipid phase, they have been proposed for the development of lipid-reduced food emulsions without the need of reducing the volume fraction of dispersed phase. The other potential application of $W_1/O/W_2$ emulsions is the encapsulation of substances in the inner water droplets for their

isolation, protection or controlled release. The limits of these systems are given by the volume of water droplets that can be retained within the oil droplets and the minimum required size of oil droplets, which should be large enough to contain smaller water droplets. These conditions imply that $W_1/O/W_2$ emulsions present new issues regarding the stability of the system, adding different destabilization processes that should be controlled and measured. Different strategies have been implemented to increase the volume and stability of the inner water droplets, such as the generation of an osmotic gradient by the addition of solutes in the dispersed aqueous phase or the inclusion of fat crystals in the dispersed lipid phase. With respect to proper applications of $W_1/O/W_2$ emulsions for the production of functional foods, different objectives have been boarded: encapsulation of vitamins, minerals or microorganisms; formulation of low-calorie foods; control of taste perception; etc. Moreover, different methods have been developed for the analysis of $W_1/O/W_2$ emulsions, including spectrophotometry, conductimetry and differential scanning calorimetry techniques.

Chapter 3 - Bread is one of the major constituents of contemporary man's diet. The main microbiological problems of bread industry are mold and bacterial spoilage (roping) of bread. Moreover, the increasing environmental deterioration results in a significant increase in the insemination level of typical raw materials used in bread production. The manufacturers' solution is the extensive use of preservatives. Nowadays, the consumers' health awareness led to the increasing demand for preservative-free food products, but with retained or improved organoleptic characteristics and extended shelf life. The healthier alternative to preservatives in bread industry is the development and introduction of sourdough starters containing selected homo- and heterofermentative lactic acid bacteria and propionic acid bacteria strains.

Fourteen lactobacilli strains were isolated from different sources and were identified to species level using biochemical (API 50 CHL) and molecular-genetic (ARDRA-analysis and 16S rDNA sequencing) methods. The studied lactobacilli strains were able to grow in flour/water environment and reached high concentrations of viable lactobacilli cells. They also inhibited the growth of typical bread saprophytic microorganisms - *Bacillus subtilis, Aspergillus niger, Penicillium* sp., *Rhizopus* sp., but did not inhibit baker's yeasts *Saccharomyces cerevisiae*.

Fourteen sourdough starters for rye and wheat bread were developed on the basis of the selected strains of homo - and heterofermentative lactobacilli with the addition of the probiotic propionic acid bacteria strain *Propionibacterium freudenreichii* ssp. *shermanii* NBIMCC 327 and *Lactobacillus sanfranciscensis*. Bread with best organoleptic characteristics was obtained with the incorporation of 7% of the four-strain starter sourdough.

For the prevention of microbial bread spoilage the percentage of the selected best performance four-strain starter sourdoughs was between 10% and 15% for the prevention of bread roping (bacterial spoilage) and between 15% and 20% for the prevention of fungal growth. The developed starter sourdoughs could be included in the kneading of the main dough in the form of liquid sourdoughs, frozen sourdoughs or freeze-dried starter concentrates.

A biotechnological scheme for the production of „LB-acidifiers" (dry sourdough) for rye and wheat bread with the best sourdough starters for rye and wheat bread was developed. The obtained „LB-acidifiers" are applied in bread production in a concentration of 3% - 5% in order to obtain bread with improved organoleptic characteristics, extended shelf life (5 days), without the addition of preservatives.

The development and application of lactobacilli sourdough starters in bread manufacture ensures the conduction of targeted fermentation process, the microbiological safety, the improved flavor and the extended shelf life without preservative addition, thus enhancing the beneficial effects of bread consumption.

Chapter 4 - Functional Food contains known biologically-active compounds which, in defined quantitative and qualitative amounts, provide a clinically confirmed and documented health benefit, and thus, providing an important source in the prevention, management and treatment of chronic nowadays diseases. Child nutrition is a worldwide concern. In South America, according to World Health Organization database (WHO), an average of 3% of children under 5 years old are underweight. The problem is particularly severe in countries such as Bolivia and Peru (http://www.who.int/nutrition/databases/infantfeeding/ en/). The numbers are even higher in other continents such us Africa and conflict zones like Afghanistan where 30% of children under 5 years old are underweight.

This chapter deals with the design and the structural and functional characterization of bioactive compounds (BC) as functional food ingredients for an industrial application in drinkable food such as chocolate milk. The BC is better if added through natural vehicles like liposomes and more suitable if made of natural products such as soy lecithin. Why liposomes? They can contain BC (PUFAs and vitamin E) and other vitamins like folic acid (FA) or vitamin C (VC) for the fortification of chocolate milk and ions such as calcium increasing nutritional value of drinkable foods. The chocolate milk has the advantage over traditional milk to provide a pleasant taste and smell for most of consumers, especially for the children's sector.

When new functional foods are designed, the characterization, stability and sensorial evaluation is a necessary step. Characterization of liposomes includes oxidative stability, flavour and appearance by using wide variety of methodologies. Oxidative stability (thiobarbituric acid and ORAC methods) followed by characterization studies is a necessary step to be performed in food-model systems in order to reach the market under local regulations and to cover health prevention. It was found that vehicles like liposomes showed significant oxidative stability and a protective effect over different hydrophobic and hydrophilic compounds. The authors will specifically touch basis on thermolabile vitamins like Folic Acid and Vitamin C.

Moreover, functional foods to enrich different levels of high-risk population (children and elderly), sensory evaluations must be performed in all final products, and they should demonstrate acceptability in general positive effects. One way is to perform sensory evaluation using commercial milk and adjoining liposomes with BC.

Currently, the application of functional foods and consumption has been gaining increasing interest among consumers and researchers because studies have been associated with a positive direct effect on human health. Indeed, the incorporation of bioactive compounds into drinkable foods can become an alternative to protect and enhance health care of two main stages in human life: children and elderly.

Therefore, the aim of this review is to describe the potential use of natural lipids as stabilizing matrices holding BC, counteracting the disadvantages of direct application and enhancing the functional properties of drinkable foods through the incorporation of BC.

Future perspectives are discussed based on the requirements to focus on studies on this subject in order to develop new functional foods applications with improved functionality and

high sensory performance with enhanced shelf life and nutritional quality and with increased consumer acceptance.

Chapter 5 - Probiotics are living microorganisms which, when ingested in certain amounts, have a positive impact on human health, mainly due to their roles in improving the balance of the intestinal microflora.

On the other hand, the prebiotic are food ingredients that may also have a positive impact in the improvement of the intestinal flora. These components, which fall into the category of fibers, are not digested in the upper gastrointestinal tract, and therefore reach the colon where they stimulate the growth and/or the activity of some types of bacteria. The term synbiotic is used for products that contain both probiotics and prebiotics, thus taking advantage of both the addition of beneficial bacteria and the encouragement of the growth of resident beneficial bacteria.

The present chapter aims to review the scientific literature related to prebiotics, probiotics and synbiotics, including their identification, properties and health benefits.

Chapter 6 - Yogurt has long been known as a product with many desirable effects for health. The excellent sensory properties and the health benefits of yogurt can be credited to the action of yogurt bacteria and their metabolites. Probiotic is a dietary supplement of live microorganism that contributes to the health of the host. The most common types of microbes that used as probiotic are lactic acid bacteria (LAB) particularly Lactobacilli, Streptococci and Bifidobacteria. They are the most important microorganisms associated with the health status of human gastrointestinal tract which justifies the reason for calling them friendly bacteria. Therefore, this study is to investigate the inclusion of probiotics in yogurt as functional food.

Chapter 7 - Yogurt supplies the organism with proteins, lipids, carbohydrates, vitamins and microelements and imports active yogurt microflora. Therefore traditional yogurt is a suitable basis for the development of new functional foods.

Starter culture development, including strain property studies and development of symbiotic relationships between the strains in the starter composition, is of paramount importance in yogurt manufacture. Any failure in the starter development will, in most cases, lead to detrimental effects on the quality of the final product. One of the aims of the present chapter is to present the authors' experience in the development of yogurt starters, including the selection of strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* and the development of symbiotic starters for traditional yogurt.

Nowadays, most consumers choose foods not only based on their flavor and direct nutritional value, but also on their potential health benefits. Functional foods fulfill the organism's nutritional needs and improve its general condition (e.g., pre- and probiotics), reduce the risk of certain diseases (e.g., cholesterol-lowering products), and can be used to treat other diseases. Development of new functional foods is a good opportunity to improve food quality and enhance its health benefits; thus, they are the subject of growing industrial and social interest in contemporary society.

Only species and strains that meet the specified requirements for probiotic strains can be included in the composition of probiotics and milk-based and non-milk-based probiotic foods. For each food type are selected microorganism strains that by performing their inherent metabolic processes are involved in flavor and aroma formation, influence the shelf life of the finished foods and contribute to the health benefits of their consumption.

Another aim of the present chapter is to present the authors' experience in the development of multistrain starters for yogurt series with the inclusion of different probiotic strains - yogurt enriched with *Lactobacillus delbrueckii* ssp. *bulgaricus*; *Lactobacillus gasseri*; *Lactobacillus acidophilus*; *Bifidobacterium* strains - *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium infantis*. The resulting yogurts retain high concentration of viable cells of the probiotic strain included, preserve their organoleptic characteristics and moderate titratable acidity for 30 days of refrigeration storage.

Proteins of plant origin present a good alternative for partial substitution of animal milk in the production of different dairy foods and beverages. Their application as substrates for lactic acid bacteria and bifidobacteria growth allows the development of non-milk-based probiotic fermented foods that bring the valuable characteristics of plants and the biological activity of probiotic microorganisms together. The authors have developed soy yogurts (soy acidophilic yogurt and soy bifid yogurt and yogurt beverages). The active cell concentration in the finished products exceeds 10^9 cfu/cm^3, and their shelf life is more than 20 days at refrigeration conditions. The organoleptic evaluation showed blinding of the soy off-flavor.

The wide range of milk-based and non-milk-based yogurts can be used as functional foods and beverages, providing the organism with both the inherent traditional yogurt health benefits and the required amount of beneficial microflora to exert its preventive role.

Chapter 8 - Food with curative properties has attracted interest among many consumers around the world due to the increasing attention paid to food-related health issues, such as food's potential ability to prevent diseases and improve consumers' mental state or quality of life. Most of the extant studies on functional food examine the role of functional food in developed Western nations which might not suitable for developing countries. Thus, the role of culture and value systems in influencing functional food consumption is poorly understood in developing countries. In Asia, foods with functional properties have been an important part of Asian culture for centuries, even though the term 'functional food' is not commonly used. For many years, Asian countries have supplemented their diets with naturally occurring substances with health-giving and curative properties. The knowledge of functional foods is often passed from generation to generation through oral traditions. In Malaysia functional food is a type of culturally-based food that is unique to ethnic group. Over time, though, pervasive cultural changes brought about by intergroup communications, economic development and greater social interaction promotes a certain level of cultural convergence in functional food consumption. In this chapter the authors will discuss the role of ethnicity and the dynamics of cultural and value changes in traditional and emerging economies, explore the convergence of cultural values in functional food consumption in multicultural societies, and outline lessons for businesses that compete in functional food markets.

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Chapter 1

USE OF EDIBLE FILMS AND COATINGS FOR FUNCTIONAL FOODS DEVELOPMENTS: A REVIEW

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ABSTRACT

New developments on edible films and coatings are gaining the interest topics of researchers in food science, consumers and the food industry. The term edible film refers to a biodegradable matrix performed by the use of natural biopolymers that could form a network. This network is applied onto the surface of foods products, spraying, dipping or used as the shell of microcapsules. Edible films have been introduced as oxygen and moisture barrier and also used as very good carriers for the delivery of several bioactive compounds such as vitamins, antioxidants, antimicrobials and probiotics in functional food systems. The most used matrices for this purpose are gelling polysaccharides (starch, cellulose, β-glucan, alginate, pectins, carrageenan, chitosan), proteins (whey, soy, gelatin, casein) and lipids. When used as carrier, the selection of the material is linked to their physicochemical properties and the interaction between the host compound and the media where it should express their activity. For example, for drugs delivery or probiotics treatment, the material should protect the active compound from acidic digestion (pH 1.2-1.5) but dissolves at around pH 7.5 in the intestine. However, some biopolymers present bioactivity by themselves, without the addition of any compound, e.g.,: chitosan and β-glucan. This review is aim to describe the alternatives used to perform functional systems by using biopolymers combined with functional compounds.

Keywords: edible films, coatings, encapsulation, probiotics, antimicrobials, antioxidants

INTRODUCTION

New developments on edible films and coatings are gaining the interest topics of researchers in food science, consumers and the food industry. The term edible film refers to a biodegradable matrix performed by the use of natural biopolymers that could form a network. Biopolymers forms networks with promising characteristics for food preservation like good barrier properties against oxygen preventing oxydation of the food packed or can be used as carriers of active compounds also to preserve foodstuff and extended it shelf-life. In addition, biopolymers are able to protect the active and functional compound from external hazards by an efficient encapsulation technique, allowing to the compound of interest, to be delivered to the target and express its functional activity when consumed. In this chapter, the most important biopolymers used for developing functional food, encapsulation techniques, and the applications of edible films and coatings containing active compunds will be described.

1. PROPERTIES AND APPLICABILITY OF EDIBLE FILMS RELATED TO FUNCTIONAL FOODS

The term “edible films” refers to a biodegradable, thin layered structures of biopolymer that can be consumed and are usually applied onto the surface of food products by dipping, spraying or brushing. In addition, an important use of edible films is in the encapsulation of flavours, polyphenols, vitamins, microorganism cells, etc (Reineccius, 2009). These films are a thin matrix preformed from a solution or dispersion of polymers of long chains. To form the film matrix it is necessary to remove the solvent from the solution or dispersion by an appropriate method in order to decrease the distance between polymers and favouring their interaction (Felton, 2013). This interaction promotes an interleaving of polymers chains that increase the viscosity of the system allowing the formation of a polymer network that will be ended with a film conformation. The application of edible films in food has previously shown to be effective for the control of shelf-life by slowing detrimental reactions e.g., enzymatic, physical and chemical by raising a thermodynamical or physical barrier that retards water vapour, oxygen and solutes mobility (Falguera et al., 2011).

Edible films are conformed by biopolymers such as polysaccharides, proteins, and lipids (Nussinovitch, 2009). Depending on the type of biopolymer used and the humidity conditions of environment, edible films could contain hydration water that acts as plasticizer of film by embedding itself between the polymers chains, spacing them, lowering the glass transition temperature, and improving flexibility (Levine and Slade, 1988). Therefore, hydration water affects the main structural and functional properties of the film such as mechanical and barrier properties (Cuq et al., 1997). In this way, edible films can be divided into three categories: hydrophilics, hydrophobics, and composites containing hydrophilic and hydrophobic components.

1.1. Hydrophilic Films

Polysaccharides and proteins interact strongly with water; therefore films made from these biopolymers are hydrophilic films. Suitable polysaccharides include cellulose derivatives, pectins, alginates, starches, chitosan and others (Bourtoom, 2008; Vieira et al., 2011). Film-forming proteins include gelatin, casein, soy protein, whey protein, wheat gluten, zein (Bourtoom, 2008; Janjarasskul and Krochta, 2010). Hydrophilic films also include films obtained from the integral cell biomass of microorganisms such as yeast, containing both polysaccharides and proteins (Delgado et al., 2016).

The charged state of hydrophilic biopolymers can be convenient for film formation, i.e., alginates and pectins are charged polysaccharides that require the addition of polyvalent ions such as calcium to facilitate film formation (Nussinovitch, 2009). Therefore, the properties of charged polysaccharides and protein-based films depend on the pH of the media, since the pH has a direct influence on the polymer charges and on the polymer conformation, changing polymer chains interactions that would affect the matrix properties (Nussinovitch, 2009). The susceptibility of hydrophilic biopolymers to the pH is the main characteristic that makes them interesting for functional food applications.

Hydrophilic films interact strongly with water, in general they show isotherms with a slight increase in the hydration water content for low values of a_w, and a significant increase for $a_w > 0.6$ (Delgado et al., 2016). This suggests a hydration mainly in forms of multilayer, with a small monolayer of hydration. In this way, the water hydration in hydrophilic film is mobile water that is not strongly bounded to the film.

The water content or sorption of water in the film affects directly the moisture barrier properties increasing water vapour permeability (Bertuzzi et al., 2007; Gontard et al., 1993). Moreover, the increasing in hydration causes an augment in elongation properties and a decrease in tensile strength and elastic modulus (Cuq et al., 1997). In this way hydrophilic films have poor control of water vapour migration but have good barrier properties to oxygen, carbon dioxide, and lipids, and also have desirable mechanical properties (Janjarasskul and Krochta, 2010). Regarding the barrier properties, they could act efficiently as selective barriers to gases in order to generate modified atmospheres.

In the hydrophilic group, there are both soluble and insoluble biopolymers in water, such as cellulose that is a highly hydroxylated polysaccharide, which is swellable but not soluble in water. This is due to high levels of intramolecular bonding and less amount of intermolecular hydrogen bonding between hydroxyl groups in and between glucopyranosyl rings within a polysaccharide chain and between adjacent polysaccharide chains.

1.2. Hydrophobic Films

Hydrophobic films are formed from a variety of lipids including fats and natural waxes (Bourtoom, 2008). Due to the low polarity of lipids, these films have a poor interaction with water and consequently their present good barrier properties to water vapour but poor barrier to oxygen and carbon dioxide (Janjarasskul and Krochta, 2010). Since lipids are polymers of short chains, the structure of the film matrix is not suitably intercalated (Nussinovitch, 2009). Furthermore, hydrophobic films are basically dehydrated in all conditions of environments; therefore they are not intrinsically plasticized such as hydrophilic films. As follows,

hydrophobic films have not appropriate mechanical properties and their use is limited because most lack sufficient structural integrity and durability (Bourtoom, 2008). Their main function is the barrier against the passage of moisture. Waxes are commonly used for coating fruits and vegetables to retard respiration and reduce moisture loss. However, lipids are useful for some capsule applications for example, the encapsulation of hydrophilic compounds by emulsification or spray chilling (Galus and Kadzinska 2015; Leonel et al., 2010).

1.3. Composite Materials

Films containing hydrophilics and hydrophobics components can be formulated to combine the advantages and lessen the disadvantages of each component. When a barrier to water vapour is desired, the lipid component can help this function while the hydrophilic component provides the necessary integrity and durability. On the other hand, hydrophilic components are a good barrier against oxygen. Composite films can be formed by a single layer or by a laminate of multilayer. The laminated is form in two stages, a first forming polysaccharide-based film or protein-based film, and then applying the lipid layer in order to overlap both layers (Kester and Fennema, 1989; Slavutsky and Bertuzzi, 2015). On the other hand, in monolayer composite films, the dispersion or emulsion of the lipid in the hydrophilic phase is achieved before the casting process (McHugh and Krochta, 1994). Properties of lipid-hydrocolloid bilayer films have been studied extensively showing good barrier properties however it has been observed separation and fracture of the lipid layer (Janjarasskul and Krochta, 2010). Monolayer composite films have also been studied however their barrier properties were lower than the observed in lipid-hydrocolloid bilayer films (McHugh and Krochta, 1994). Interesting mixtures between hydrophilic and hydrophobic are those lipids emulsified among a hydrophilic matrix. These systems are performed for delivery of lipophilic and hydrophilic drugs and other bioactive components (Mc Clements and Li, 2010).

1.4. Applicability

Over the last decades the production and research on edible films with good barrier and mechanical properties has gained the attention of many sectors, consumers and industry. Recently, edible films and coatings have been introduced as efficient carriers for the delivery of several bioactive or functional compounds e.g., vitamins, antioxidants, probiotics in food systems (Kanmani and Lim, 2013; López de Lacey et al., 2012), and protect the content against the external exposure (heat, oxygen, acids) that may reduce the activity of the functional compound. So they can deliver the correct amount of the desired compound to be ingested by the consumer.

The environmental friendly management of industrial wastes, and the growing interest in the economical valorisation of industrial by-products, make scientists and industries to look for alternative sources and new functionalities of some biopolymers and the search for innovative processing conditions as well as potential novel applications, such as gelatine from the meat and fish industry, collagen or spent yeast from brewer industry. Table 1 shows a resume of biopolymers mostly used in functional foods applications.

2. USE OF EDIBLE FILMS IN ENCAPSULATION TECHNOLOGIES REGARDING FUNCTIONAL FOOD

Encapsulation has been used extensively to entrap functional components in a carrier to impart protection against oxidation, isomerization, degradation during storage or processing, extending the shelf-life and protecting components against nutritional loss. In addition, encapsulation could be used to control the release of functional or bioactive components when ingested in the body; this is, the encapsulated component should remain intact in the stomach and then release in the intestine over a physiological pH values (Chen et al. 2006; McClements and Li 2010; Kumar Anal &Shing 2007) or control the release of an additive in a progressive manner to extend the shelf life of food. Additional uses of encapsulation are for masking flavors, colors or odors coming from the functional component, or for enhancing the viability of bacteria, where the capsulation facilitates handling of cells and allows a controlled dosage.

Traditional carriers are food grade biopolymers, in particular those hydrophilic ones, such as gelatin, modified starch, maltodextrin, arabic gum, chitosan, alginates, carrageenan, pectin, carboxymethyl cellulose (CMC). Each carrier has advantages and disadvantages in terms of cost and encapsulation efficiency. Indeed, the encapsulation technologies used determine the extent of protection obtained and the stability of the functional compound during storage. The most frequently used methods are coacervates, spray drying, freeze drying, ionic gelation, emulsion, extrusion, among other techniques

2.1. Simple and Complex Coacervates

Coacervation is based on the ability of charged polymers to interact with water to form a liquid, neutral, polymer rich phase called coacervate (Munin and Lévy, 2011). There are two methods, simple and complex coacervates, the process is identical but they differ except for the way in which the phase separation is carried out. The first one a desolvatation agent is added to produce the phase separation, while the complex coacervation involves complexation between two oppositely charged polymers and is formed a dense coacervate that wraps as a uniform layer the core material and both are diluted in a diluted phase (Jyothi et al. 2010). Figure 1 shows the scheme of complex coacervation. Parameters of these techniques are: pH, temperature, ionic strength, molecular weight and polymer concentration. The coacervates are stabilized by thermal treatments, crosslinking or desolvatation techniques. The three basic steps involved in complex coacervation are: 1) formation of three immiscible phases; 2) deposition of the coating; 3) rigidization of the coating. This method is useful for the encapsulation of high value active molecules or for unstable substances, as is the case for polyphenols. For example, an extract of yerba mate (*Ilex paraguariensis*) which contains 62.11 ± 1.16 mg of gallic acid per gram, was encapsulated by using complex coacervation between calcium alginate and chitosan, this technique was compared with ionic gelation (calcium alginate) that will be explained in this section (Dealdino et al., 2008). Gelatin coacervates complexed with anionic polymers in the form of microcapsules are of special interest as they can entrap functional components in a carrier and provide protection against oxidation or degradation during storage (Gómez-Guillen et al., 2011).

Table 1. Biopolymers mostly used for functional food applications

Biopolymer	Nature	Monomer	Applications	Ref
Starch	Hydrophilic/ Polysaccharide No Charged	Glucose α (1-4)	Biodegradable packaging material. Encapsulation of volatile and hydrophobic compounds.	Lu et al. (2009), Conde-Petit (2006)
Cellulose	Hydrophilic/ Polysaccharide No Charged	Glucose β (1-4)	Microencapsulation	Desai et al. (2005)
β-D-glucan	Hydrophilic/ Polysaccharide No Charged	Glucose β (1-4), branched (1-6)	Encapsulates antioxidants Biodegradable films	Bishop et al. (1998), Salari et al. (2013), Novak et al. (2012)
Pectin	Hydrophilic/ Polysaccharide Charged	Galacturonic acid Rhamnose Galactose Arabinose	Biodegradable films Microencapsulation hydrophobic and hydrophilic compounds	Pérez-Espitia et al. (2014)
Chitosan	Hydrophilic/ Polysaccharide Charged	Randomly distributed β (1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit)	Biodegradable films, Antimicrobial Microencapsulation	Ruiz-Navajas et al. (2013), Chávarri et al. (2010)
Gums	Hydrophilic/ Polysaccharide Charged	Guar gum (Mannose Galactose) Arabic gum (Galactose Arabinose Rhamnose Glucuronic acid)	Microencapsulation Edible coating	Sarkar et al. (2011), Narsaiah et al. (2014), Ali et al. (2010)
Alginates	Hydrophilic/ Polysaccharide Charged	Mannuronic acid Guluronicacid Linear (1-4)	Active antimicrobialedible films, Encapsulation of probiotic bacteria	Pranoto et al. (2005), Sultana et al. (2000)
Carrageenan	Hydrophilic/ Charged	Galactose 3,6 Anhydrogalactose Linear polymer, alternating α-(1-3) and β-(1-4) linkages	Encapsulation Biodegradable active antimicrobial film	Kadam et al. (2010), Hambleton et al. (2009), Campos et al. (2011)
Casein	Hydrophilic/Protein Charged – pH dependant	Amino acids Peptidic linkage	Encapsulation of hydrophobic and hydrophilic compounds, Biodegradable films	Augustin et al. (2014), Arrieta et al., (2014)
Whey	Hydrophilic/Protein Charged – pH dependant	Amino acids Peptidic linkage	Wall material for encapsulation of volatiles, hydrophobic and hydrophilic compounds, probiotics	Rosenberg et al. (1996), Augustin et al. (2014)
Gelatin	Hydrophilic/Protein Charged – pH dependant	Amino acids Peptidic linkage	Biodegradable packaging materials, microencapsulating agents	Gómez-Guillén et al. (2011), Augustin et al. 2014
Soy proteins	Hydrophilic/Protein Charged – pH dependant	Amino acids Peptidic linkage	Biodegradable films Microencapsulation	Ciannamea et al. (2015), Jun-Xia (2011)
Cross-linked triglycerides and waxes	Hydrophobic	Fatty acids	Encapsulation of hydrophilic compounds and yeast	Jean et al. (2014)

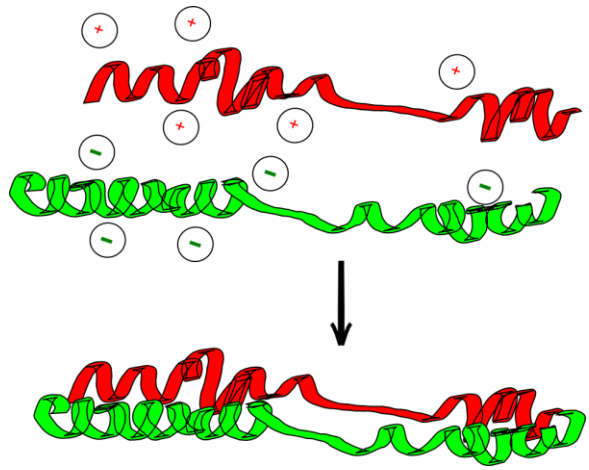

Figure 1. Complex coacervation for encapsulation scheme.

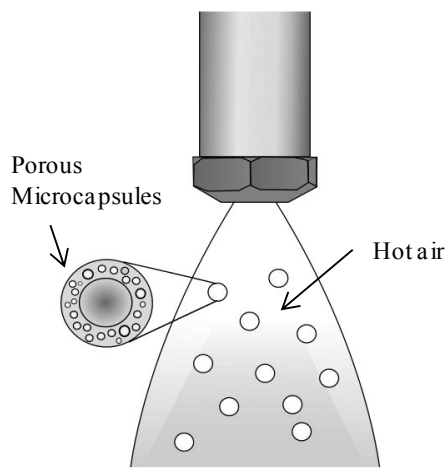

Figure 2. Spray-drying method for encapsulation scheme.

2.2. Spray Drying

Spray-drying is a routine process in the food industry to convert liquids into dry powders. This technique is useful to produce microcapsules by the formation of particles from a dispersion of active compound in a solution of coating agent (Huq et al., 2013). This technique applied to the development of microcapsules consists on a liquid formulation containing the coating agent and the bioactive or functional compound, afterwards the solvent is atomized into droplets via either a nozzle using compressed gas to atomize the liquid feed, or a rotary atomizer using wheel rotating high speed. In the spray drying method, a heated process gas leads to the evaporation of the solvent from the droplets. The formed droplets are porous microcapsules and the active compounds are trapped in the particle matrix (See Figure 2). This technique is widely used in the industry for the production of microspheres or microcapsules with a size between 1-100 μm. This technique is relatively low cost, flexible,

and leads to the production of high quality and stable particles, making this technique the most used in the food industry. In this technique many biopolymers were used such as proteins: sodium casein and gelatin, hydrocolloids: Arabic gum, starch and hydrolysed starch: maltodextrins, lactose (Gallardo et al., 2013; Gharsallaoui et al., 2007). It is necessary a solids concentration around 20-30%, so it is often encounter it in industries coupled to a concentration operation such as evaporation. Probiotics and prebiotics can be encapsulated through this method (Peighambardoust et al., 2011; Fritzen-Freire et al., 2012) and efforts in researches are pointed to improve process conditions in order to achieve the highest cell viability. The reason that spray drying is interesting to encapsulate bacteria in comparison of freeze drying (despite of the elevated temperature conditions used) is that this technology is cheaper than the other one, it is compatible with continuous flow operation and equipment is very well spread in factories. There are some variables that can be controlled to obtain different particle sizes, like spray air flow, concentration level and rate of feed and use of surfactant agents.

A derived technique from spray drying but that uses a cooling system in place of drying air is spray cooling. This technique allows encapsulating hydrophilic compounds, especially low molar mass compounds that diffuse very quickly in water, using lipids as wall materials. A suspension or emulsion containing a hydrophilic dispersed phase onto a molten lipid massive phase is atomized in a cold chamber with a temperature below of melting point of lipids used as wall material, that can also contain an active compound such as α-tocopherol (Gamboa et al., 2011; Leonel et al., 2010).

2.3. Freeze Drying

Freeze-drying, also known as lyophilization, is one of the most used processes for the protection of thermo-sensitive and unstable molecules. It is a dehydration operation at low temperature consisting in eliminating water by sublimation of the frozen product. Biopolymers used as carrier in this technique are maltodextrin 20DE, gum arabic and tapioca starch (Murali et al., 2015). Mixtures of arabic gum, sucrose and gelatin were also studied (Kaushik and Roos 2007). Indeed, HP-β-cyclodextrin and β-cyclodextrin were used as coating materials to encapsulate polyphenols from blue berries (Wilkowska et al., 2016).

2.4. Ionic Gelation

This technique is a simple and mild method based on the complexation of positively charged polymers when coming in contact with specific polyanions to form inter and intramolecular cross-linkages and form hydrogel beads, also called gelispheres (Hu et al., 2008), or negatively charged polymers in contact with polycations, calcium is the most used cation for gellification of the majority of the negatively charged polymers, see Figure 3. Gelispheres are spherical crosslinked hydrophilic polymeric systems capable of extensive gelation and swelling in simulated biological fluids and the release of any drug or bioactive compounds controlled by polymer relaxation. In the internal ionic gelation, the hydrogel beads are produced by dropping a drug-loaded polymeric solution into the aqueous solution of polyvalent cations. The cations diffuse into the drug-loaded polymeric drops, forming a

three dimensional lattice of ionically crosslinked moiety. Polymers used for this technique are: alginates, gellan gum, chitosan, pectin and carboxymethyl cellulose. The biopolymers contain certain anion/cations on their chemical structure; these anions/cations form a network by their combination with the counter ion (Patil et al., 2012). In the case of chitosan due to their positive net charge, an anion is used to generate nano/microparticles; the most frequently used is tripolyphosphate (Hosseini et al., 2015).

An alternative method to obtain microspheres and avoid the release of the functional compound to the obtention media, is by dropping the dispersion of the selected polymer and an insoluble salt of calcium (calcium carbonate) in a lipid phase that contains a lyposoluble acid (e.g., acetic acid). When the drops enter in contact with acetic acid, pH goes down, calcium carbonate is solubilised and becomes available to interact with polymer and make possible the gelation process (Liu et al., 2007).

2.5. Extrusion Method

Extrusion method is a simple and cheap method mostly used for probiotic cells encapsulation since it makes cell injuries minimal and causes relatively high viability of cells (Huq et al., 2013). In this technique a hydrocolloid solution is prepared, then the cells are added and the solution is dripped through a syringe needle or nozzle. The droplets are allowed to fall into a hardening solution. In this technique, alginate, k-carrageenan, mixtures of k-carrageenan and locust bean gum, or xanthan and gellan, alginate plus corn starch and whey proteins have been used as wall materials for encapsulation of lactobacilli and bifidobacteria. The size of the microcapsules is affected by the nozzle size. The diameter of the obtained alginate beads is also increased as the concentration of sodium alginate increases, but the alginate concentration does not significantly influence the numbers of free cells (Rokka and Rantamaki, 2010).

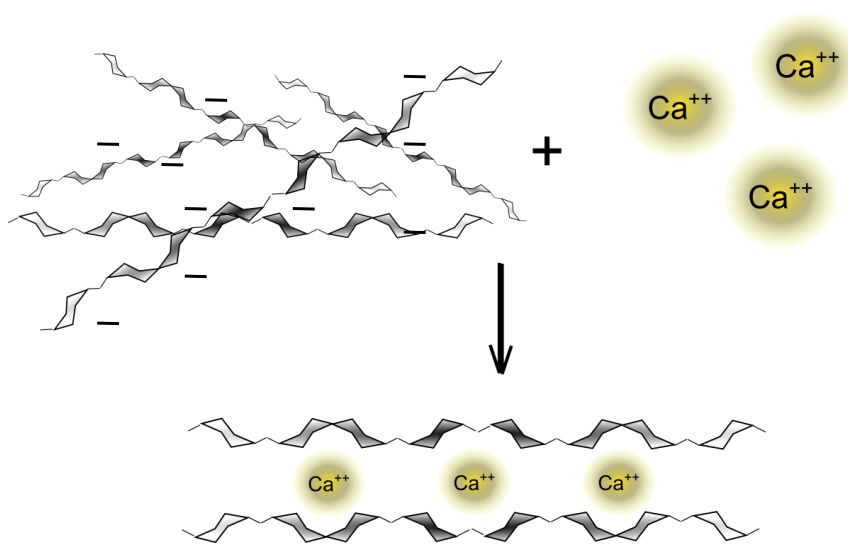

Figure 3. Ionic gelification method for encapsulation scheme.

2.6. Emulsion Method

This technique is also useful for microencapsulation of probiotic bacteria. Emulsion can be easy scale-up and the obtained micropasules are smaller than those obtained by extrusion (Munin and Edwards-Lévy, 2011). However, this method requires more cost for performance compared with the extrusion method due to need of using vegetable oil for emulsion formation. In this technique, a small volume of cell/polymer slurry (as a dispersed phase) is added to the large volume of vegetable oil (as a continuous phase). Resulting solution becomes well homogeneous by proper stirring/agitating, till water-in-oil (w/o) emulsion forms. In order to obtain a better emulsion, Tween 80 is recommended as the best choice (Galus and Kadzinska, 2015). These w/o emulsions are useful to encapsulate hydrophilic compounds, such as cells slurryes or hydrophilic compounds.

3. EDIBLE MATERIALS APPLICATION IN PROBIOTICS FUNCTIONAL FOODS

Probiotics are defined as live organisms that when administered in adequate amounts (>6-7 log cfu/g) confer health benefits to the host (FAO/WHO 2002). Although the functionality of the probiotics depends on the strain, health benefits including regulation of the gastrointestinal tract, stimulation of the immune system, reduction of serum cholesterol levels and lactose intolerance and prevention of cancer and cardiovascular disease have been reported (Saad et al., 2013). Lactic acid bacteria (LAB) are used for more than 4000 years for food fermentations. Nowadays, these bacteria are still highly used for those applications but the interest of using them as probiotic bacteria gain the attention of consumer and food industries (Huq et al., 2013). These microorganisms benefit human health by improving the balance of intestinal microbiota and by strengthening mucosal defences against pathogens (Fritzen-Freire et al., 2012).

LAB are the most important probiotic microorganisms and they are gram positive, rod-shaped, non-spore-forming, catalase-negative organism, acid tolerant, aero-tolerant and strictly fermentative; lactic acid is the major end product of sugar fermentation. LAB used as probiotics are *Lactobacillus acidophilus, Lactobacillus amylovorous, Lactobacillus casei, Lactobacillus crispatus, Lactobacillus delbrueckii, Lactobacillus gasseri, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus*, among others bacteria (Anal and Singh, 2007). Other probiotic microorganisms commonly used in food formulations are the bifidobacteria, also gram-positive and rod-shaped but are strictly anaerobic. These bacteria can grow at pH in the range 4.5–8.5. The most recognized species of bifidobacteria that are used as probiotic organisms are *Bifidobacterium adolescentis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium lactis and Bifidobacterium longum*. Some other microorganism demonstrate probiotics effects such as *Bacillus cereus* var. toyoi, *Escherichia coli* strain *nissle*, *Propionio bacterium freudenreichii*, and some types of yeasts, e.g., *Saccharomyces cerevisiae* and *Saccharomyces boulardii* (Holzapfel et al., 2001).

As comment before, the use of probiotics in the food industry has grown remarkably in the last decades mostly in dairy products, such as yogurt, milk, ice cream, cheese; juices and

beverages (Gobbetti et al., 2010). During production a significant loss of probiotic viability may occur due to heat, mechanical or osmotic stress that induces cellular injuries (Bustos and Bórquez, 2013). There are several strategies to overcome these processing obstacles and reach the maximum viability of probiotics, all along the production cycle and consumption; this is during processing, storage, under gastric juice and intestine bile salt conditions. Encapsulation segregates the cells from adverse environment, thus potentially reducing cell injury. Encapsulation has been used as a technology that can provide protection against the sensitive probiotic cultures, improving their stability and viability in food products and performing the target delivery in gastrointestinal tract. There is a need for encapsulation of probiotic bacteria to survive human gastric juice in the stomach, where the pH can be as low as 2 (Huq et al., 2013). In this section some examples of the application of edible films for the incorporation of probiotics the consumer's diet are described.

As mentioned in previous sections, encapsulation was one of the main strategies to preserve cell viability of probiotic bacteria. However, applications of probiotic edible films in food matrix are a challenging practice, due to the wide range of detrimental processes that happened during food processing and storage. Strategies should overcome osmotic, heat, acid induced stresses and mechanical injuries that may happened during food preparation and storage (Fu and Chen, 2011).

Gelatin was used as biopolymer to encapsulate LAB and bifidobacteria by using extrusion and spray-drying technology, and results demonstrated that the survival of bacteria cell against harmfull conditions during processing and against aggressive conditions of stomach (Weissbrodt and Kunz, 2007). Combination of alginate and gelatin were used to immobilize *Lactobacillus casei* ATCC 393 cells and the survival of probiotic bacteria after drying at 4°C was described by Li et al. (2009).

Soukulis et al. (2014) developed probiotic bread by the application of a film forming solution based on two formulations: 1% w/w of sodium alginate and a binary blend of 0.5% w/w sodium alginate and 2% w/w whey protein concentrate, plasticizer was added in both formulations. In this work, *Lactobacillus rhamnosus GG* was used as probiotic bacteria and added to the film forming solutions. A small amount of probiotic edible film forming solution was applied and uniformly distributed by brushing on the crust of the bread and dried. Viability of bacteria cell during the air drying was a critical and the presence of protein in the film forming solution reduced the viability during drying and storage. It was demonstrated that depending the type of edible film used, the viability and stability of probiotics cells varied, and higher viability was observed in those breads coated with the blend of sodium alginate and protein film forming solutions compared to the coating based on sodium alginate alone. However, when testing the bread coated with the probiotic edible film under simulated gastrointestinal fluids, viability of bacteria cells was higher when using the sodium alginate edible film this is due to an ionic setting mechanism. On the contrary, the aggregates formed by alginate-whey protein did not provide sufficient protection against drastic gastric juice.

Moreover, the type of material used for encapsulation may produce some injuries of bacteria cells due to osmotic stress. For example, some reports revealed that polysaccharides such as pectin, cellulosics or alginates could impact the viability of bacteria cells both throughout the drying process and storage period (Soukulis et al., 2014; Yonekura et al., 2013). Burgain et al. (2013) described the in-vitro interactions between probiotics, in particular *Lactobacilli* and milk proteins and they demonstrated that there was an interaction through the adhesive features of bacteria, such as exopolysaccharides or proteins, with whey

proteins via electrostatic, steric, short-range forces, or biospecific interactions, making an stable dairy food system with optimum viability of bacteria cells. Altamirano et al. (2012) also studied the application of probiotic edible films on bread crust but probiotics were first microencapsulated in a mixture solution conformed by whey protein isolates, CMC, pectin, inulin and agave sap, and microcapsules were incorporated in a starch solution that will be used to coat the bread surface (Rodríguez-Huezo et al., 2007). In this research, different probiotic coatings (dispersed or multilayer) were applied onto the surface of partially baked breads and they found that, microencapsulated *L. acidophilus* survived after baking and storage time. Therefore, encapsulated *L. acidophilus* can be applied to bread surface through edible coatings, leading functional bread with similar characteristics to common bread, but with additional healthy benefits.

Prebiotics were first defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health." This definition was later refined, focusing on particular microorganisms (Gibsonet al., 2004): "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits to host well-being and health." This last definition, target to Lactobacilli and bifidobacteria (Slavin, 2013). Food ingredients should possess several characteristics to be considered as prebiotics such as:

- Resist gastric acidity, hydrolysis by mammalian enzymes, and absorption in the upper gastrointestinal tract;
- Be fermented by the intestinal microflora
- Selectively stimulate the growth and/or activity of intestinal bacteria potentially associated with health and well-being.

The symbiotic combination of prebiotics with probiotic strains promotes colonization in the intestinal tract inhibiting the growth of human or animal pathogens and promoting bifidogenicity (Mugambi et al., 2012). Reports have been published by the combination of prebiotics and probiotics in a microencapsulated system and in the particular case of anhydrobiotics (viable probiotics stabilized in a dried format) have conferred a beneficial effect on cell viability.

An alternative to increase viability of probiotics cells during spray drying process is indeed by the addition of prebiotics. The most studied prebiotics are inulin and fructo-oligosaccharides (FOS) and the mixture of both ingredients is known as FOS enriched inulin, sorbitol, mannitol, lactulose, xylitol and raffinose (An et al., 2007). The drawback of using prebiotics together with the probiotics in a microcapsule is that the physical characteristic of the microcapsule may change. Frotzen-Freireet al., (2012) evaluate the effect of prebiotic agents (inulin, FOS and their mixture) on the viability of *Bifidobacterium BB-12* microencapsulated by spray drying. Results suggested that the viability was higher when using prebiotic microcapsuled than those with skim milk as protector of the bacteria cells.

4. ANTIMICROBIALS AND ANTIOXIDANTS EDIBLE FILMS

Besides exerting a barrier action against O_2, CO_2, water vapour and presenting good mechanical properties for such purposes, edible films may present antimicrobial and antioxidants properties. In both cases, is well described the addition of bioactive compounds with antimicrobial and/or antioxidant activity in the polymer matrix. Regarding antimicrobial properties, there are two alternatives to achieve this property, by the addition of the antimicrobial as an additive of the polymer matrix, or the biopolymer (matrix) presents antimicrobial properties by itself, this is the case of chitosan that is described as follows.

4.1. Chitosan

Marine resources are well recognized as sources of bioactive compounds with a desired biological activity with a great application potential in functional foods [Quinna et al., 2013]. Chitosan and chitin are functional biopolymers which are present in marine crustacean and, chitosan on the other hand it produced also by microorganism (*Aspergillus niger, Mucorrouxii, Penecilliumnotatum*) (Devlieghere et al., 2004). Chitosan is an N-diacethylated form of chitin. The free amine group ($-NH_2$), responsible of it antimicrobial activity as we will describe later, is protonated as ($-NH_3^+$) in the acidic conditions of stomach (Sajomsang, 2010). Chitosan, as commented before presents antimicrobial activity, and this activity will depend on the pH of the media, molecular weight, degree of deacetylation, temperature, food components, among others. It is not very well known the mechanism of the antimicrobial activity, but among the several hypotheses, the most feasible is a change in cell permeability due to interactions between the polycationic chitosan and the electronegative charges of the cell surfaces. This interaction leads to the leakage of intracellular electrolytes and proteinaceous constituents (Papineau et al., 1991).

Develieghere et al., (2004) study the antimicrobial activity of chitosan, with a deacetylation degree of 94% and low molecular weight (43 kDa) was evaluated against psychrotropic foodborne bacteria and the influence of food composition (oil, salt and protein concentration). They observed that the presence of proteins may interfere the antimicrobial activity of chitosan if the pH of the media is higher to proteins isoelectric point, where proteins are negatively charged and could compete with negatively charges from cell surface of microorganisms. The same happened with NaCl, where Cl^- can neutralize the positive charges of chitosan and Na^+ may compete with positive charges (Chung et al., 2003). However, chitosan gets solubilized with the presence of salts. Regarding the presence of fats, besides the complex that might be established, the positive charges are still available for the antimicrobial activity. Chitosan performance sometimes is better than biocides, its antimicrobial activity at 0.5, 1 and 2% against *S. aureus* was better than a commercial biocidal based on hydrogen peroxide. Goy et al. (2016) used chitosan as precursor of trimethylchitosan (TMC), and both materials chitosan and TMC were evaluated as antimicrobial agents against *E. coli* and *S. aureus* (food born bacteria and hospital-acquired pathogens) as a function of polymer concentration. Chitosan, owing to its promising properties like excellent biodegradability, biocompatibility, its general recognition as safe

(GRAS) and ability to form films, gels, beds, fibers and particles, this polysaccharide has been used in the development of drug delivery system.

4.2. Addition of Antimicrobials and Antioxidants as Functional Compounds to Edible Matrices

Carvacrol the major component of the essential oil from some species such as oregano, thyme, majoran, and is recognized as a safe food additive and Keachaoon and Yoksan (2011) prepared nanospheres by using chitosan as shell and carvacrol as bioactive compound. Since carvacrol is a volatile compound which easily evaporates and/or decomposes when using it during food processing or antimicrobial film preparation, encapsulation inside a biopolymer matrix was an alternative to increase its shelf life and maintain its functional properties. This compound demonstrated antimicrobial (Arrieta et al., 2014) and antioxidant activity (Ramos et al., 2014) when used in biodegradable matrices. The loading of carvacrol into chitosan particles was successful and the antimicrobial activity of the nanospheres was good against G*ram* (+) and *Gram* (-) bacteria. Release of carvacrol varied depending on the pH of the media, and it was observed that the release was relatively quickly in an acidic solution, followed by alkaline and neutral media, respectively (Keachaoon and Yoksan, 2011). Other example is described by Acevedo-Fani et al. (2015), who investigated the incorporation of three different essential oils from thyme, lemongrass and sage as source of antimicrobial agents in nanoemulsions trapped into alginate matrices, thyme essential oil demonstrated the best growth inhibition effect against *E. coli* with around four log decades of growth reduction.

According to the growing interest in using the so called functional food for preventing illnesses, natural antioxidants are known to be beneficial against chronic diseases including cardiovascular disease and certain types of cancers (Dillard and German, 2000; Hertog et al., 1997). Polyphenols have been extensively investigated as phytochemicals antioxidants. They are valuable compounds with scavenging properties towards radical oxygen species. These abilities make polyphenols interesting for the treatment of various diseases like inflammation or cancer, but also for anti-ageing purposes in cosmetic formulations, or for nutraceutical applications. However, in many cases, the phenolics have an unpleasant astringent and bitter taste, which limits their use in food or in oral medications; indeed, they present unstable properties, they are sensitive to light and heat. Moreover, polyphenols often present a poor biodisponibility mainly due to low water solubility (Vidal et al., 2004). Thus, encapsulation is a promising alternative to avoid these problems.

Lycopene is the pigment principally responsible for the characteristic deep-red color of ripe tomato fruits and tomato products. It has attracted attention due to its biological and physicochemical properties, especially related to its effects as a natural antioxidant (Chiu et al., 2007). Lycopene extract from tomato pulp waste is highly susceptible to oxidation and isomerization reactions. Therefore, Chiu et al. (2007) formulated microcapsules by using porcine skin type-A gelatin and poly(γ-glutamic acid) as carriers of the natural antioxidant, in order to protect it from degradation. Gelatin is positively charged at pH<PI (isoelectric point) and it must be prone to form complexes with anionic polysaccharides such as poly (γ-glutamic acid) (PGA), which a biodegradable and nontoxic polymer synthetized from microorganism Bacillus species. PGA tends to ionize completely at pH>9 because of

dissociation of carboxylic acid groups. The release of lycopene from the microcapsules occurred rapidly at pH 5.5 and 7.0, while no lycopene was released at pH 2.0 and 3.5, which means that the unstable constituents can remain intact in the stomach and then be released into the intestine, which should enhance the bioavailability of lycopene. Following this trend, Lee et al. (1996) used chitosan and sodium alginate as coating material for the encapsulation of guaifenesin in medicinal syrups and they found that the release of the drug was higher at pH 8.8 than pH 4.8. This phenomenon was explained as most carboxyl groups of sodium alginate and amino groups of chitosan remain ionized at pH 4.8, which may lead to the formation of strong network through electrostatic interaction that may prevent the release of the drug from capsules under those conditions (low pH).

Gelatine was also used as carrier for polyphenols by means of the development of active packaging materials as an active gelatine film to cover food products and extend its shelf-life. The gelatine would enhance the stability of the natural extract and control the release to the covered food product; indeed the biopolymer will mask the odour and taste that vegetal extract often present. Tea polyphenols was encapsulated in chitosan nanoparticles and incorporated in gelatinefilms. The presence of nanoparticles reduced tensile strength and oxygen permeability of gelatine films but increased water vapour permeability. The active films demonstrated antioxidants properties as expected since the oxidation of fish oil was retarded (Bao et al., 2009).

Catechins are powerful natural antioxidants but the major drawback is that they are very unstable in alkaline conditions encountered in biological fluids, and in some experimental protocols. That is why research teams studied encapsulation to bypass this limit to the application (Dube et al., 2010). Catechin and (-)-epigallocatechin were immobilized within chitosan- tripolyphosphate-nanoparticles. After 24 hours, the measured antioxidant activity was 88.3% and 73.4%, respectively. After 24 hours, 50% of the encapsulated catechin was degraded, while 8 hours were enough to degrade the same amount of free catechin. On the other hand, epigallocatechin was much more unstable because after 40 min, more than a half was denatured. Regarding other phenolic extract, such as yerba mate extracts (*Ilex paraguariensis*), were encapsulated by simple (alginate) and complex coacervation (alginate-chitosan) methods (Dealdino et al., 2008). This study demonstrated that the antioxidant activity was higher than 85% and was maintained when phenolics were encapsulated in alginate beads; however when alginate beads were coated with chitosan only 50% of the activity was maintained. This behavior was attributed to the possibility of solubilization of the active compound in the chitosan solution. Therefore, is clear the influence of the material used for the capsule wall on the release of natural antioxidants present in the yerba mate extract. The powerful antioxidant properties of compounds presents in yerba mate extract have gained the interest of researchers to reduce tumors proliferation in in vitro studies (de Mejía et al., 2010).

Complex coacervation was also used for propolis (polyphenol-rich mixture) encapsulation and though limit the problems related to propolis solubility and pronounced taste (Nori et al., 2011). The encapsulation performed with pectin and soy protein appeared as an interesting alternative. The result obtained in this study is a powder, easily dispersible in liquids other than alcohol, with antioxidant and antimicrobial properties, and from which the release of the active material can be controlled. Gelatin-pectin based coacervates microparticles used to entrap polyphenols and flavonols. Those compounds react under oxidizing conditions to form covalent cross-links with gelatine. The formed structure presents

good mechanical and thermal stability (Strauss and Gibson, 2004). Another way to incorporate polyphenols or other lypophilic compounds in films is emulsifying them, using vegetable oils and Galus and Kadzinska (2015) have well reviewed multiple applications of emulsified edible films.

A low cost-high volume microencapsulation process for the encapsulation of essential oils and flavours is using yeast cells. Due to their light colour, bland taste and availability in large quantities, yeast cells (*Saccharomyces cerevisiae*) is a promising alternative to encapsulate and protect bioactive compounds. Additionally, during processing, no additives but only water, yeast and the active compound are used. This means, that baker's yeast has emerged as a convenient host for the development of drug delivery systems.

Shi et al. (2007) used yeast cells to encapsulate chlorogenic acid (CGA) as a water soluble antioxidant. CGA is one of the most naturally existed phenolic compounds. It has many beneficial properties due to its ability to interact with reactive oxygen species. However, due to its chemical configuration, CGA may undergo oxidation by gamma irradiation or may be involved in transesterification reactions during storage and processing. In vitro release studies were performed in simulated gastric fluid (HCl) and results showed that the release of the antioxidant was higher at this low pH than in buffered solution pH 7.4 or water. Results are consistent with yeast cell wall structure, which is composed by mannoproteins, β-1,3-glucan, β-1,6-glucan and chitin [Moran, 2004]. This outer protein layer and plasma membrane are the permeability cell barrier, and they can be destroyed by acid or base, thus making the release of CGA in simulated gastric fluid and buffer (pH 7.4) faster than in water. Results demonstrated also that more than 95% of the CGA was released within 2 h in the acidic solution. Bishop et al. (1998) and Nelson (2002) suggested that the bilayer membrane of yeast cell may act as liposome during microencapsulation of essential oils and allowed the stabilization of oil droplets within the cell, providing stable products. Yeast cells can protect the bioactive compound against light and oxygen during storage. Recently, the use of yeast cells to encapsulate bioactive compounds was also tested by Salari et al. (2013) used *Saccharomyces cerevisiae* cells as carrier for berberine. Berberine is the most significant alkaloid present in Barberry, a famous plant with well-known medicinal properties, like antiplatelet effects, reduces fever, it has good effect on inflammatory diseases (Fatehi-Hassanabad, 2005; Yesilada and Küpeli, 2005). The encapsulation efficiency was quite good, since the loaded berberine was up to 42%. In addition, to the application of yeast cell as capsules, coatings were also performed by an acid treatment process of yeast cell wall (Kasai et al., 2000). In this way, a delivered system was performed by adding certain drug to the polymer matrix. The films obtained under this methodology presented very good oxygen barrier, though an efficient protection of the bioactive compound will be achieve by using this system. Acetaminophen (AAP) was used as compound to be coated and its release from AYC-coated tablets showed a sigmoidal release profile with an initial lag time; furthermore, it was possible to control the lag time and the release rate of AAP by varying curing time and temperature (Yuasa et al., 2000).

Delgado et al. (2016) also demonstrated the possibility to perform biodegradable films by using whole yeast biomass by applying thermal and homogenization processes, with the potentiality of adding bioactive compounds within the polymer matrix.

There are some biopolymers that could offer a beneficial and functional activity without the addition of any bioactive or functional compound. Since the molecule is conformed in such a way that could interact with microorganisms, like chitosan, already explained or may

present other beneficial effects such as mycotoxins adsorption, stimulation of the immune system, anticancer or wound healing activity, like the polysaccharide β-glucan does. For this, it is important to highlight the promising properties of these microbial polysaccharides as functional biopolymer. In addition, the β-glucans are abundant and they could be obtained from spent yeast collected from the residues from the brewer industry.

β-(1,3)/(1,6)-glucans are the main structural polysaccharides of cell walls of yeast and they have demonstrated many therapeutic properties (Chen and Seviour, 2007). β-D-glucans are single ordered helical structures existing as single polymer strands with helical conformation. They display many advantageous effects, including anti-microbial, anti-inflammatory, anti-carcinogenic effects and they accelerate wound healing (Bzducha-Wróbel et al., 2014) and yeast β-D-glucans belongs to a class of drugs known as "biological response modifiers," since they modified the biological response of the host by the stimulation of the immune system. In this way, they are non-toxic to the cells of the host organism since they do not attack the infective or tumor-agent, they just activate the immune system (Sandula et al., 1995). The European Commission included β-glucan to the list of novel food components according to the Regulation no. 258/98 of the European Parliament, since 2011 (EC Decision of 24 November 2011). The stimulation of the immune system of black tiger shrimps of β-glucan obtained from spent yeast was investigated in vitro and in vivo. In vitro results, demonstrated that β-glucan extracted from autolysed yeast cells significantly enhanced phenoloxidase (PO) activity of black tiger shrimp hemolymph as compared to controls without added glucan. Also in vivo, an oral administration of 0.2% (w/w) in diets for 3 days significantly increased the PO-activity of the shrimps (Suphantharika et al., 2003).

Besides many recommended practices exist to avoid molds development, with its concomitant mycotoxigenesis during crop growth, harvesting and storage, contamination still occurs and several strategies have been tested in order to keep out mycotoxins from the food chain (Yiannikus et al., 2004). Management of mycotoxins includes many strategies such as prevention, monitoring, avoidance, decontamination, detoxification, and animal treatments (Jouany, 2007). Some of the strategies, such as removal or destruction of contaminated food and fed, are expensive and unrealistic (Pasikatan and Dowell, 2001). An effective strategy is the use of binding agents such as activated carbon, clays, bentonites or organic compounds such as polysaccharides (cellulose, yeast cell wall, bacteria glucomannans, peptidoglycans) and synthetic polymers (Schatzmayr et al., 2006; Dawson et al., 2001). The addition of these adsorbents to food and feeds contaminated with mycotoxins would reduce their bioavailability in the digestive tract and their detrimental effects on animals. It is well reported that yeast cell walls are powerful mycotoxin binders. The polysaccharides (glucan and mannans), proteins and lipids from the cell wall present different accessible adsorption centers and binding mechanisms (e.g., hydrogen bonds, ionic, or hydrophobic interactions).

El-Naggar and Thabit (2014) evaluated β-D-glucan, from yeast cell wall, as a natural material to reduce mycotoxins in feed without reducing nutritional value, with no harm to human or animal health. These authors studied the efficiency of the β-D-glucan against some toxigenic Fusarium isolates in vitro and in vivo, resulting in a higher adsorption process in comparison with clays and calcium propionate. Another study from Yiannikouris et al. (2004) demonstrated that β-(1,3 and 1,6)-D-glucans and related alkaline-extracted fractions isolated from the cell wall of *Saccharomyces cerevisiae* are able to adsorb mycotoxins such as zearalenone with an affinity of up to 50%. Other in vitro studies have shown that the yeast cell wall is also able to bind zearalenone and fumonisin (Devegowda et al., 1998). According

to Joannis-Cassan et al. (2011), the cell wall from baker's yeast can adsorb up to 62% of OTA depending on the mycotoxins concentration and yeast composition and aflatoxine B1. Indeed, alkaline and water extraction of yeast cell wall gives such a glucanable to adsorb Ochratoxin A (OTA), the alkali-insoluble fraction presented a reduced ability for this purpose (Piotrowska and Masek, 2015).

CONCLUSION

Throughout this chapter it was referenced different possibilities and alternatives for using edible films and coatings as biomaterials for functional foods application. The use of these materials is important due to their low environmental impact, they come from renewable resources and they are non-toxic. Biopolymers belong, of course, to the biodegradable materials group, and are represented by proteins and polysaccharides, that due to their intrinsic conformation present such versatility that are used for many application and formulations. The application of these biomaterials includes the development of food contact materials, with good barrier to oxygen, acceptable mechanical properties and able to carry active compounds in their matrix and, indeed they present the unique capacity to encapsulate bioactive or functional compounds with the aim of protecting them from external hazards and allow the compounds to fulfill the desired function.

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Chapter 2

DOUBLE EMULSIONS: POTENTIAL APPLICATIONS FOR THE ELABORATION OF FUNCTIONAL FOODS

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ABSTRACT

Double or water-in-oil-in-water ($W_1/O/W_2$) emulsions are complex systems consisting of an aqueous phase dispersed in a lipid phase, which in turn is dispersed in a continuous aqueous phase. Because these systems include water within the dispersed lipid phase, they have been proposed for the development of lipid-reduced food emulsions without the need of reducing the volume fraction of dispersed phase. The other potential application of $W_1/O/W_2$ emulsions is the encapsulation of substances in the inner water droplets for their isolation, protection or controlled release. The limits of these systems are given by the volume of water droplets that can be retained within the oil droplets and the minimum required size of oil droplets, which should be large enough to contain smaller water droplets. These conditions imply that $W_1/O/W_2$ emulsions present new issues regarding the stability of the system, adding different destabilization processes that should be controlled and measured. Different strategies have been implemented to increase the volume and stability of the inner water droplets, such as the generation of an osmotic gradient by the addition of solutes in the dispersed aqueous phase or the inclusion of fat crystals in the dispersed lipid phase. With respect to proper applications of $W_1/O/W_2$ emulsions for the production of functional foods, different objectives have been boarded: encapsulation of vitamins, minerals or microorganisms; formulation of low-calorie foods; control of taste perception; etc. Moreover, different methods have been developed for the analysis of $W_1/O/W_2$ emulsions, including spectrophotometry, conductimetry and differential scanning calorimetry techniques.

Keywords: $W_1/O/W_2$ emulsion, encapsulation, lipid-reduced food, stability

INTRODUCTION

Double or water-in-oil-in-water ($W_1/O/W_2$) emulsions are multi-compartmentalized systems consisting of an aqueous phase (W_1) dispersed as small droplets in a lipid phase (O), which is simultaneously dispersed in a continuous outer aqueous phase (W_2). A schematic representation is shown in Figure 1. Also, oil-in-water-in-oil ($O_1/W/O_2$) emulsions can be obtained and employed in specific applications, but the most commonly used and reported type is the $W_1/O/W_2$ system. The presence of an emulsifier with low hydrophilic/lipophilic balance (HLB) in the lipid phase is usually required to stabilize the inner water droplets of the $W_1/O/W_2$ emulsion. Common examples of such lipophilic emulsifiers are sorbitan esters (Spans), monoglycerides, phospholipids and polyglycerol polyricinoleate (PGPR). The latter surfactant has been the most widely used for the preparation of $W_1/O/W_2$ emulsions due to its high emulsifying properties, which is attributed to the excellent water-binding capacity of the long hydrophilic polyglycerol chain (Wilson et al., 1998). In turn, the oil droplets (containing the inner water droplets) should be stabilized with a hydrophilic emulsifier (i.e., with higher HLB value) included in the continuous aqueous phase. Such is the case of proteins or non-ionic surfactants like polyethoxylated sorbitan esters (Tweens). Thus, because of their amphiphilic nature, the lipophilic and hydrophilic emulsifiers tend to be located at the inner and outer interfaces, respectively, forming the corresponding interfacial films (Dickinson and McClements, 1995). Soluble polysaccharides have also been added in the outer aqueous phase to act as stabilizing agents due to their thickening/gelling properties (Dickinson, 2011).

It has been paid special attention to $W_1/O/W_2$ emulsions because of their particular characteristic of having an internal compartment, presenting the interesting ability to control the release of certain chemicals initially placed therein. Thus, various industries have shown particular interest in these systems. For instance, $W_1/O/W_2$ emulsions have been applied in cosmetics and pharmaceuticals for the slow release and targeted delivery of hydrophilic drugs, such as vaccines, vitamins, enzymes and hormones (Laugel et al., 1996; Gallarate et al., 1999; Vlaia et al., 2009). With regard to food industry, the application of $W_1/O/W_2$ emulsions follows different objectives. They have been proposed for the elaboration of low-calorie food products by lipid-reduction without the need of reducing the volume fraction of dispersed phase (de Cindio and Cacace, 1995). Hence, it is possible to produce reduced-fat products with similar physicochemical and sensory properties as full-fat products, e.g., appearance, texture, mouthfeel, and flavor. Other potential applications of $W_1/O/W_2$ emulsions in food systems are the isolation, protection and controlled release of hydrophilic compounds, such as minerals and vitamins, by their encapsulation in the inner water droplets (Auweter, 2001).

The application of $W_1/O/W_2$ emulsions in food industry requires the elaboration of products with good stability until their consumption. The actual use of $W_1/O/W_2$ emulsions in food industry is still scarcely developed probably because they are highly susceptible to a variety of factors, generating destabilization mechanisms that are responsible for the system breakdown. Some of these processes are similar to those observed in conventional O/W emulsions, but others belong exclusively to $W_1/O/W_2$ emulsions (Florence and Whitehill,

1981; Dickinson and McClements, 1995; Garti, 1997a, 1997b). These destabilization processes should be controlled and measured. Particularly, it is important controlling the volume and stability of inner water droplets in order to achieve and maintain the functionality of the food system. Different strategies have been proposed to improve the stability of $W_1/O/W_2$ emulsions, taking into account the limitations imposed by food safety regulations (Muschiolik, 2007).

Although the subject is not a novelty, the studies on $W_1/O/W_2$ emulsions have increased during the last years, providing new perspectives. The aim of this review is to summarize the general aspects of $W_1/O/W_2$ emulsions regarding their applications in the food area. New characterization methods and strategies for the improvement of stability and inner water retention are informed herein.

PREPARATION METHODS OF $W_1/O/W_2$ EMULSIONS

The most commonly reported procedure for the formation of $W_1/O/W_2$ emulsions is the two-stage homogenization method, using conventional rotor-stator and/or high pressure valve homogenizers, among others. This method is the most widely used due to the possibility to obtain $W_1/O/W_2$ emulsions in a systematic and better controlled way, with reproducible droplet size distribution and known composition. The first stage consists on the preparation of a primary W_1/O emulsion in which a lipid phase, including a lipophilic emulsifier, is homogenized with an aqueous phase by the application of high energy; and the second stage involves the dispersion of this W_1/O emulsion in another aqueous phase (W_2) with a hydrophilic emulsifier, using lower homogenization energy (Matsumoto and Kang, 1989). It should be taken into account that the higher the energy applied in the second homogenization stage the lower the yield, which is defined as the percentage of aqueous phase of primary W_1/O emulsion that remains entrapped in the final $W_1/O/W_2$ emulsion. Thus, the homogenizing conditions employed in the second stage should be less energetic than those used for the preparation of the primary W_1/O emulsion, in order to avoid or reduce the loss of inner water droplets and obtain large enough oil droplets to contain smaller water droplets. Moreover, the lipophilic:hydrophilic emulsifiers ratio should be higher than 10:1 to achieve a yield superior to 90%, though the use of some surfactant must be limited in food systems. The yield also depends on the volume fractions of dispersed phase in the primary W_1/O and final $W_1/O/W_2$ emulsions (Dickinson and McClements, 1995; Garti, 1997a).

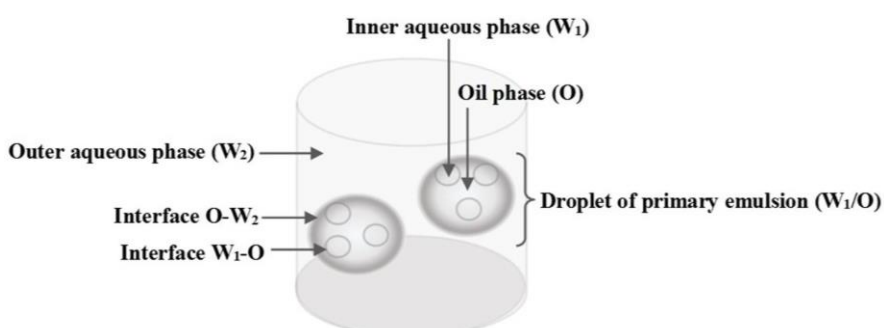

Figure 1. Schematic representation of a $W_1/O/W_2$ emulsion.

$W_1/O/W_2$ emulsions can also be prepared via a phase inversion process. In this case, an aqueous solution with a hydrophilic emulsifier is gradually added to a previously formed W_1/O emulsion, increasing the volume fraction of water to a certain degree. This procedure is also known as a catastrophic inversion method (Jahanzad et al., 2009). Another type of phase inversion process is the temperature method, in which a change in the affinity of surfactants to their corresponding phase is produced during heating or cooling (Morais et al., 2008, 2009). In both procedures, the $W_1/O/W_2$ emulsion is obtained as an intermediate stage before the system is totally inverted into a simple O/W emulsion. The actual obtaining of $W_1/O/W_2$ emulsions by this preparation method is doubtful in some cases. Moreover, it is difficult to control the distribution of the emulsifiers within the two interfaces as they migrate between the phases, causing destabilization of the system.

Mechanical agitation is a one-step emulsification method, where an aqueous solution containing a hydrophilic emulsifier is vigorously stirred together with an oil phase containing a large amount of lipophilic emulsifier. The high total emulsifier concentration promotes oil droplet elongation, due to the low interfacial tension; and at the same time the high lipophilic emulsifier content induces the formation of a W/O emulsion. Then the $W_1/O/W_2$ emulsion is formed spontaneously as a result of local fluctuations in the curvature of the interface, from convex to concave and vice versa, with respect to the oil phase (Dickinson and McClements, 1995; Hong et al., 2012). In some instances, where the aim was to obtain a W/O emulsion, an unexpected formation of a $W_1/O/W_2$ emulsion occurred even though only a lipophilic emulsifier was employed as surfactant (Márquez et al., 2007). Recently, Clegg et al. (2016) have reviewed three approaches for the preparation of $W_1/O/W_2$ emulsions in a single step: microfluidic production, droplet stabilization using block copolymers and droplet stabilization using colloidal particles.

Alternative non-conventional techniques for the preparation of $W_1/O/W_2$ emulsions were proposed by other authors. Vigie (1992) indicated that it is possible to obtain a $W_1/O/W_2$ emulsion using a lamellar phase dispersion process. This procedure involves the formation of liposome-like vesicles with non-ionic surfactants and can be carried out when the constituents form a lamellar phase by mixing emulsifiers with water in definite proportions. Membrane emulsification is a mild process where a W_1/O emulsion is pressed through a microporous membrane while the continuous aqueous phase flows along the membrane surface; then oil droplets containing water droplets are formed at pores, detaching at a certain size (van der Graaf et al., 2005). Another alternative method consists on the use of specially treated micropipettes for the production of a large droplet of oil in water, which is subsequently injected with smaller water droplets (Hou and Papadopoulos, 1997).

APPLICATIONS OF $W_1/O/W_2$ EMULSIONS IN FUNCTIONAL FOODS

The strategic use of $W_1/O/W_2$ emulsions for the formulation of foods basically points to two main objectives: lipid reduction and encapsulation of hydrophilic compounds. Due to the nature and composition of these systems, they can be applied to obtain low-calorie emulsified products. The historical problems of obtaining lipid-reduced food systems are usually linked to unfavorable changes in organoleptic properties like taste and texture. $W_1/O/W_2$ emulsions have been purposed as a likely solution because they allow the preparation of systems with

same volume fraction of dispersed phase but lower lipid content, because of the partial replacement of oil/fat by inner water droplets (De Cindio and Cacace, 1995). In this way, the loss of taste and rheological changes can be diminished because the surface area of lipid phase is maintained. Different works have been performed regarding the application of $W_1/O/W_2$ emulsions for the elaboration of specific lipid-reduced foods, including white fresh cheese-like products (Lobato-Calleros et al., 2006, 2008), stirred yogurt (Lobato-Calleros et al., 2009), dairy cream substitute (Márquez and Wagner, 2010) and meat systems (Cofrades et al., 2013, 2014). Apart from a modification of lipid quantity, the lipid quality can be improved by the use of alternative oils/fats with healthier fatty acid profiles (Jiménez-Colmenero, 2013).

The presence of inner water droplets inside oil droplets allows the encapsulation of hydrophilic compounds, giving the possibility of enhancing the nutritional value and the functionality of the developed food. Minerals and vitamins are among the most common nutrients whose encapsulation by a $W_1/O/W_2$ emulsion was proposed. An example of this application is the isolation of calcium chloride in inner water droplets to avoid its interaction with soybean proteins in the continuous aqueous phase; in this way, a soymilk based emulsion was fortified with calcium while the destabilization of the system was prevented (Márquez and Wagner, 2010). Another approach was the microencapsulation of ascorbic acid to protect this unstable vitamin and to mask its acidic taste (Comunian et al., 2013). Other authors assayed the encapsulation of magnesium (Bonnet et al., 2009) and B-group vitamins (Benichou et al., 2007; O'Regan and Mulvihill, 2010). The encapsulation of microorganisms was also proposed, for example, as a method to protect acid lactic bacteria against gastrointestinal tract conditions (Shima et al., 2006; Pimentel-González et al., 2009). The controlled release of substances is another interesting application of $W_1/O/W_2$ emulsions (Pays et al., 2002). For instance, Vaziri and Warburton (1994) studied the encapsulation and release of chloroquine phosphate (an antimalarial) in order to mask its unpleasant taste. $W_1/O/W_2$ emulsions were also designed to control the delivery and release of flavors during consumption (Malone et al., 2003).

Beyond their potential applications in food industry, $W_1/O/W_2$ emulsions present limitations that should be taken into account. A typical problem is the need of relatively large oil droplets to contain smaller water droplets, which may give an undesirable oily taste to the food product. Because the smaller the oil droplets size the higher the loss of inner water droplets, optimum emulsification conditions must be found. The generation of very small water droplets is always desirable; but the employed amount of lipophilic emulsifier should not exceed levels given by regulations for food safety. Other factors such as volume fractions of water and oil droplets or applied energy and time for the second homogenization stage should be controlled in order obtain an acceptable taste and a reasonably good yield (Dickinson and McClements, 1995; Muschiolik, 2007; Jiménez-Colmenero, 2013).

DESTABILIZATION PROCESSES OF $W_1/O/W_2$ EMULSIONS

The application of $W_1/O/W_2$ emulsions for the elaboration of food systems demands acceptable physic stability, in order to keep their characteristics until its consumption. $W_1/O/W_2$ emulsions are subjected to similar destabilization processes to those observed in

O/W systems: creaming, flocculation and coalescence of oil droplets (Garti, 1997b). The relatively large oil droplets usually obtained in $W_1/O/W_2$ emulsions tend to favor gravitational separation by creaming (Dickinson and McClements, 1995). However, depending on the inner water retention of the system, creaming could be reduced due to the increased density of oil droplets containing water droplets. Moreover, the presence of a lipophilic emulsifier in the lipid phase may lead to competitive adsorption at the outer interface in emulsions stabilized with proteins as hydrophilic emulsifier (Gülseren and Corredig, 2012). In this way, protein displacement can occur, weakening the interfacial film and modifying the repulsive interaction between droplets. These changes can favor the collision and fusion of oil droplets by coalescence when the lipid phase is totally liquid and the aggregation by partial coalescence when the lipid phase is partially composed by fat crystals (Fredrick et al., 2010).

Because of the presence of an aqueous phase dispersed in the lipid phase, $W_1/O/W_2$ emulsions present additional processes regarding the stability of inner water droplets. These changes would lead to the release of dispersed aqueous phase over time, preventing the effective application of the system. The typical destabilization processes of inner water droplets are illustrated in Figure 2 and were described by Florence and Whitehill (1981). The sequential expulsion of inner water droplets toward the continuous aqueous phase occurs as a coalescence process, but in this case the collision involves one water droplet and the outer interface (Figure 2a). A proper coalescence process leading to the fusion of two or more inner water droplets can also take place, so that the number of water droplets decreases and their individual size increases (Figure 2b). The existence of an osmotic unbalance, because of different solute concentrations in the dispersed and continuous aqueous phases, can lead to the diffusion of water toward one or the other aqueous phase. Water transportation through the oil layers occurs by solubilization into reverse micelles of lipophilic emulsifier (Matsumoto et al., 1980). A higher solute concentration in the continuous aqueous phase favors the loss of dispersed aqueous phase (Figure 2c); and when the direction of the osmotic gradient is the opposite, water can be incorporated into the oil droplets, increasing the volume fraction of inner water droplets and sometimes provoking their coalescence (Figure 2d). All these processes eventually could lead to the complete loss of dispersed aqueous phase, turning the $W_1/O/W_2$ emulsion into an O/W system.

The diffusion of water from one aqueous phase to the other, as a consequence of an osmotic unbalance, not only modifies the volume fraction of dispersed aqueous phase but also the volume fraction of total dispersed phase in the $W_1/O/W_2$ emulsion. The loss of inner water droplets leads to shrinkage of oil droplets and, thus, a diminution of the volume fraction of total dispersed phase. These changes tend to reduce the viscosity of the emulsion and could promote destabilization by creaming. On the other side, swelling of oil droplets occurs when water is incorporated into them, increasing the volume fraction of total dispersed phase and the viscosity of the system. An extreme case of the latter phenomenon would be an excessive swelling of oil droplets, leading to the complete rupture of the $W_1/O/W_2$ emulsion and the conversion to an O/W system. However, in the practice this event hardly occurs because the osmotic unbalance tends to be equilibrated with the diffusion of water from the continuous aqueous phase; and when the volume fraction of total dispersed phase is relatively high, oil droplets become too packed and do not have space to keep on swelling (Dickinson and McClements, 1995).

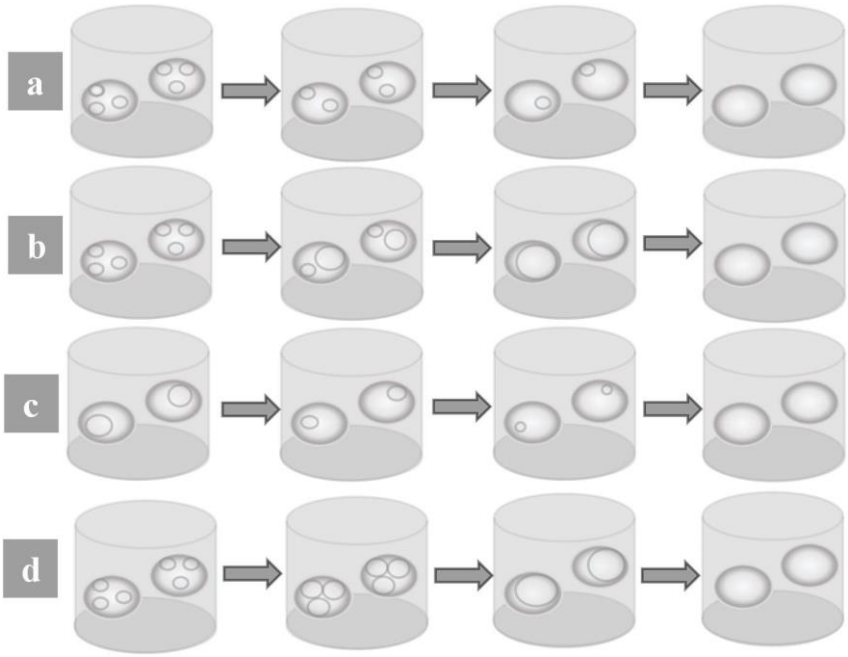

Figure 2. Destabilization processes of inner water droplets in $W_1/O/W_2$ emulsions. a) Expulsion of inner water droplets. b) Coalescence of inner water droplets. c) Release of dispersed aqueous phase due to higher solute concentration in continuous aqueous phase. d) Incorporation of dispersed aqueous phase due to lower solute concentration in continuous aqueous phase.

METHODS FOR THE ANALYSIS OF STABILITY AND ENCAPSULATION EFFICIENCY

Different methods can be applied to study the stability of $W_1/O/W_2$ emulsions. Creaming, flocculation and coalescence of oil droplets can be measured by traditional methods used for the analysis of O/W emulsions, such as light scattering, rheology and microscopy. These methods can also be used to detect variations of volume fraction of total dispersed phase, due to the loss or gain of dispersed aqueous phase, analyzing microstructural and rheological changes. The viscoelasticity of the emulsion gives additional information related to the inner water retention, because the higher the volume fraction of dispersed aqueous phase the higher the elastic character of the system (Pal, 2007). However, a precise calculation of inner water retention and its variation over time demand alternative techniques because the dispersed aqueous phase is hidden in the lipid phase and its quantification and qualification is more complicated. In this way, different methods have been developed to measure the encapsulation efficiency, which is a parameter used as a percentage quantification of retained water and/or solutes in the dispersed aqueous phase (Su et al., 2008; Sapei et al., 2012). It should be taken into account that the releases of water and solute are not necessarily equivalent because they can migrate toward the continuous aqueous phase by different processes. The loss of encapsulated hydrophilic compounds can occur by the previously described mechanisms involving the release of dispersed aqueous phase (Figure 2); but some solutes can also diffuse toward the continuous aqueous phase without water release (Lutz et

al., 2009). Thus, the encapsulation efficiency value depends on whether released water or solute is measured, and in the latter case, the nature of the solute.

The inclusion of a marker in the dispersed aqueous phase whose release can be measured in the continuous aqueous phase is a method usually employed for the estimation of encapsulation efficiency in $W_1/O/W_2$ emulsions. For instance, the addition of a dye of known concentration in the dispersed phase of the primary W_1/O emulsion enables the release quantification of this dye by spectrophotometry (Su et al., 2006; O'Regan and Mulvihill, 2009; Matos et al., 2013). The disadvantage of this method is the need to destruct the system by centrifugation to separate the continuous aqueous phase and measure its coloration, because inner water droplets could be released in the process. Moreover, the obligatory inclusion of a dye may have an effect on the stability of inner water droplets; and its application in food emulsions is limited because of the turbidity given by their complex composition, interfering with the measurement by spectrophotometry.

Another method for the calculation of encapsulation efficiency, maybe more proper for food emulsions, is conductimetry. In this case, electrolytes (e.g., sodium chloride) are included in the dispersed aqueous phase and the conductivity is measured in the continuous aqueous phase to calculate the released salt (Frasch-Melnik et al., 2010; Sapei et al., 2012). As it was previously mentioned, the nature of the salt could have an effect on the obtained data; for instance, Lutz et al. (2009) observed that sodium chloride was released more slowly than sodium ascorbate. Results obtained by our research group using the conductimetry method are presented in Figure 3a, showing an analysis of $W_1/O/W_2$ emulsions prepared with sodium caseinate as hydrophilic emulsifier, PGPR as lipophilic surfactant and sodium chloride solution as dispersed aqueous phase. As it was expected, the encapsulation efficiency increased with increasing PGPR concentration. The variation of this parameter with time can also be appreciated. These data were confirmed by optical microscopy, as a higher volume fraction of inner water droplets was observed at higher PGPR content (Figure 4). Nevertheless, $W_1/O/W_2$ emulsions without electrolytes in dispersed aqueous phase can not be analyzed by conductimetry.

A promising method for the estimation of encapsulation efficiency in food emulsions could be differential scanning calorimetry (DSC), as it does not require the presence of specific makers or solutes. This technique allows the quantification of the dispersed aqueous phase by the detection and measurement of the corresponding exothermic peak during the cooling of sample, because the inner water droplets freeze at lower temperatures than the continuous aqueous phase, as a consequence of delayed ice nucleation. DSC can be also used to determine the relative size and distribution of inner water droplets due to a variation of their freezing temperature (Schuch et al., 2013, 2014). An example of DSC thermograms of $W_1/O/W_2$ emulsions is shown is Figure 3b; it can be observed an increase of the exothermic peak area of dispersed aqueous phase with increasing PGPR concentration, showing the same tendency as previously indicated by conductimetry for the same systems (Figure 3a). The limitation of this method is, in certain $W_1/O/W_2$ emulsions, the incapability to obtain a defined exothermic peak of the dispersed aqueous phase; in such cases the analysis of DSC data would be qualitative more than quantitative. Moreover, a transfer of water from the inner to the outer frozen aqueous phase can occur during the cooling of the emulsion, attributed to the difference of vapor pressures between ice and undercooled water at the same temperature (Potier et al., 1992).

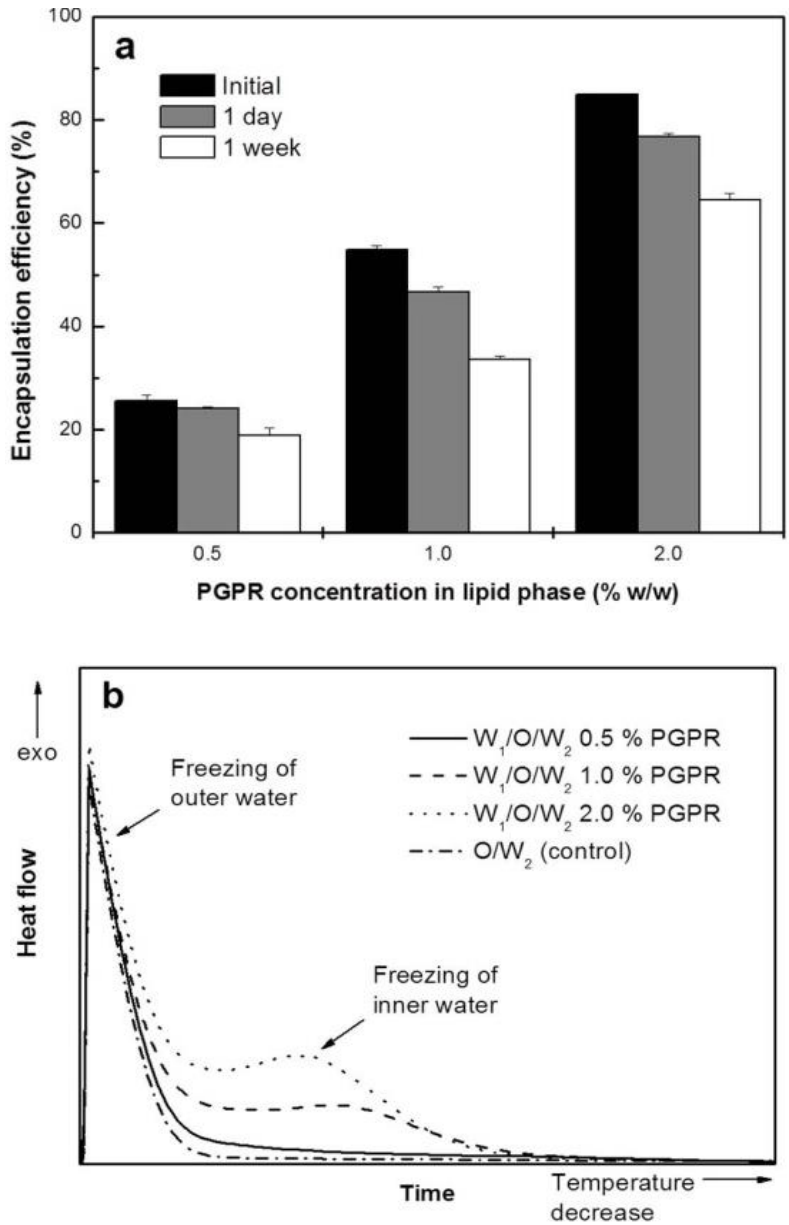

Figure 3. Analysis of $W_1/O/W_2$ emulsions prepared by the two-stage method: the primary W_1/O emulsion was composed by 25 g dispersed aqueous phase (containing 0.6% w/w sodium chloride) and 75 g lipid phase (sunflower oil with varied PGPR concentration); and 40 g of this W_1/O emulsion was mixed with 60 g continuous aqueous phase (W_2, containing 1.0% w/w sodium caseinate and 0.2% w/w xanthan gum). a) Estimation of encapsulation efficiency at different storage times by conductimetry. b) DSC thermograms of initial emulsions (2.5ºC/min cooling rate).

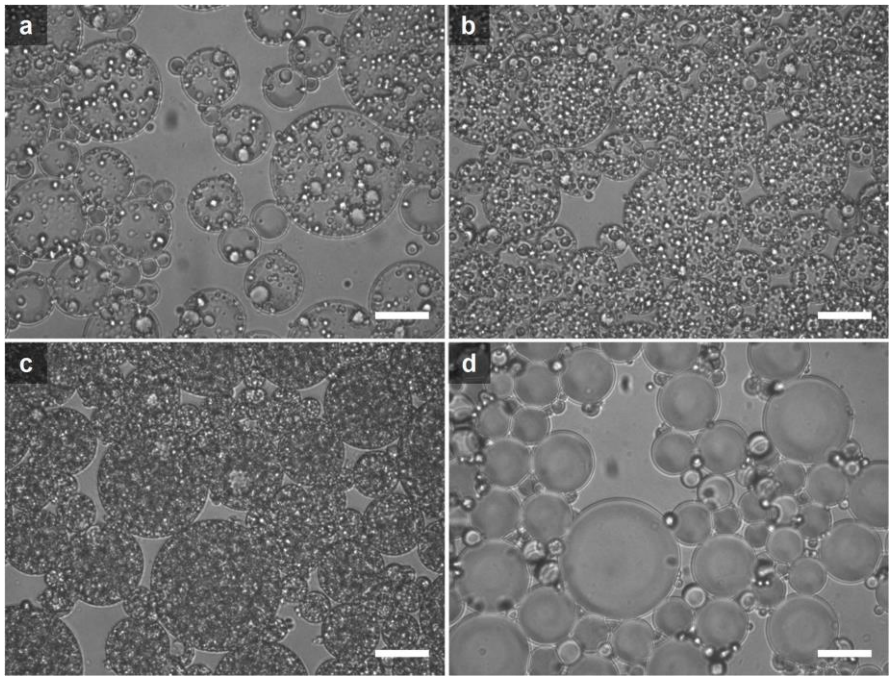

Figure 4. Optical micrographs (400×) of $W_1/O/W_2$ emulsions (analyzed in Figure 3). a) 0.5% PGPR in lipid phase. b) 1.0% PGPR in lipid phase. c) 2.0% PGPR in lipid phase. d) O/W_2 emulsion (control). Bar = 20 μm.

STRATEGIES FOR THE IMPROVEMENT OF STABILITY AND ENCAPSULATION EFFICIENCY

Although several applications of $W_1/O/W_2$ emulsions have been proposed for the development of functional foods, the food industry is still reticent to their actual use. Some reasons may be found in the stability issues of these systems and the limited possibilities of improvement due to the need to respect food safety conditions. To overcome these limitations, different strategies have been designed. But first of all, the formulation and preparation conditions of $W_1/O/W_2$ emulsions should be optimized. Creaming, flocculation and coalescence of oil droplets could be reduced by the addition of stabilizing agents such as hydrocolloids (e.g., xanthan gum, gelatin, pectin, etc.), increasing the viscosity of the continuous aqueous phase and decreasing the mobility of the dispersed phase. To prevent expulsion and coalescence of inner water droplets, the selection of an effective lipophilic emulsifier is required, also taking into account the maximum concentration allowed by food regulations. And the osmotic pressures of dispersed and continuous aqueous phases should be equilibrated in order to avoid the transport of water from one aqueous phase to the other (Dickinson and McClements, 1995; Garti, 1997b; Muschiolik, 2007). Then, after all these destabilizing factors are controlled, new strategies could be designed for further improvement of $W_1/O/W_2$ emulsion stability.

The increase of encapsulation efficiency and its maintenance over time have become key objectives boarded by several researches. Sapei et al. (2012) formulated $W_1/O/W_2$ emulsions

for food applications with PGPR and polysorbate 80 as lipophilic and hydrophilic emulsifiers, respectively, and stabilized the inner water droplets by the inclusion of sodium chloride and/or gelatin in dispersed aqueous phase. Apart from balancing the osmotic pressures, the presence of salt prevented the coalescence of inner water droplets because electrolytes would increase the adsorption density of the lipophilic emulsifier at the inner interface and reduce the interfacial tension, as it was previously observed in W/O emulsions (Aronson and Petko, 1993; Márquez et al., 2010). The addition of gelatin also increased the stability of the system and enhanced the encapsulation efficiency due to the gelation of the dispersed aqueous phase. Furthermore, the authors reported a synergistic action of the salt and the gelling agent in stabilizing the inner water droplets.

The inclusion of fat crystals in the dispersed lipid phase of $W_1/O/W_2$ emulsions has also been considered to increase the stability of inner water droplets. Frasch-Melnik et al. (2010) prepared a primary W_1/O emulsion with a lipid phase composed by sunflower oil and including 1.25% saturated monoglyceride and 2.5% tripalmitin; potassium chloride was added in the dispersed aqueous phase to measure its release. Then this W_1/O emulsion was diluted with sunflower oil and mixed with an aqueous phase containing 1% sodium caseinate (W_2), where different concentrations of glucose or sodium chloride were added in order to generate various osmotic gradients. The obtained $W_1/O/W_2$ emulsion presented good stability despite the osmotic unbalance given by higher solute concentration in the continuous aqueous phase, attributed to the stabilizing effect of fat crystal shells. Only 20% potassium chloride was released within 6 weeks. However, when the direction of the osmotic gradient was the opposite, the stability of the system decreased due to fat crystal re-arrangement and breaking of shells.

An interesting method for the preparation of $W_1/O/W_2$ emulsions with high volume fraction of inner water droplets was designed by Leal-Calderon et al. (2012), using food-based ingredients. The fabrication of the system consisted on the preparation of a $W_1/O/W_2$ emulsion by the two-stage homogenization method including relatively high solute concentration in both aqueous phases and at the same level. Afterwards the obtained emulsion was diluted with pure water, leading to the osmotic swelling of oil droplets by the transport of water from the continuous to the dispersed aqueous phase. According to the authors, $W_1/O/W_2$ emulsions with more than 90% dispersed aqueous phase within oil droplets can be obtained by this method and they proposed it for the formulation of food systems with enhanced encapsulation capacity and reduced fat content.

The formulation of $W_1/O/W_2$ emulsions with increased encapsulation efficiency and stability should always take into account certain limitations if they are meant to be applied for the elaboration of food systems. Particularly, PGPR is the most widely used emulsifier for the preparation of the primary W_1/O emulsion because of its effectiveness, but its employment is limited by food regulations (Wilson et al., 1998). The employed concentration of this surfactant can be reduced by the addition of other components whose combination with PGPR increases the stability of inner water droplets. Su et al. (2006) reported a possible synergistic effect of sodium caseinate (when added in the dispersed aqueous phase) and PGPR on the stabilization of inner water droplets. The authors indicated that PGPR concentration can be reduced from 4 to 2% if 0.5% sodium caseinate is added in the dispersed aqueous phase. In this way, the encapsulation efficiency and the stability of the $W_1/O/W_2$ emulsion was not affected while the PGPR content was diminished. The addition of calcium chloride in the dispersed aqueous phase also allowed the reduction of PGPR concentration

without affecting the stability of the primary W_1/O emulsion (Márquez et al., 2010), so that calcium salt not only contributed its nutritional value but also acted as a functional ingredient in $W_1/O/W_2$ emulsions prepared with soybean milk (Márquez and Wagner, 2010).

CONCLUSION

The potentiality of $W_1/O/W_2$ emulsions for the elaboration of functional foods has been demonstrated by several authors. Different strategies were implemented to overcome the limitations of these systems and new techniques were developed in order to analyze and improve their stability and encapsulation efficiency. Promising results were obtained in different studies and the future of $W_1/O/W_2$ emulsions in food industry could be a forthcoming reality.

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Chapter 3

FUNCTIONAL BREAD: DEVELOPMENT OF SOURDOUGH STARTERS TO IMPROVE BREAD QUALITY

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ABSTRACT

Bread is one of the major constituents of contemporary man's diet. The main microbiological problems of bread industry are mold and bacterial spoilage (roping) of bread. Moreover, the increasing environmental deterioration results in a significant increase in the insemination level of typical raw materials used in bread production. The manufacturers' solution is the extensive use of preservatives. Nowadays, the consumers' health awareness led to the increasing demand for preservative-free food products, but with retained or improved organoleptic characteristics and extended shelf life. The healthier alternative to preservatives in bread industry is the development and introduction of sourdough starters containing selected homo- and heterofermentative lactic acid bacteria and propionic acid bacteria strains.

Fourteen lactobacilli strains were isolated from different sources and were identified to species level using biochemical (API 50 CHL) and molecular-genetic (ARDRA-analysis and 16S rDNA sequencing) methods. The studied lactobacilli strains were able to grow in flour/water environment and reached high concentrations of viable lactobacilli cells. They also inhibited the growth of typical bread saprophytic microorganisms - *Bacillus subtilis, Aspergillus niger, Penicillium* sp., *Rhizopus* sp., but did not inhibit baker's yeasts *Saccharomyces cerevisiae*.

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Fourteen sourdough starters for rye and wheat bread were developed on the basis of the selected strains of homo - and heterofermentative lactobacilli with the addition of the probiotic propionic acid bacteria strain *Propionibacterium freudenreichii* ssp. *shermanii* NBIMCC 327 and *Lactobacillus sanfranciscensis*. Bread with best organoleptic characteristics was obtained with the incorporation of 7% of the four-strain starter sourdough.

For the prevention of microbial bread spoilage the percentage of the selected best performance four-strain starter sourdoughs was between 10% and 15% for the prevention of bread roping (bacterial spoilage) and between 15% and 20% for the prevention of fungal growth. The developed starter sourdoughs could be included in the kneading of the main dough in the form of liquid sourdoughs, frozen sourdoughs or freeze-dried starter concentrates.

A biotechnological scheme for the production of „LB-acidifiers" (dry sourdough) for rye and wheat bread with the best sourdough starters for rye and wheat bread was developed. The obtained „LB-acidifiers" are applied in bread production in a concentration of 3% - 5% in order to obtain bread with improved organoleptic characteristics, extended shelf life (5 days), without the addition of preservatives.

The development and application of lactobacilli sourdough starters in bread manufacture ensures the conduction of targeted fermentation process, the microbiological safety, the improved flavor and the extended shelf life without preservative addition, thus enhancing the beneficial effects of bread consumption.

Keywords: sourdough, starter, *Lactobacillus*, *Propionibacterium*, preservative, mold spoilage, roping of bread, probiotic

INTRODUCTION

1. Characteristics of Functional Foods

Food is a source of energy for all living organisms. It serves both to satisfy hunger and to deliver certain mineral elements, growth factors, promoters and regulators of various biological functions, through which the physical and mental well-being of the organism is improved (Takachi et al., 2008; Betoret et al, 2011).

According to the Functional Food Science in Europe (FuFoSE), in a coordination with the International Life Sciences Institute (ILSI), Europe "a food product can only be considered functional if together with the basic nutritional impact it has beneficial effects on one or more functions of the human organism, thus either improving the physical and general conditions, or/and decreasing the risk of the evolution of diseases."

Functional foods can improve the general health state of the organism (e.g., pre- and probiotics), reduce the risk of certain diseases (e.g., cholesterol-lowering products), and can be used to treat other diseases.

The development of new functional foods is a good opportunity to improve food quality to positively affect human health. These food products with added value are the subject of growing industrial and social interest in contemporary society.

Functional foods are developed from foods that have normal nutritional value by applying one or more of the following approaches:

- eliminating a food component, which is known to have a negative effect on the body;
- increasing the concentration of food components to the point at which the projected beneficial effects of their consumption will be exhibited or increasing the concentration of non-food ingredients to a level that is known to have a beneficial effect;
- adding components which are not present in most foodstuffs, and which are not nutrients but have proven favorable effect on the body;
- replacing food components whose intake is in surplus and has negative effects with food components with known beneficial influence;
- increasing the bioavailability or stability of a food component, which is known to exhibit positive effect or reduce the risk of developing a disease.

Along with the necessary nutrients for the body functional foods contain ingredients that have beneficial effects on human health. Lactic acid bacteria play a key role in food fermentations, contributing to the development of desirable organoleptic properties of the final product and its microbiological safety (Smaoui et al., 2010; Cizeikiene et al., 2013).

2. Lactobacilli as Starter Cultures in Food Production

Species of the *Lactobacillus* genus are associated with the production of functional foods, because of their preservative effect, due to acidification, and/or because their inclusion in food improves the flavor, texture and nutritional characteristics of the obtained food products (Stiles, 1996). Lactic acid bacteria play a crucial role in the preservation and microbial safety of fermented foods (Caplice and Fitzgerald, 1999), and provide microbial stability of the end products of fermentation (Mensah et al., 1991). Since lactic acid bacteria naturally occur in various food products, they have traditionally been used as natural food biopreservatives. Food protection is due to the production of organic acids (lactic acid, acetic acid, propionic acid, sorbic acid, benzoic acid), carbon dioxide, ethanol, hydrogen peroxide and diacetyl (De Vuyst and Vandamme, 1994a; Atrih et al., 2001), antifungal compounds such as fatty acids (Corsetti et al., 1998b) or phenyllactic acid (Lavermicocca et al., 2000), bacteriocins (De Vuyst and Vandamme, 1994a; Eswranandam et al., 2004), and antibiotics such as reutericyclin, nisin (Holtzel et al., 2000). Probiotic preparations which contain a high concentration of viable cells of the microorganism supporting the organism's health are also included in the group of functional foods. There is a variety of pre- and probiotic products available on the market, but the application of probiotic cultures in foods other than fermented dairy foods is a new trend in functional nutrition (Sharma et al., 2014).

Not all strains of lactobacilli may be included in the composition of functional foods. For each product type are selected suitable microorganism strains involved in the formation of flavor, aroma and influencing the shelf life of the finished foods by performing their inherent metabolic processes.

3. Basic Bread Components

Bread is one of the most consumed products worldwide with annual production of 9 billion kilograms (Heenan et al., 2008; Plessas et al., 2011a). It is an important ingredient of everyday human diet in almost every country. It is a source of nutrients, especially carbohydrates, fibers, proteins and minerals (magnesium, phosphorus, iron) (Kopec et al., 2011). The main raw materials for its production are flour, water, salt and baker's yeasts *Saccharomyces cerevisiae*.

Flour is the main raw material in bread production. It modulates the specific characteristics of bread and bakery products. It consists of proteins (gliadins and glutenins), starch and other carbohydrates, ash substances, fibers, lipids, water and minor amounts of vitamins, minerals and enzymes. Wheat flour is the most commonly used type of flour.

Water is necessary for dough formation and it determines its texture. Water dissolves salt and sugars and assists the dispersion and propagation of the yeast cells. Water is also necessary for starch and sucrose hydrolysis as well as starch gelatinization. Water contributes to dough swelling during baking by water evaporation (Giannou et al., 2003; Chavan and Jana 2008).

Sugars are normally used by yeasts during the early stages of fermentation. More sugars, which are transformed to gas under the action of flour enzymes, are released after that.

Sodium chloride (salt) strengthens the gluten, controls the activity of the yeasts and thus controls bread volume. A small amount of salt in the dough improves the flavor and promotes amylase activity, which helps maintaining a constant stock of maltose as an yeast substrate (Giannou et al., 2003; Chavan and Jana, 2008).

Lipids favor dough handling and contribute to flavor improvment of the product. They also help to preserve the quality, softness and moisture, as well as the good texture of bread (Giannou et al., 2003; Chavan and Jana 2008, Chavan and Chavan, 2011).

Bread quality depends on several factors: the internal parameters of flour, such as carbohydrates (Collar, 1996), gluten (Callejo et al., 1999), mineral elements (Emodi and Scialpi, 1980), lipid content (Collar et al., 1998) and endogenous enzyme activity (Martinez-Anaya, 1996a); and the external parameters related to bread production technology, such as temperature, stages and degree of fermentation (Spicher and Bruemmer, 1995), water activity (Berland and Launay, 1995), redox potential and additives (Ravi et al., 2000), and inclusion of nutritional or rheological enhancers, such as milk ingredients (Kenny et al., 2000), which affect the quality of the final product. The effect of these factors can be direct or indirect, but they all influence the microflora, whether it comes in the form of a commercial starter or by conventional processes involving the use of sourdough.

Bread freshness depends on the flavor, the appearance and the crispness of the crust, the hardness of the crumb and the bread volume. Bread flavor, however, is considered the most important feature for consumers as a criterion for eligibility of the products (Plessas et al., 2011b).

The most important quality characteristics of fermented wheat bread are big volume, soft and elastic crumb structure, good shelf life and microbiological safety of the product (Cauvain, 2003; Chavan and Jana, 2008).

Bakery products have a very short shelf life and their quality depends on the period of time between baking and consumption (Arendt et al., 2007).

4. Microbial Spoilage of Bread and Bakery Products

Spoilage of bread and bakery products is mainly due to the growth of molds, the main species belonging to the genera *Aspergillus*, *Fusarium* and *Penicillium*. In addition to the major economic losses arising from mold growth, an additional problem is the potential production of mycotoxins, which pose a health risk (Gerez et al., 2009).

Roping of bread is a widespread bacterial spoilage of bread, caused mainly by *Bacillus subtilis* and *Bacillus licheniformis* (Collins et al., 1991) that come from raw materials, the breadbaking atmosphere and the equipment surfaces (Bailey and Von Holy, 1993) and is still a major economic problem in bread production industry (Nielsen, 2003).

Roping of bread becomes noticeable within 12-24 hours after taking the bread out of the oven. Only vegetative forms of bacteria are destroyed during bread baking (Nowicki et al., 1988). Bacterial spores can survive the baking process in which the temperature in the center of the crumb does not exceed 97-101°C (Corsetti and Settanni, 2007). Spoilage is initially noticed as unpleasant odour, followed by the appearance of discolored, sticky and soft bread crumb caused by the degradation of the starch and proteins by microbial amylases and proteases, as well as by the production of extracellular polysaccharides (Mentes, 2007). Strains of both *Bacillus* species can cause foodborne diseases if their concentrations exceed 10^5CFU/g (Corsetti and Settanni, 2007).

Although it is unlikely for foodborne diseases associated with poor quality bread consumption to occur due to the greasy appearance of the bread crumb with high concentrations of *B. subtilis* and *B. licheniformis*, its consumption is possible to induce diarrhea and vomiting (Rosenkvist and Hansen, 1995).

Loss of bread freshness along with the increase in the hardness of the crumb during storage, resulting in a loss of acceptable appearance for the consumer is the process known as bread staling (Hebeda et al., 1990). Changes during bread staling include changes in both the crumb and the crust (D'Appolonia and Morad, 1981). Changes occur in the texture of the crumb - it becomes more rigid, more crumbly and opaque. The staling of the crust is usually caused by moisture migration from the crumb to the crust (Lin and Lineback, 1990), resulting in the crust becoming soft, with a rubbery consistency, which in general is less unpleasant than the changes in crumb (Arendt et al., 2007).

The deterioration of the ecological environment in the world is the reason for the usage of flour highly inseminated with mold and *Bacillus* sp. spores. Products with very short shelf life, due to the occurrence of fungal and bacterial spoilage are obtained. This leads to major economic losses for producers while consumers are exposed to serious health risks. These problems are particularly acute in warmer months.

5. Preservatives in Bread Production

In addition to the general physical methods for food preservation (cooking, cold storage, storage in modified atmosphere, drying, freeze-drying) (Farkas, 2001), protection of bakery products from spoilage caused by molds is achieved primarily through inactivation of contaminating spores using (1) infrared and microwave radiation; (2) fungal inhibitors such as ethanol and propionic, sorbic, benzoic and acetic acid and their salts; (3) appropriate

packaging techniques such as modified atmosphere packaging, and (4) inclusion of sourdough (Guynot et al., 2005; Corsetti and Settanni, 2007).

Nowadays, preservatives such as lactate, acetate and propionate, which inhibit the growth of molds, and thus improve food safety and extend food shelf life are added to foods to prevent bread spoilage (Gould, 1996).

Acetic acid (E260) is included in quantities of 0,20 to 0,30%. Acetic acid causes a pH decrease, thus inhibiting bacterial growth, since the optimum pH values for the growth of most bacteria, including the genus *Bacillus*, are in the weakly acidic and neutral pH range. Acetic acid has an inhibitory effect on the growth of fungi and yeasts, but in comparison with the action of other preservatives, its influence is weak. Complete inhibition of fungal and yeast growth occurs at concentrations of 3,50 to 4,00% acetic acid. It should be noted that higher concentrations of salt in bread enhance the inhibitory effect of acetic acid due to the reduction of the water activity (a_w).

Calcium propionate (E281) is the second most important preservative used in bread production. It is included in quantities of 0,10 to 0,32%. It is easily absorbed in the digestive tract, because of its good water solubility. It has been shown that propionic acid inhibits mold and *Bacillus* sp. spores, but it does not inhibit yeast growth, so it is traditionally applied as a bread preservative. Undissociated calcium propionate has antimicrobial activity, which largely depends on bread pH. Its antimicrobial activity is manifested at higher pH values ($pH \geq 7$). Therefore in the permissible concentrations calcium propionate manifests itself as a weak preservative. This means that it needs to be added to food in higher concentrations in order to exhibit satisfactory antimicrobial action. Some fungi, such as *Penicillium* sp., grow in the presence of 5% propionate in the medium. Nevertheless, calcium propionate is incorporated in order to suppress the growth of fungi and bacteria of the species *Bacillus subtilis*, whose spores cause bread roping. The propionate antifungal activity is higher than that of lactate and acetate. In addition to the effect of propionic acid on the cytoplasmic pH, propionate is also converted to propionyl-CoA, which inhibits pyruvate dehydrogenase and thereby inhibits glucose metabolism in fungi (Zhang et al. 2010).

According to the law within the European Parliament and Council Directive № 95/2/EC, propionic acid can be added to bread in amounts no more than 3000 ppm (European Union, 1995). However, recent studies have shown that under these conditions, propionic acid is not effective against common microorganisms causing bread spoilage (Lavermicocca et al., 2000). Furthermore, the reduction of preservatives to sub-inhibitory levels can stimulate the growth of spoilage molds and/or production of mycotoxins. Due to the low propionate antimicrobial activity, it needs to be introduced in higher concentrations in order to prevent fungal spoilage and to suppress the growth of mycotoxin-producing molds. The high concentrations of calcium propionate, however, slow down dough fermentation which can be compensated by increasing the amount of yeast or by increasing the fermentation time. Moreover, propionate affects bread flavor and aroma which is undesirable for bread and bakery products. Besides, the presence of calcium propionate in high concentrations inhibits microbial enzymes and blocks microbial metabolism. The propionic acid obtained by calcium propionate dissolution inhibits the growth of body cells and causes migraine.

Potassium sorbate (E202) together with calcium propionate is used as a preservative in bread and bakery products at concentrations of 0,1 to 0,3%. The antimicrobial activity of potassium sorbate is associated with inhibition of the action of various enzymes in microbial cells, such as enzymes involved in the carbohydrate metabolism – enolase, lactate

dehydrogenase, malate dehydrogenase, isocitrate dehydrogenase, ketoglutarate dehydrogenase, succinate dehydrogenase, fumarase and aspartase, catalase and peroxidase; and destruction of the cell walls of the vegetative forms of microorganisms.

In comparison with calcium propionate, potassium sorbate exhibits a significantly higher inhibition activity. It is added to flour during dough kneading. Especially good effect is achieved when using freeze-dried baker's yeasts. The same effect as in calcium propionate is observed when using pressed yeasts, because a delay of dough fermentation and an extension of the fermentation process occur due to potassium sorbate's negative effect on yeast growth. Presently, to overcome these problems encountered in bread production too much baker's yeasts (dry baker's yeasts or pressed baker's yeasts) - up to 4-5% are being used. Upon absorption of bread ingredients in the human body, from the yeast cells are absorbed primarily proteins, carbohydrates, lipids, but not their nucleic acids. The diet of modern man is high in energy and the consumer's needs of energy are met by energy from the breakdown of proteins, carbohydrates and lipids. There is no need to use nucleic acids as an energy source. However, uric acid is obtained if nucleic acids break down, and the accumulation of uric acid in the body causes gout disease.

Less frequently in the production of bread is used ***sodium benzoate (E211)***, which is imported in quantity of up to 0,15 – 0,25% mainly to suppress fungal and wild yeast growth, but it is a preservative with a proven carcinogenic effect.

Very rarely in the production of bread and bakery products is applied ***sulfur dioxide (E220)***. It has a bleaching effect.

Every chemical preservative has a pH optimum of action. If it is introduced into food that does not have this optimum pH value, it would not execute its preventive role. But when it it intaken together with a given food in the digestive tract where the pH varies from 1.8 - 2.0 to 7, the preservative would eventually find its optimal pH value and would influence not only the pathogenic and saprophytic microflora but also the beneficial microflora in the gastro-intestinal tract. Maintainance of the balance of the microflora in the stomach and intestines determines the health status of the individuals to a significant extent.

The reduction of the preservative content to sub-inhibitory levels might stimulate the growth of spoilage molds (Marin et al., 1999) and/or production of mycotoxins (Bullerman, 1985).

The addition of sourdough is the best approach to prevent bread spoilage that meets consumers' demand for natural food without preservative addition (Rosenquist and Hansen, 1998; Corsetti and Settanni, 2007).

A synergistic effect in sourdough fermented with antifungal strains of *Lactobacillus plantarum* in combination with calcium propionate (CAP) for the production of wheat bread was established (Ryan et al., 2008). Sourdough inclusion allows the reduction of CAP levels by about 30% without negatively affecting bread shelf life (Moroni et al., 2009).

6. Sourdough

Sourdough is a mixture of flour (wheat, rye, rice, etc.) and water, which is fermented by the action of lactic acid bacteria and yeasts (Vogel et al., 1999; De Vuyst and Ganzle, 2005). These microorganisms typically originate from the flour, dough ingredients or from the environment.

Sourdough is a food ecosystem that (I) allows the selection of lactic acid bacteria strains that are adapted to the sourdough environment and (II) is inhabited by lactic acid bacteria species that are specific for the sourdough (Dal Bello et al., 2005; De Vuyst and Neysens, 2005; Gobbetti et al., 2005). The adaptation of certain lactic acid bacteria to the sourdough environment comprises (I) a central metabolism and/or transport of specific sourdough carbohydrates (maltose and fructose), maltose being the most common fermentable carbohydrate, and fructose being an important alternative electron acceptor; (II) activated proteolytic activity and/or arginine deaminase pathway; (III) defined stress responses, and (IV) production of antimicrobial compounds (De Vuyst and Vancanneyt, 2007).

There are significant pieces of evidence of the positive effects of sourdough use in bread production, including improvements in the bread volume and the crumb structure (Corsetti et al., 2000; Crowley et al., 2002), the flavor (Thiele et al., 2002), the nutritional value (Liljeberg and Bjorck, 1994; Liljeberg et al., 1995) and the shelf life (Corsetti et al., 1998b; Lavermicocca et al., 2000, 2003; Dal Bello et al., 2006; Arendt et al., 2007) due to the slowing of the staling and the prevention of fungal and bacterial spoilage (De Vuyst and Vancanneyt, 2007).

The positive effect of sourdough on bread volume is the result of the better gas-retention ability of the gluten in sourdough-containing dough (Gobbetti et al., 1995a, 1995b), the dissolving of pentosans (Corsetti et al., 2000), and the altered activity of endogenous enzymes. This is due to sourdough use and the subsequent low pH (Clarke et al., 2003) and the more rapid alcohol fermentation in the presence of lactic acid bacteria (Gobbetti et al., 1995a, 1995b; Katina et al., 2006). The improvement of the specific bread volume is associated with reducing the staling rate (Maleki et al., 1980), which is proved by the slow reduction of the crumb softness in sourdough bread during storage (Corsetti et al., 2000; Crowley et al., 2002; Arendt et al, 2007). These positive effects are associated with the metabolic processes of the microorganisms in the composition of the sourdough, such as lactic acid fermentation, proteolysis, production of exopolysaccharides and synthesis of antimicrobial and volatile compounds (Arendt et al., 2007; Corsetti and Settanni, 2007; Moroni et al., 2009).

Microorganisms in sourdough are traditionally allowed to grow naturally, but starters with selected and defined composition are used to control the process of sourdough preparation and to optimize the benefits of its application. The trend is the development of new and improved starters for optimization of sourdough fermentation, leading to the preparation of better-quality bread. Therefore the selection of the microorganisms used is important for providing high-quality sourdough and easier control of the process of sourdough preparation. The associations of yeasts and lactic acid bacteria are self-defending and self-controling (Plessas et al., 2008).

7. Sourdough Starters in Bakery Industry

The proper selection of strains is the main requirement to design sourdough starters for bread (Stoltz and Böcker, 1996). The strain selection and starter design are mainly based on acidification, proteolysis and the synthesis of volatile compounds during sourdough fermentation (Collar, 1996; Corsetti et al., 1998a; Hammes and Gänzle, 1998; Gobbetti et al., 2005). Moreover, functional sourdough starters must be well adapted to the sourdough

environment and to the sourdough fermentation process in order to ensure stability and consistency of the dough (De Vuyst, 2000; De Vuyst and Neysens, 2005). Although the species belonging to the genera *Pediococcus*, *Leuconostoc* and *Weissella* are isolated from sourdough, the majority of the indigenous sourdough strains belong to the genus *Lactobacillus*.

Selected homo- and heterofermentative lactic acid bacteria strains are applied as components of sourdough starters. They are involved in dough fermentation and in the formation of the flavor and aroma complex of the finished bread. They are also responsible for the prolonged shelf life by protecting bread from bread roping and mold spoilage.

Heterofermentative lactic acid bacteria have a favorable effect on the structure of the dough, and of the bread. They also produce carbon dioxide which further increases bread volume. Although heterofermentative lactic acid bacteria generate less carbon dioxide than baker's yeasts, they are included in sourdough starter composition because they lower bread acidity and give bread a specific flavor (Bratowanova, 1991). Lactic acid bacteria have a key role in dough protection from contamination and spoilage (Bogatireva al., 1996 Bogatireva al., 1999).

Bread produced with sourdough starters containing only homofermentative lactic acid bacteria species, bears no specific flavor. The growth of heterofermentative cultures alone results in a large amount of acetic acid, which gives the bread pungent odour and sour flavor. Bread with best quality is obtained by the joint use of homo- and heterofermentative lactic acid bacteria in a certain ratio (Corsetti et al., 2001).

The formation of rich flavor is controlled by limiting the quantity of the sourdough used (5-10g/100g dough) in the preparation of the main dough. This approach, however, limits the amount of flavor precursors, as well as important flavor compounds, in the final dough coming from the sourdough (Katina et al., 2006).

An alternative is to adjust the processing conditions to produce maximum amounts of aromatic precursors such as amino acids and flavor volatile compounds with minimal acidification (Katina et al., 2004). The improved volume and shelf life of sourdough bread would depend on the nature and intensity of the acidification process (Clarke et al., 2003; Katina et al., 2006).

The selection of pure cultures consists in the use of separate species or combination of species specific to the sourdough fermentation process and having the ability to grow under these conditions. It is particularly important to manage to maintain the starter cultures for prolonged use when using pure cultures. The application of highly active yeast and lactic acid starters in bread production requires selection of active strains on one hand, and development of methods for maintaining the production cultures, on the other.

Baker's yeasts are associated with lactic acid bacteria in sourdough, the ratio of baker's yeasts/lactic acid bacteria being typically 1 : 100 (Gobbetti et al., 1994a; Ottogalli et al., 1996). Yeasts found in sourdough belong to more than 20 species (Rossi, 1996; Stolz, 1999; Gullo et al., 2002). The dominant species *Saccharomyces cerevisiae* (Gobbetti et al., 1994a; Corsetti et al., 2001; Vernocchi et al., 2004a; Vernocchi et al., 2004b) are often introduced in bread production by the addition of baker's yeasts (pressed of freeze-dried) (Corsetti et al. 2001). Typical yeasts associated with lactic acid bacteria in the composition of sourdough are *Saccharomyces exiguus*, *Candida humilis* (previously named as *Candida milleri*), and *Issatchenkia orientalis* (*Candida krusei*) (Gobbetti et al., 1994a; Succi et al., 2003; Vernocchi et al., 2004a; Vernocchi et al., 2004b). Other yeast species found in the sourdough ecosystem

are: *Pichia anomala* (known as *Hansenula anomala*), *Saturnispora saitoi* (known as *Pichia saitoi*), *Torulaspora delbrueckii*, *Debaryomyces hansenii* and *Pichia membranifaciens* (Gobbetti et al., 1994a; Succi et al., 2003).

There is a great diversity of lactic acid bacteria isolated from spontaneously fermented sourdough: *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, *Lactobacillus farciminis* (obligate homofermentative), *Lactobacillus plantarum*, *Lactobacillus homohiochii*, (facultative heterofermentative), *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus fermentum*, *Lactobacillus hilgardii*, *Lactobacillus sanfranciscensis*, *Lactobacillus viridiscens*, *Lactobacillus panis* and *Lactobacillus pontis* (obligate heterofermentative) (Wiese et al., 1996; Pepe et al., 2004). *Lactobacillus sanfranciscensis*, *Lactobacillus brevis* and *Lactobacillus plantarum* are the lactobacilli species most frequently isolated from sourdough (Valmorri et al., 2006; Corsetti and Settanni, 2007).

Besides the dominant lactic acid bacteria and yeast species, which are typically isolated in large quantities and contribute largely to the fermentation process, secondary microflora in a lower concentration is also possible to exist in spontaneous sourdough fermentation. The secondary microflora comprises lactic acid bacteria of the species *Lactobacillus alimentarius*, *Lactobacillus acidophilus*, *Lactobacillus fructivorans*, *Lactobacillus fermentum*, *Lactobacillus reuteri* and *Lactobacillus pontis* (Gobbetti et al., 1994a; Vogel et al., 1996; Corsetti et al., 2001) and yeast species such as *S. exiguus*, *Candida krusei* and *Candida milleri* (Rossi, 1996). These species influence the sourdough ecosystem directly or indirectly affecting the dominant microflora (Paramithiotis et al., 2006).

The stable co-metabolism between lactic acid bacteria and yeasts is characteristic for many foods, which allows the use of substrates that are normally non-fermentable (e.g., starch) from various microorganisms and thereby increase the microbial adaptability to complex food ecosystems (Gobbetti et al., 1994b; Gobbetti and Corsetti, 1997; De Vuyst and Neysen, 2005; Corsetti and Settanni, 2007).

The stability of mature sourdough depends on the microflora of the environment (the flour and the other ingredients used, the intrinsic microflora, etc.), the metabolic activity (amylase activity of the flour and/or microorganisms, the ability for cofactor regeneration, and the ability to produce energy by the microorganisms included in the composition of the sourdough starter, etc.), as well as the specific technological parameters of the process (chemical and enzymatic composition of the flour, fermentation and storage temperature, pH, redox potential, dough hydration, dough yield, number of steps of daily back-slopping of the sourdough, fermentation time between back-slopping, the use of starter cultures and/or baker's yeasts, etc..) (Gobbetti et al., 2005; Arendt et al., 2007; Corsetti and Settanni, 2007; Ganzle et al., 2007). As a consequence of the heterogeneity of these environmental determinants, mature sourdoughs differ in species diversity and metabolic activity (De Vuyst et al., 2002; De Vuyst and Neysens, 2005).

Fermented sourdough can affect health through several mechanisms: 1) modulation of the complex of dietary fiber and the subsequent fermentation pattern; 2) production of exopolysaccharides with prebiotic properties, and 3) production of metabolites from the fermentation of lactic acid bacteria that affect the intestinal microflora (Poutanen et al., 2009).

With the inclusion of starters pH falls much faster, so the entire production process is accelerated, resulting in economic benefits for the manufacturer. The majority of natural starters contain isolates of the desired microorganisms normally found in the substrates fermented (Holzapfel, 2002; De Vuyst and Vancanneyt, 2007; Edema and Sanni, 2008).

The use of baker's yeast in the production of rye bread does not exclude the use of sourdough. In rye bread a lowering of the pH is necessary in order to bake the bread (Hammes and Ganzle, 1998; Salovaara, 1998). Rye and wheat flour differ - rye flour contains high levels of pentosans. In rye doughs proteins play a minor role in the process of forming the dough structure compared to the same role in wheat doughs because pentosans suppress the formation of the gluten network (Cauvain, 1998). Solubility and swelling of pentosanes increase at low pH values which are typical for sourdough (Hammes and Ganzle, 1998). Furthermore, under the acidic conditions enzyme activities are partially inactivated, particularly amylase activity in rye flour (Seibel and Brummer, 1991). This is an important aspect, since rye starch is gelled at a relatively lower temperature 55-70±1°C, which coincides with the temperature range for maximum α-amylase activity (Cauvain, 1998). In poor quality rye flour amylase activity is so high that the crumb of the baked bread might completely be hydrolysed. Excessive amount of α-amylase in rye flour not only makes the crumb sticky, but at higher levels, there is a reduction in the bread volume (Hammes and Ganzle, 1998). Acidification has a positive effect on the structure of starch granules, which leads to an increased waterbinding capacity (Hammes and Ganzle, 1998). Acidification of rye dough improves its physical properties, making it more elastic and extensible and gives the sour aftertaste that is typical of rye bread (Arendt et al., 2007).

8. Metabolic Activity of Sourdough Starter Cultures

There are three sources of enzymes in the biotechnology of bakery products: endogenous enzymes of the flour; enzymes associated with the metabolic activity of the dominant organisms (yeasts and lactic acid bacteria) and exogenous enzymes that are intentionally added to the composition of the dough.

Industrial or exogenous enzymes are used as natural supplements (Mutasaers, 1996). New enzymes with new and/or improved technological effects are currently used. The trend is to use complex mixtures of enzymes that act synergistically and can increase the individual effects on the various flour components (Mutasaers, 1996). The positive effects of exogenous enzymes relate primarily to changes in the texture, the hardness, the staling and the flavor of the baked goods (Martinez-Anaya, 1996a).

Sourdough addition affects the effectiveness of exogenous enzymes during fermentation due to the lowering of the pH. Enzymes also interact with the metabolic activity of lactic acid bacteria in the composition of the sourdough by releasing the available nutrients or modifying other environmental factors (Gobbetti, 2008; Martinez-Anaya and Devesa, 2000).

The selection of the most useful combination of sourdough lactic acid bacteria and exogenous enzymes is of great importance in modern bread biotechnology (Di Cagno et al., 2003).

8.1. Proteolysis

The protein fraction of wheat and rye flour is crucial for bread quality. Proteolysis during sourdough fermentation is among the main phenomena that affects sourdough bread quality (Ganzle et al., 2008). Lactic acid bacteria play a minor role in protein hydrolysis (Wieser et al., 2008). Proteolysis provides precursor compounds for the formation of aromatic volatile compounds during baking, as well as substrates for the microbial transformation of amino

acids into aromatic precursors (Thiele et al., 2002). Gluten proteins in wheat flour determine dough rheology, its gas-retention ability and bread volume (Arendt et al., 2007). The aim is to obtain pores with a relatively small size (1 or 2 mm) in bakery products, bearing in mind that large pores or uneven pore distribution in the crumb are undesirable (Cauvain, 1998).

Proteolysis and the further amino acid transformation by sourdough lactic acid bacteria contributes to their competitiveness (production of ATP, regeneration of cofactors and/or response to acid stress) and flavor activity (Ganzle et al., 2007; Vermeulen et al., 2007). Most sourdough lactic acid bacteria, in particular *Lactobacillus sanfranciscensis*, do not have extracellular protease activity and favor the uptake of peptides instead of amino acid transport (Thiele et al., 2003). Primary proteolysis is carried out by the endogenous enzymes of wheat or rye, which are activated at low pH values. The further peptide hydrolysis to amino acids is carried out by the intracellular peptidases of lactic acid bacteria in a strain-specific manner - the type and the amount of the released amino acids depend on the fermenting strain (Di Cagno et al., 2002). Proteolysis carried out by lactic acid bacteria causes softening of the dough in comparison with chemically acidified doughs (Di Cagno et al., 2002; Moroni et al., 2009).

It was found that the conduction of limited proteolysis during sourdough fermentation improves bread flavor with no adverse effects on the texture and the volume (Thiele et al., 2002). Amino acids and peptides influence fermented food flavor and are important precursors for flavor volatile compound formation. Amino acids are substrates for microbial transformations or are converted into flavor components during baking; thus limited proteolysis during fermentation improves bread flavor (Thiele et al., 2002). The aroma of the bread crumb is determined mainly by microorganism fermentation products, while flavor and aromatic products obtained by thermal effects dominate in the crust (Kirchhoff and Schieberle, 2001).

In addition to the effects of sourdough on the structure and rheology of the gluten proteins that make up the dough protein network, sourdough addition also influences gas formation. Having in mind that gas formation by microorganisms is required in order to obtain fermented bread, the impact of sourdough on gas formation must be considered. In sourdough bread carbon dioxide is produced by both lactic acid bacteria and yeasts, and the contribution of each group to the total volume of gas varies depending on the type of sourdough starter and the technology used to prepare the dough (Hammes and Ganzle, 1998; Arendt et al., 2007).

8.2. Hydrolysis of Phytic Acid

Phytic acid is known as the storage form of phosphorus in seeds. Since it is highly negatively charged (six anionic groups), it acts as a chelating agent and binds to nutritional minerals (Nolan and Duffin, 1987), by preventing their absorption and thus reducing their bioavailability. Phytic acid forms a complex with the alkaline groups of the protein amino acids, resulting in their decreased bioavailability (Dvorakova, 1998).

It has been shown that phytase supplements prevent the formation of such complexes or help their faster dissolution, thereby phytases improve protein digestibility (Kies et al., 2006).

Phytases are widespread in plant materials such as wheat and rye flours. Their level depends on the plant variety and the harvest, but are generally insufficient to significantly reduce the quantity of phytic acid (Cossa et al., 2000; Corsetti and Settanni, 2007).

Phytases are produced by many microorganisms, including baker's yeasts (Turk et al., 2000) and sourdough lactic acid bacteria (Lopez et al., 2000; Reale et al., 2004). Phytases, whose activity is optimal at sourdough pH (pH about 4.5) (Fretzdorff and Brummer, 1992), hydrolyze IP6 to IP5, and then to lower myo-inositol phosphate esters (IP4-IP1), which are less likely to bind with minerals to form mineral complexes (Sandberg et al., 1999).

The pH optimum of the wheat phytase is pH=5.0, the pH optimum of the yeast phytase is pH=3.5 (Turk et al., 1996). The moderate lowering of the pH to pH=5.5 during sourdough fermentation is sufficient to reduce the phytate content of wholemeal wheat flour with approximately 70% under the action of the endogenous wheat flour phytase (Leenhardt et el., 2005; Poutanen et al., 2009).

According to Chaoui, Fais and Belhcen, 2003 through the combination of selected yeast and lactic acid bacteria strains it is possible to achieve high level of phytate biodegradation; the best combination being *Saccharomyces cerevisiae* / *Lactobacillus plantarum* / *Leuconostoc mesenteroides*. These results highlight the microbial potential to improve the cereal nutritional quality (Corsetti and Settanni, 2007).

8.3. Production of Organic Acids

Production of organic acids and carbohydrate metabolism depend to varying degrees on the composition of the microbial starters and the parameters of the fermentation process, such as flour ash content, dough yield, fermentation time and temperature and concentration of NaCl (Martinez-Anaya et al., 1994; Gobbetti et al., 1995a, 1995b; Rouzaud and Martinez-Anaya, 1997; Meignen et al., 2001).

Selected strains of homo- and heterofermentative lactic acid bacteria are applied as components of sourdough starters. The selected strains absorb the substrates with the formation of lactic acid and acetic acid, thus acidifying the medium (pH, total titratable acidity (TTA)) (Collar, 1996; Hammes and Ganzle, 1998; Corsetti et al., 1998a). Acetate production in heterofermentative metabolism is important for the formation of bread flavor. The ratio of lactic acid and acetic acid, called fermentation factor (FQ), is an important factor that can influence the flavor profile of the finished bread (Corsetti and Settanni, 2007) and is influenced by the fermenting microorganisms, the fermentation temperature and the flour type (Hansen and Schieberle, 2005). It is very important for the structure of the final products. The acetic acid produced by the heterofermentative lactic acid bacteria is causing the tighter and firmer gluten, while lactic acid can gradually form a more flexible gluten structure (Lorenz, 1983). As for whole-grain rye bread with sourdough, the optimal FQ is in the range of 1.5-4.0 (Spicher, 1983). The molar ratio between lactic acid and acetic acid in bread is considered to be optimal in the range between 2.0 and 2.7 (Hammes and Ganzle, 1998). The attention drawn to the increase the acetic acid content is also due to its antimicrobial effect against microorganisms causing bread roping and mold spoilage (Rosenquist and Hansen, 1998).

Various organic acids produced during sourdough fermentation improve bread flavor, support gluten swelling and increase the gas-retention ability, leading to the preparation of products with good texture and volume. To summarize, organic acids function as natural dough improvers (Park et al. 2006). They have a strong impact on dough kneading. Dough with a low pH requires shorter time for kneading (Hoseney, 1994). The pH of mature sourdoughs varies depending on the nature of the fermentation process and the starter used, but for wheat sourdough it is between pH=3.5 and pH=4.3.

The production of suitable end products during sourdough fermentation depends on the availability of soluble carbohydrates. In wheat flour the concentration of maltose, sucrose, glucose and fructose is very low and ranges from 1.5 to 2 g/100 g. Important changes in the carbohydrate fractions occur during sourdough fermentation, resulting from the enzymatic activities of the flour and of the metabolic conversions catalyzed by microbial enzymes (Collar, 1996; Martinez-Anaya, 1996b). Carbohydrate metabolism is species-specific, more precisely – strain-specific. It also depends on the co-presence of yeasts and the processing conditions (Gobbetti et al., 1994b; Martinez-Anaya and Rouzaud, 1996a; Rouzaud and Martinez-Anaya, 1997). Soluble carbohydrates that remain after microbial fermentation participate in the Mayyar reaction of non-enzymatic browning of the crust during baking, contributing to the organoleptic characteristics of the final bread (Collar, 1996). In most industrial production practices different amounts of yeasts along with starter sourdoughs are added to bread dough to slow bread staling and flavor loss (Hammes and Ganzle, 1998). Baker's yeast enzymes influence the metabolic activity of the sourdough microflora during the process of breadmaking.

Besides the direct impact of low pH on the dough characteristics, the secondary effects of acidification include changes in the activity of cereal or bacterial enzymes (Arendt et al., 2007).

Sourdough fermentation is essential for obtaining good bread flavor since the comparison between chemically acidified bread and sourdough bread shows that sourdough bread has better quality (Kirchhoff and Schieberle, 2002).

8.4. Production of Exopolysaccharides

The addition of plant polysaccharides is a common practice in bread production aiming at improving bread texture and shelf life. Exopolysaccharides produced *in situ* are more effective than the exopolysaccharides added. In addition, the metabolic activity of lactic acid bacteria during sourdough fermentation yields other metabolites, including mannitol, glucose and acetate, which improve bread quality (Korakli et al., 2003; Arendt et al., 2007).

About 30 lactobacilli species are described as exopolysaccharide producers. The most pronounced among them are *Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus brevis, Lactobacillus curvatus, Lactobacillus delbrueckii* ssp. *bulgaricus, Lactobacillus helveticus, Lactobacillus rhamnosus, Lactobacillus plantarum, Lactobacillus johnsonii*, etc. The highest exopolysaccharide yield was obtained with *Lactobacillus rhamnosus*, which is a well described probiotic microorganism (Badel et al., 2011).

Exopolysaccharides produced by lactic acid bacteria in sourdough improve dough rheology and bread texture. They are used to replace or reduce the amount of the more expensive hydrocolloids added for improvement of bread texture. Moreover, some of the exopolysaccharides produced by lactic acid bacteria exhibit prebiotic properties (Gibson and Roberfroid, 1995; Arendt et al., 2007).

Two classes of exopolysaccharides produced by lactic acid bacteria are distinguished: extracellular homopolysaccharides composed of only one monosaccharide type; and heteropolysaccharides with (non)symmetrically repeating units (De Vuyst et al., 2001).

Glucan and fructans produced by fermenting lactic acid bacteria strongly influence the quality of wheat bread when it comes to bread volume and flaky appearance (Di Cagno et al., 2006; Lacaze et al., 2007).

Homopolysaccharide production by sourdough lactic acid bacteria performs two functions: improvement of bread structure and bread nutritional value (Moroni et al., 2009).

8.5. Antimicrobial Substances

The antimicrobial activity of sourdough is due to the lactic acid, the acetic acid, the carbon dioxide, the diacetyl, the ethanol, the hydrogen peroxide and the bacteriocins produced by lactic acid bacteria during sourdough fermentation (Mentes et al., 2007).

The main characteristics of an antimicrobial substance in order to be active under the conditions of the food environment, are to be produced in active concentration and its effect not to be masked by food components.

Bacteriocins are antimicrobial peptides or short-chain proteins that have bactericidal or bacteriostatic effect against microorganisms that are closely related to the producing strains (De Vuyst and Vandamme, 1994b; Schillinger and Holzapfel, 1996).

Bacteriocin-producing strains are protected against their own bacteriocins. Each bacteriocin-producing strain has its own specific protein expressed simultaneously with the bacteriocin production, which guarantees the producer's immunity (Nes et al., 1996). Bacteriocins contribute to the competitiveness of the producing strains in the fermented food ecosystem (Caplice and Fitzgerald, 1999).

Some bacteriocins produced by sourdough lactic acid bacteria show ability to inhibit food-transmitted pathogens. They might also inhibit spoilage bacteria including *Listeria monocytogenes, Bacillus subtilis* and *Staphylococcus aureus*, which is why the application of bacteriocin-producing strains as starter cultures, contributes to the production of safer food. Moreover, bacteriocins allow the reduction of the amount of chemical preservatives used in food production (Messens and De Vuyst, 2002; Corsetti and Settanni, 2007), including bread production.

According to Schnurer and Magnusson (2005), the basic antifungal compounds produced by various lactic acid bacteria are: lactic acid and acetic acid, carbon dioxide, diacetyl, hydrogen peroxide, hexanoic acid, 3-hydroxy fatty acids, phenyllactic acid, cyclic dipeptides, reuterin and fungicins (Corsetti and Settanni, 2007).

There are a number of confirmations of the antifungal properties of some lactobacilli, for example *Lactobacillus acidophilus* (Batish et al., 1990b), *Lactobacillus casei* (Gourama, 1997), *Lactobacillus coryniformis* ssp. *coryniformis* (Magnusson and Schnurer, 2001), *Lactobacillus pentosus* (Okkers et al., 1999), *Lactobacillus plantarum* (Lavermicocca et al., 2000; Laitila et al., 2002; Strom et al., 2002; Lavermicocca et al., 2003), *Lactobacillus rhamnosus* (Stiles et al., 2002), *Lactobacillus salivarius* (Stiles et al., 1999), *Lactobacillus sanfranciscensis* (Gobbetti and Corsetti, 1997), *Lactobacillus lactis* ssp. *lactis* (Roy et al., 2001) and *Lactobacillus lactis* ssp. *lactis* var. diacetylactis (Batish et al., 1990a).

To a great extent the antifungal capacity of lactic acid bacteria is due to the production of antifungal protein or proteinaceous compounds. Other species, such as *Lactobacillus plantarum* and *Lactobacillus sanfranciscensis*, produce specific organic acids (3-phenyl-L-lactic acid and caproic acid, respectively) that have antifungal properties (Corsetti et al., 1998b; Strom et al., 2002; Lavermicocca et al., 2003).

9. Formation of Bread Aroma

The aroma of sourdough baked bakery products is affected by the raw materials, the sourdough fermentation, the starter culture type and the impregnation and baking conditions. Aromatic substances are removed or generated during the whole process of bread production. Microbial and enzymatic conversion of carbohydrates, amino acids and lipids in dough leads to the formation of flavor compounds associated with the aroma of the crumb, i.e., alcohols, esters and carbonyl compounds, while the aroma of the crust is affected by the thermal effects that occur during baking (Moroni et al., 2009).

There are two categories of aromatic compounds produced during sourdough fermentation. The first one includes non-volatile compounds, including organic acids produced by homo- (Gobbetti et al., 1995a) and heterofermentative lactic acid bacteria (Gobbetti et al., 1995b), which acidify the medium, reduce the pH and contribute to the flavor of the bread dough (Barber et al., 1985). The second one comprises volatile compounds of sourdough bread, which include alcohols, aldehydes, ketones and esters. All these compounds are produced as a result of biochemical reactions during sourdough fermentation and contribute to the formation of bread taste (Chavan and Chavan, 2011).

Sourdough bread has higher content of volatile substances and achieves higher scores of the final product, compared with bread chemically acidified with lactic acid and acetic acid (Hansen and Hansen, 1996).

Lactic acid bacteria are able to catalyze reactions such as deamination, transamination and decarboxylation, thus also contributing to the formation of the taste of the final product (Moroni et al., 2009).

The most important factors that regulate amino acid levels in wheat dough are dough pH, fermentation time and the consumption of amino acids by fermenting microflora (Arendt et al., 2007).

Together with proteins, *lipids* significantly affect bread quality. Lipid oxidation which occurs during flour storage and dough kneading, causes the formation of (E)-2-nonenal and other aldehydes, which are key aromatic compounds in wheat and rye bread (Hansen and Schieberle, 2005; Vermeulen et al., 2007). During sourdough fermentation the concentration of these aldehydes is reduced by the alcohol dehydrogenase activity of lactic acid bacteria, particularly that of heterofermentative lactic acid bacteria (Vermeulen et al., 2007; Moroni et al., 2009).

Besides the differences in acetic acid production, the differences in the overall flavor profile of the final bread are due to the dominant lactobacilli species. The ethyl acetate content is higher in whole wheat (Damiani et al., 1996) and rye sourdough (Lund et al., 1989), fermented under the action of heterofermentative lactic acid bacteria as compared to sourdough fermented under the action of homofermentative lactic acid bacteria. The same trend is observed for hexyl acetate in whole rye sourdough. The content of aldehydes is higher in rye sourdough fermented with homofermentative cultures (Lund et al., 1989). The diacetyl content is higher in sourdough involving homofermentative, compared to sourdough with heterofermentative cultures (Lund et al., 1989; Damiani et al., 1996). However, because of the evaporation during baking, the amounts of alcohols, esters or diacetyl in sourdough bread is much lower than in the respective dough (Hansen and Hansen, 1996; Lund et al., 1989; Corsetti and Settanni, 2007).

The aromatic component concentrations before and after fermentation are proof that the main influence of microorganisms on sourdough aroma comprises an increase or a decrease in the concentration of specific volatile substances that are already present in the flour. Therefore, careful selection of starter cultures is essential to change the flavor of floury products (Vogel et al., 2002; Corsetti and Settanni, 2007).

Nowadays, sourdoughs are used in the production of plain bread; special traditional bread (sourdough is used as a natural agent providing fermentation because of its high quality); traditional rye bread, cakes and biscuits, pizza and other sweet baked goods (De Vuyst et al., 2009).

10. Basic Technological Factors in Bread Production

The optimization of industrial processes requires a thorough understanding of the factors that determine microbial metabolism, the stability of the microflora and the influence of the process parameters. The endogenous factors in cereal products (carbohydrates, nitrogen sources, minerals, lipids and free fatty acids, and enzyme activities) and the technological parameters (temperature, dough yield (DY), oxygen, fermentation time and number of steps of daily back-slopping of the sourdough) significantly influence sourdough microflora and the characteristics of the resulting sourdough bakery products (Hammes and Ganzle, 1998).

Sourdough consistency varies. Sourdough fermentation is carried in the form of a solid dough or a liquid suspension of flour and water. The ratio between flour and water is called dough yield (DY) and is defined as:

Dough yield (DY) = (amount of flour + amount of water) * 100 / amount of flour

The DY value of sourdough significantly affects sourdough flavor and aroma. The tighter the sourdough is (lower DY value), the greater the amount of acetic acid and the lower the amount of lactic acid produced are.

The rate of acidification is also influenced by sourdough DY. The higher the DY value, the faster the acidification is, probably due to the better distribution of the produced organic acids in the medium (Spicher and Stephan, 1999).

Temperature is a very important factor as it influences DY more than the rate of acidification. It also influences the microbial composition of the sourdough. If the back-slopping technology is applied, whereby a portion from the sourdough from the previous day is used to inoculate the fresh flour/water mixture on the next day, fermentation temperature is crucial to sourdough stability as part of the microflora may be lost during sourdough back-slopping, if no control is applied (Spicher and Stephan, 1999).

Low temperatures have positive influence on the growth of contaminants; thus they influence the properties of the final dough (Lonner et al., 1995). Therefore, it is important to use starter cultures that are fully adapted to the sourdough environment and the fermentation conditions applied (De Vuyst, 2000; Neysens and De Vuyst, 2005).

11. Types of Sourdough

Sourdoughs are classified into three types based on the type of technology applied for their production. Each sourdough type is characterized by specific microflora that includes lactic acid bacteria (De Vuyst and Neysens, 2005):

A. Type I Sourdough

Type I sourdoughs are used in fermentation processes in three stages (Hammes and Ganzle, 1998). *Lactobacillus sanfranciscensis, Lactobacillus pontis, Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus paralimentarius* and *Lactobacillus rossiae* are the predominant lactic acid bacteria in these sourdoughs (Vogel et al., 1999, Messens and De Vuyst, 2002, De Vuyst et al., 2009).

Type I sourdoughs are characterized by a continuous, daily back-slopping to retain the microorganisms in an active state, i.e., high metabolic activity, primarily in terms of fermentation, i.e., gas production. The process is carried out at room temperature (20-30 ± 8°C) and the pH is about 4.0 (De Vuyst and Neysens, 2005).

Lactobacillus sanfranciscensis, Lactobacillus pontis, Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus paralimentarius and *Lactobacillus plantarum* are frequently selected for traditional type I sourdoughs (De Vuyst and Vancanneyt, 2007).

B. Type II Sourdough

The industrialization of the rye bread bakng process and the demand for faster, more efficient, controllable, large-scale sourdough fermentation led to the development of type II sourdoughs, which are semiliquid preparations. Sourdough processes with continuous back-slopping and long single-stage fermentation ensure more reliable and flexible manufacturing. A typical process with type II sourdough continues 2-5 days at an elevated fermentation temperature (usually 30±8°C) for its acceleration (Hammes and Ganzle, 1998; De Vuyst and Neysens, 2005).

Liquid sourdough is preferred because of the following technological and analytical advantages: (1) easier control and reproducibility during operating conditions; (2) easier control of the fermentation parameters (e.g., temperature, pH, dough yield) and easy addition of nutrients (e.g., vitamins, peptides, carbohydrates) to satisfy the needs of the microorganisms; (3) higher suitability of the microbial metabolism in order to obtain optimal organoleptic profile; (4) higher suitability for use as a natural starter without changing the current bread formulations; and (5) higher suitability for use in a variety of technologies to produce various bakery products.

In liquid sourdough it is very easy to control the acidity, the release of amino acids and the production of various aromatic components. Preliminary production studies also show that the rheological properties of liquid sourdough are significantly influenced by the type and quantity of flour and the fermentation temperature (Carnevali et al., 2007).

In comparison with conventional sourdough, liquid sourdough is an unstable natural system, because the microorganisms are more sensitive to the changing environmental conditions. A positive aspect of the liquid system resides in the fact that the parameters of the environment (eg. high nutrient content) are homogeneous in the entire system.

The completely different parameters of the fermentation process with type II sourdough lead to the formation of a different microbial ecosystem in terms of composition and

population dynamics. The predominant species are obligate homofermentative species - *Lactobacillus acidophilus, Lactobacillus delbrueckii, Lactobacillus amylovorus* (rye), *Lactobacillus farciminis* and *Lactobacillus johnsonii*; and obligate heterofermentative species - *Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus frumenti, Lactobacillus pontis, Lactobacillus panis, Lactobacillus reuteri*, and *Weissella* (*Weissella confusa*) (Vogel et al., 1999; Muller et al., 2001).

Since these sourdoughs are stored fresh until use (up to one week), they cannot be produced in large quantities. In industry, they are also applied for the production of dried sourdough preparations (De Vuyst and Neysens, 2005).

C. Type III Sourdough

Type III sourdoughs are dried sourdoughs, in powder form. They contain the most common lactic acid bacteria that are resistant to drying and are able to survive in this form, i. e. heterofermentative strains of *Lactobacillus brevis*, facultative heterofermentative strains of *Lactobacillus plantarum* and homofermentative strains of *Pediococcus pentosaceus*. The drying process (spray drying or tumble drying) also increases sourdough shelf life and turns it into a product for later use. Dried sourdoughs are comfortable and easy to use and provide standardized end products. They are distinguished by color, flavor and acid content (Stolz and Bocker, 1996).

Unlike type I sourdough, type II sourdoughs and type III sourdoughs require the addition of baker's yeasts (*Saccharomyces cerevisiae*) for fermentation (De Vuyst and Neysens, 2005).

Type 0 sourdough is not prepared with application of sourdough, so the main fermenting agents are yeasts (Corsetti and Settanni, 2007).

Fresh bakery products have a relatively short shelf life due to the occurrence of microbial spoilage and, especially, due to physical and chemical changes that lead to rapid staling (Selomulyo and Zhou, 2007).

Due to the increasing consumer demand for fresh bread, new technologies to avoid these unwanted changes and to extend bread shelf life have been developed (Fik and Surówka, 2002).

Frozen dough technology allows producers to offer fresh bread at any time of the day, thereby eliminating staling and problems with storage (Fik and Surówka, 2002). However, the use of frozen dough poses problems such as prolonged final fermentation time, smaller bread volume and deterioration of bread characteristics (Wolt and D'Appolonia, 1984). Water crystallization causes either physical damage to the gluten network (Sharadanant and Khan, 2003) or leakage of the yeast cell membranes (Mazur, 1961). Furthermore, during freezing and frozen storage, the yeasts can release reducing compounds, such as glutathione, which also contribute to the weakening of the gluten network and reduce the gas-retention ability (Ribotta et al., 2003).

The inclusion of additives such as hydrocolloids, emulsifiers and antioxidants in bread can solve the problems associated with frozen dough (Selomulyo and Zhou, 2007). Hydrocolloids favor the production of softer bread by increasing the water retention in the dough (Kim et al., 2008). Emulsifiers bind to the hydrophobic protein surface, stimulating gluten aggregation in the dough which in turn leads to improved bread texture and increased

bread volume (Kamel and Ponte, 1993). Oxidants compensate for the release of reducing compounds from the yeast cells, improve the structure and the volume of the final bread and increase the dough strength (Selomulyo and Zhou, 2007; Minervini et al., 2011).

The objectives achieved through the sourdough application are significant extension of bread shelf life, increase in bread nutritional value and better organoleptic characteristics of bread. The increase in the shelf life is due to the higher values of the acidity and the higher concentration of organic acids in comparison to bread produced with yeasts alone. The improvement in the organoleptic characteristics is due to the presence of significant amounts of volatile and non-volatile compounds which improve bread flavor. However, the production of sourdough bread is a very sensitive method that depends on various parameters which must be controlled. The most important among them are fermentation pH, fermentation temperature and selection and maintainance of sourdough starter strains with specific and desired properties (Plessas et al., 2011a).

The purpose of the present work was the development and application of sourdough starters including selected lactobacilli and propionic acid bacteria strains to obtain bread with extended shelf life without the addition of preservatives.

MATERIALS AND METHODS

1. Microorganisms

1.1. In the present work were used 14 strains of the genus *Lactobacillus*, isolated from different sources: *Lactobacillus* LBRH9, *Lactobacillus* LBRH10 of human origin; *Lactobacillus* LBRZ6, *Lactobacillus* LBRZ7, *Lactobacillus* LBRZ8, *Lactobacillus* LBRZ12, isolated from spontaneously fermented vegetables; *Lactobacillus* LBRC11, isolated from homemade cheese; *Lactobacillus* Z10, *Lactobacillus* Z14, *Lactobacillus* PX3, *Lactobacillus* RN5, *Lactobacillus* X1, *Lactobacillus* X2, *Lactobacillus* F3, isolated from spontaneously fermented sourdough, which were isolated by spread plating on MRS-agar and LAPTg10-agar. The strain *Lactobacillus sanfranciscensis* R and the probiotic strain *Propionibacterium freudenreichii* ssp. *shermanii* NBIMCC 327, part of the collection of the Department of "Microbiology" at the University of Food Technologies, Plovdiv, Bulgaria were also used in the research.

1.2. Test microorganisms: Saprophytic microorganisms: *Bacillus subtilis* (food isolate), *Saccharomyces cerevisiae* (baker's yeasts), *Penicillium* sp. (food isolate), *Rhizopus* sp. (food isolate), *Aspergillus niger* (food isolate). They were cultured at 30°C on LBG-agar.

2. Media

2.1. MRS-broth (Scharlau) – for the culturing of lactobacilli strains and sourdough starters.

2.2. MRS-agar - to determine the concentration of viable *Lactobacillus* cells.

2.3. LAPTg10 - broth. Composition (g/dm^3): peptone - 15; yeast extract - 10; tryptone - 10; glucose - 10. pH was adjusted to 6.6 - 6.8 and Tween 80 - 1cm^3/dm^3 was added. Sterilization - 20 minutes at 121ºC. – for the culturing of lactobacilli.

2.4. LAPTg10 - agar. Composition (g/dm^3): LAPTg10-broth medium, agar - 20. Sterilization - 20 minutes at 121ºC. - to determine the concentration of viable lactobacilli cells.

2.5. LBG - agar. Composition (g/dm^3): tryptone – 10, yeast extract - 5, NaCl – 10, glucose – 10, agar - 20. pH was adjusted to 7.5. Sterilization - 20 minutes at 121°C. - to determine the concentration of viable saprophytic cells and to examine the antimicrobial activity against saprophytic microorganisms.

2.6. msLAPTg10 - agar. Composition (g/dm^3): peptone - 15; yeast extract - 10; tryptone - 10; soluble starch - 10, Tween 80 - 1 cm^3/dm^3, agar - 20. pH = 6.6 - 6.8. Sterilization - 121°C for 20 minutes. - to determine the presence of amylolytic activity.

2.7. Sterile skimmed milk with titratable acidity of 16-18ºT. Composition (g/dm^3): skimmed milk powder (Scharlau) - 100. Sterilization - 15 minutes at 118ºC. - to determine the presence of proteolytic activity.

2.8. Elective medium for Propionibacterium sp. Composition (g/dm^3): tryptone - 10; yeast extract - 10; Na-lactate (freshly prepared) – 10, KH_2PO_4 - 2.5; $MnSO_4$ - 0,005; agar - 20; pH=6.8. Sterilization - 20 minutes at 121°C. – to determine the number of viable propionic acid bacteria cells.

2.9. Hydrocolloid matrices: pectin + sodium alginate / 1:1 / - 1,2% solution of sodium alginate, and 4% solution of pectin. The concentrations of the hydrocolloid solutions were determined on the basis of the physicochemical parameters of the hydrocolloids used. The application of hydrocolloids of plant origin was in compliance with the requirements for physiological activity, safety and microbial stability (Willemer, 1983; Denkova et al., 2014a).

3. Methods

3.1. Physicochemical Methods

3.1.1. Determination of the Titratable Acidity

The Thorner method was used to determine the acidification-ability of microorganisms. One °T (Thorner degree) is equal to 1 cm^3 0,1 N NaOH, spent on the neutralization of an equivalent amount of organic acids contained in 100 cm^3 of culture medium. The method is based on the titration of the sample with 0,1 N NaOH. For that purpose 10 cm^3 of each test sample were mixed with 20 cm^3 of distilled water. The mixture was titrated with 0,1 N NaOH using phenolphthalein as an indicator until the appearance of pale pink color that persisted over one minute.

3.1.2. Determination of Total Titratable Acidity of Sourdough / Yeast Dough (AACC, 1975)

3.1.3. Determination of Total Titratable Acidity of Bread Crumb (AACC, 1975)

3.2. Microbiological methods

3.2.1. Culturing and Storage Conditions

All *Lactobacillus* strains were isolated from a single colony and were grown in MRS-broth medium for 24 h in order to obtain pure cultures.

3.2.2. Determination of the Number of Viable Microorganism Cells

Appropriate tenfold dilutions of each sample in saline solution were prepared. The last 3 tenfold dilutions were spread plated on the appropriate solid medium (MRS-agar or LAPTg10-agar – for the enumeration of lactic acid bacteria; LBG-agar – for the enumeration of saprophytic microorganisms) or pour plated (elective medium for *Propionibacterium* sp.- for the enumeration of propionic acid bacteria). The inoculated plates or tubes were cultured at optimum temperature for the growth of the corresponding microorganisms - 37°C or 30°C for the lactic acid bacteria and the propionic acid bacteria or 30°C for the saprophytic microorganisms until the formation of countable single colonies.

3.2.3. Determination of the Antimicrobial Activity of the *Lactobacillus* Strains against Saprophytic Microorganisms – Well-Diffusion Method

To determine the antimicrobial activity of the studied *Lactobacillus* strains against saprophytic microorganisms were used culture liquid (CL), acellular supernatant without pH adjustment (ASN) and neutralized (with 1N NaOH) acellular supernatant (NASN) (pH = 6.5) prepared from 48-hour culture of the studied strain. The antimicrobial activity against the following test microorganisms was examined: bacteria *Bacillus subtilis*; yeasts *Saccharomyces cerevisiae* (baker's yeasts), molds *Aspergillus niger*, *Penicillium* sp., *Rhizopus* sp. Yeasts and molds were grown in an incubator at 30°C on LBG-agar for 3 to 7 days, while bacteria were propagated for 3 days at 37°C. Spore suspensions of each of the test microorganisms (10^6-10^7 CFU/cm^3) were prepared and were used to inoculate melted and cooled to 40°C LBG-agar (obtaining concentration of 10^5CFU/cm^3 in the inoculated medium), then the inoculated LBG-agar was poured in Petri dishes. Wells (6 mm) were prepared after the solidification of the agar. 0,06 cm^3 of CL, ASN or NASN were pipetted in the wells and the Petri dishes with the test microorganisms were incubated at 30°C or at 37°C. The antimicrobial activity was determined by measuring the diameters of the inhibition zones in mm.

3.2.4. Determination of the Antimicrobial Activity of the Starter Sourdoughs against Saprophytic Microorganisms – Well-Diffusion Method (Denkova et al., 2014b)

The well-diffusion method was used to determine the antimicrobial activity of the starter sourdoughs. A dilution in a ratio of 1:1 of a sourdough: saline solution of each of the sourdoughs was prepared. The antimicrobial activity was tested against the following saprophytic test microorganisms: bacteria - *B. subtilis*, *B. mesentericus*; yeasts - *Saccharomyces cerevisiae* (baker's yeasts), molds - *Aspergillus niger*, *Penicillium* sp., *Rhizopus* sp. Spore suspensions of each of the test microorganisms (10^6-10^7 CFU/cm^3) were prepared and were used to inoculate melted and cooled to 40°C LBG-agar (obtaining concentration of 10^5CFU/cm^3 in the inoculated medium), then the inoculated LBG-agar was poured in Petri dishes. Wells (6 mm) were prepared after the solidification of the agar. 0.06

cm^3 of the dillusions were pipetted in the wells of the plates and the plates with the test microorganisms were incubated at 30°C for 24-48 hours, and then the inhibition zones in mm were reported.

3.2.5. Determination of the Resistance to Preservatives – Well-Diffusion Method

To determine the resistance of the studied *Lactobacillus* strains or the test saprophytic microorganisms to the preservatives most commonly used in bread production (calcium propionate and potassium sorbate) pour plating of the studied strain in Petri dishes was conducted. After the solidification of the medium wells wells (d = 6mm) were prepared. 60 µl of the control (buffer with pH = 5) or of the solutions of the preservatives at a concentration of 0.1%, 0.2% and 0.3% prepared in the same buffer (pH=5) were pipetted into the wells. The Petri dishes were incubated at 30°C or 37ºC for 24 to 48 hours, and then the inhibition zones in mm were reported.

3.2.6. Determination of the Presence of Amylolytic Enzymes – Well-Diffusion Method

15 cm^3 of melted msLAPTg10-agar was pour plated in Petri dishes and after the solidification of the medium wells (d=6mm) were prepared. 60µl of the 24-hour cultures of the studied *Lactobacillus* strain were pipetted into the wells. The results were reported as diameters of the clear zones in mm on the 24^{th} or the 48^{th} hour of incubation at the optimum temperature for the growth of the studied *Lactobacillus* strain.

3.2.7. Determination of the Presence of Proteolytic Enzymes – Well-Diffusion Method

Sterile skimmed milk was added to sterile melted LAPTg10-agar (10 cm^3 of skimmed milk per 100 cm^3 of sterilized LAPTg10-agar medium) and the mixture was pour plated in Petri dishes (15 cm^3 of mixture in each Petri dish). After the solidification of the medium wells (d=6mm) were prepared. Fresh cultures in the exponential growth phase of the studied *Lactobacillus* strains were used. For each strain were prepared 3 samples:

CL = culture liquid – 24-hour culture suspension of the strain.

ASN = acellular supernatant – it was obtained by centrifuging the culture suspension, and the resulting supernatant was transferred to a new tube.

CSSS = cell suspension in saline solution - obtained by centrifugation of the culture suspension of the studied *Lactobacillus* strain and the biomass sludge was washed once with saline solution, followed by resuspension in saline solution to the initial volume of the sample.

60µl of each sample were pipetted into the wells. The results (the diameters of the clear zones of hydrolysis of the casein in mm) were recorded on the 48^{th} hour of incubation at the optimum temperature for the studied *Lactobacillus* strain.

3.2.8. Determination of the Microbiological Status of The Freeze-Dried Starter Concentrates - acc. BS 1670-82 and Ordinance № 5 of the MH - SG 39/84, BS EN ISO 4833 (Denkova et al., 2014a).

Indicators:

- lactic acid bacteria and propionic acid bacteria - CFU/g;
- general number of mesophilic aerobic microorganisms - CFU/g;
- *Escherichia coli* in 0,1 g of product;
- sulfite reducing clostridia in 0,1 g of product;
- *Salmonella* sp. in 25,0 g of product;
- *Staphylococcus aureus* in 1,0 g of product;
- spores of microscopic molds, CFU/g;
- yeasts, CFU/g;

3.3. Technological Methods

3.3.1. Batch Culturing

Batch culturing was carried out in a laboratory bioreactor with a working volume of 1,5dm^3. The bioreactor was equipped with control device "Sartorius A2," which included a controlling unit for agitation rate, temperature, pH and Eh (redox potential).

The batch culturing was carried out in MRS-broth without pH adjustment. The culture medium was sterilized at 121ºC for 20 min. After cooling to 39-40ºC the medium in the bioreactor was inoculated with 5% (v/v) inoculum of the wheat or rye sourdough starter. The process of culturing was carried out at 150 rpm, without aeration at 30°C for 24 hours. Analysis of the total number of viable cells of lactic acid bacteria and propionic acid bacteria (CFU/cm^3) was conducted at the end of the fermentation process.

3.3.2. Preparation of Sourdough Starters and Approbation of Starter Sourdoughs in Bread Production

A. Preparation of Single-Strain Cellular Suspensions for the Inoculation of the Flour/Water Mixture (Denkova et al., 2014c)

10 cm^3 MRS-broth aliquots were inoculated with each *Lactobacillus* strain (1%) and incubated at optimum temperature for the growth of the corresponding strain (30°C or 37°C) for 24 h. Then, the biomass was collected by centrifugation (6000xg, 15 min, 4°C), washed twice with PBS-buffer and the biomass sludge was resuspended to the initial volume with sterile saline solution. The obtained cell suspension was used to inoculate the flour/water mixture to obtain single-strain sourdough.

B. Preparation of Multi-Strain Cellular Suspensions for the Inoculation of the Flour/Water Mixture (Denkova et al., 2014c)

For the production of sourdough with the multi-strain starters the 24-hour cultural suspensions of each of the *Lactobacillus* strains, included in each starter combination, were mixed according to the proportions given in "Development of sourdough starters for wheat and rye bread" and homogenized. The lactobacilli cells were harvested by centrifugation at 5000 x g for 15 minutes, washed twice with PBS-buffer and the biomass sludge was resuspended to the initial mixed suspension volume with sterile saline solution. Then the cellular suspension was mixed in the respective ratio with the 24-hour culture suspension of *P. freudenreichii* ssp. *shermanii* NBIMCC 327 in milk and the obtained homogenized

mixture was used for inoculation of the flour/water mixture for the production of multi-strain sourdough.

C. Preparation of Single-Strain Sourdoughs (Denkova et al., 2014b)

The ability of the *Lactobacillus* strains to grow well in flour/water environment, to achieve high concentrations of viable cells and to increase the total titratable acidity was examined by the preparation of single-strain sourdoughs. The cellular suspension of each *Lactobacillus* strain was obtained using the procedure described above and used to inoculate the flour/water mixtures. The changes in the concentration of viable cells of lactic acid bacteria, yeasts and molds and in the total titratable acidity of the sourdoughs were monitored by daily back-slopping over a period of 96 h of culturing at the optimum growth temperature for each strain (30°C or 37°C) according to the following scheme:

(1) first day: 44% flour: 56% tap water and 10% of the corresponding single-strain cellular suspension;

(2) second to fifth day: 25% sourdough from the previous day: 75% fresh mixture flour/water. The fresh mixture was prepared in a ratio: 44% flour/ 56% water.

The number of lactic acid bacteria, yeasts and molds was determined by appropriate tenfold dilutions and spread plating on LAPTg10-agar medium for the enumeration of lactic acid bacteria or LBG-agar for the enumeration of molds and yeasts. Total titratable acidity was determined by a standard method (AACC, 1975).

D. Development of Sourdough Starters for Wheat and Rye Bread

During the development of sourdough starters for wheat and rye bread were developed the following combinations:

- ***Two-strain combination*** (for wheat bread) – *L. brevis* LBRZ7 : *L. paracasei* LBRC11 = 7: 3;
- ***Four-strain combination*** (for wheat bread) – *L. plantarum* LBRZ12 : *L. paracasei* LBRC11 : *L. brevis* LBRZ7 : *L. fermentum* LBRH9 = 2 : 1 : 1 : 1;
- ***Combination 1.1.*** (for wheat bread) – *L. paracasei* LBRC11 : *L. plantarum* F3 : *L. brevis* LBRZ7 : *L. fermentum* Z14 = 1 : 1 : 1 : 1;
- ***Combination 1.2.*** (for wheat bread) – *L. paracasei* LBRC11 : *L. plantarum* F3 : *L. brevis* LBRZ7 : *L. fermentum* Z14 = 1 : 2 : 1 : 1;
- ***Combination 2.1.*** (for wheat bread) – *L. paracasei* PX3 : *L. plantarum* X2 : *L. brevis* LBRZ7 : *L. fermentum* LBRH10 = 1 : 1 : 1 : 1;
- ***Combination 2.2.*** (for wheat bread) – *L. paracasei* PX3 : *L. plantarum* X2 : *L. brevis* LBRZ7 : *L. fermentum* LBRH10 = 1 : 2 : 1 : 1;
- ***Combination 3.1.*** (for wheat bread) – *L. paracasei* RN5 : *L. plantarum* F3 : *L. brevis* LBRZ7 : *L. fermentum* Z14 = 1 : 1 : 1 : 1;
- ***Combination 3.2.*** (for wheat bread) – *L. paracasei* RN5 : *L. plantarum* F3 : *L. brevis* LBRZ7 : *L. fermentum* Z14 = 1 : 2 : 1 : 1;
- ***Combination 4.1.*** (for wheat bread) – *L. paracasei* RN5 : *L. plantarum* X2 : *L. brevis* LBRZ7 : *L. fermentum* LBRH10 = 1 : 1 : 1 : 1;

- ***Combination 4.2.*** (for wheat bread) – *L. paracasei* RN5 : *L. plantarum* X2 : *L. brevis* LBRZ7 : *L. fermentum* LBRH10 = 1 : 2 : 1 : 1;
- ***Combination 5.*** (for rye bread) – (*L. paracasei* RN5 : *L. plantarum* X2 : *L. brevis* LBRZ7 : *L. fermentum* LBRH10 = 1 : 2 : 1 : 1) : *L.sanfranciscensis* R = 2 : 1;
- ***Combination 6.*** (for rye bread) – (*L. paracasei* RN5 : *L. plantarum* X2 : *L. brevis* LBRZ7 : *L. fermentum* LBRH10 = 1 : 2 : 1 : 1) : *L.sanfranciscensis* R : *Propionibacterium freudenreichii* ssp. *shermanii* NBIMCC 327 = 2 : 1 : 1;
- ***Combination 7.*** (for wheat bread) – (*L. paracasei* RN5 : *L. plantarum* X2 : *L. brevis* LBRZ7 : *L. fermentum* LBRH10 = 1 : 2 : 1 : 1) : *L.buchneri* LBRZ6 = 2 : 1;
- ***Combination 8.*** (for wheat bread) – (*L. paracasei* RN5 : *L. plantarum* X2 : *L. brevis* LBRZ7 : *L. fermentum* LBRH10 = 1 : 2 : 1 : 1) : *L.buchneri* LBRZ6 : *Propionibacterium freudenreichii* ssp. *shermanii* NBIMCC 327 = 2 : 1 : 1;

E. Preparation of Sourdoughs with Multi-Strain Starters (Denkova et al., 2014b)

The cellular suspensions of the starter combinations were obtained using the procedure described above and used to inoculate the flour/water mixtures. The changes in the concentration of viable cells of lactic acid bacteria, yeasts and molds and in the total titratable acidity of the sourdoughs were monitored by daily back-slopping over a period of 96 h of culturing at 30°C according to the following scheme:

(1) first day: 44% flour: 56% tap water and 10% of the multi-strain cellular suspension;

(2) second to fifth day: 25% sourdough from the previous day: 75% fresh mixture flour/water. The fresh mixture was prepared in a ratio: 44% flour/ 56% water.

The number of lactic acid bacteria, propionic acid bacteria, yeasts and molds was determined by appropriate tenfold dilutions and spread plating on LAPTg10-agar medium for the enumeration of lactic acid bacteria or LBG-agar for the enumeration of molds and yeasts or pour plating on elective medium for *Propionibacterium* sp. for the enumeration of propionic acid bacteria. Total titratable acidity is determined by a standard method (AACC, 1975)

F. Approbation of the Starter Sourdoughs in the Production Laboratory (Denkova et al., 2014b)

'Mother' doughs with 5%, 7%, 10%, 15% or 20% of the 96-hour starter sourdoughs were prepared. Each dough was prepared with 1.5% NaCl, 2% yeast starter, the respective percentage of starter sourdough and tap water (the amount of water was determined by the water absorption of the flour type). The dough was kneaded in a mixer: slow kneading (1000 rpm) for 4 min and fast kneading (1400 rpm) for 10 min, after which the dough was rested for about 10 min in a proofer in order for its elastic properties to be improved. Loaves were formed and placed in the forms. Then followed leavening for about 40-45 min at 30°C and 80 ± 5 RH. In the production laboratory wheat and rye bread with starter sourdough as well as control bread (bread without starter sourdough) were baked, cooled and evaluated. Baking was carried out at 225 ± 5°C for 30 min in a deck oven. Loaves were were allowed to cool for 120 min at room temperature before evaluation.

G. Evaluation of the Baked Bread (Denkova et al., 2014c)

The baked bread with different amounts (in %) and types of starter sourdough was evaluated by seven trained judges on the basis of six criteria: aroma, flavor, crumb softness, crumb colour, crust colour and loaf volume. A scale of 0 (worst quality) to 10 (best quality) was used for each criterion.

H. Determination of Bacterial Spoilage of the Baked Bread (Denkova et al., 2014b)

The determination of the appearance of bacterial spoilage was performed by 10 judges in the production laboratory and was evaluated according to a scale of I-IV, with each of the degrees corresponding to the following descriptions:

I. barely noticeable (pleasant fruity odour);
II. weak (change in the odour – distinct);
III. medium (moisty, sticky crumb, sharp odour);
IV. strong (unpleasant odour, brown-yellow crumb).

I. Determination of Mold Spoilage of the Baked Bread (Denkova et al., 2014b)

The determination of the appearance of mold spoilage was performed by 10 judges in the production laboratory and was evaluated according to the appearance of single mold colonies.

J. Lyophilization (Freeze-drying) (Denkova et al., 2014a)

The freeze-dried sourdough starter concentrates were obtained by a three-stage technology:

1. Primary processing of the cellular suspensions – the cellular suspensions of the two sourdough starters were diluted, equilibrated, dosed and immobilized by inclusion in the polymer matrix that acts as a cryoprotectant.
2. Freezing – it was performed in chambers with forced convection of the air environment at a temperature of -30°C to -35°C for 12-15 hours.
3. Freeze-drying – it was performed in a vacuum sublimation installation "Hochvakuum-TG -16.50" with contact heating of the plates at the ICFT - Institute of Cryobiology and Food Technologies, Sofia, Bulgaria.

After lyophilization, the freeze-dried concentrates were digested in granulator "Erveka." The digested lyophilized bioproducts were packed in three layer aluminum foil, sealed under vacuum.

3.3.4. Biotechnological Scheme for the Production of "LB-Acidifiers" ("LB-Acidifier" for Rye Bread and "LB-Acidifier" for Wheat Bread)

1. Enrichment with the following steps: laboratory starter - small inoculator - inoculator - first bioreactor - second bioreactor;

Each bioreactor was with a volume of 200 dm^3, the agitation in the inoculators was conducted with pumps, while the agitation in the two bioreactors was performed with a mechanical stirrer and the transfer between them occurred after the filling of the previous container and reaching total titratable acidity of around 230-300°N. The medium used in the

four containers contained soy flour, wheat flour and sugar. Culturing was carried out at a temperature of 27-32°C.

2. Preparation of starter sourdough with the cultural suspension obtained after the second bioreactor;

3. Submerge culturing in dough-like environment in a container - development of the culture under anaerobic conditions;

4. Forming mature sourdough in the form of dough crumbs;

5. Molding of the crumbs in the form of fusilli by an extruder;

6. Drying in an industrial dryer;

7. Grinding of the fusilli into small granules;

8. Packing and storage in plastic bags;

9. Expedition;

10. Application.

4. Statistical Analysis

Data from quadriplicate experiments were processed using MS Office Excel 2010, using statistical functions to determine the standard deviation and the maximum error of assessment at levels of significance $\alpha < 0,05$.

RESULTS AND DISCUSSION

1. Characteristics of the Lactobacilli Strains Isolated from Different Sources by the Methods of Conventional and Molecular Taxonomy

14 strains of the genus *Lactobacillus* were isolated from various spontaneously fermented foods and were identified by applying biochemical (API 50 CHL) and molecular genetic (ARDRA-analysis and sequencing of the gene for 16S rRNA) methods (Table 1).

2. Technological Features of the Newly Isolated *Lactobacillus* Strains

2.1. Examination of the Amylolytic Activity of the Newly Isolated Lactobacillus Strains

Dough is characterized by high starch content, the breakdown of which occurs under the action of amylolytic enzymes. Thus, it is important to know the capabilities of the lactobacilli strains intended to be included in the composition of sourdough starters to degrade startch. The presence of amylolytic activity in the newly isolated strains of the genus *Lactobacillus* - *L. fermentum* LBRH9, *L. fermentum* LBRH10, *L. buchneri* LBRZ6, *L. brevis* LBRZ7, *L. brevis* LBRZ8, *L. brevis* X1, *L. plantarum* LBRZ12, *L. paracasei* LBRC11, *L. paracasei* PX3, *L. paracasei* RN5, *L. plantarum* X2, *L. sanfranciscensis* R was examined by the well-diffusion method (Table 2).

Table 1. Identification of the newly isolated *Lactobacillus* strains by applying biochemical (API 50 CHL) and molecular-genetic (ARDRA-analysis and sequencing of the gene for 16S rRNA) methods

Strain	Species identification
Z10	*Lactobacillus acidophilus*
LBRZ7	*Lactobacillus brevis*
LBRZ8	*Lactobacillus brevis*
X1	*Lactobacillus brevis*
LBRZ6	*Lactobacillus buchneri*
LBRC11	*Lactobacillus casei* ssp. *rhamnosus*
PX3	*Lactobacillus casei* ssp. *paracasei*
RN5	*Lactobacillus casei* ssp. *rhamnosus*
LBRH9	*Lactobacillus fermentum*
LBRH10	*Lactobacillus fermentum*
Z14	*Lactobacillus fermentum*
F3	*Lactobacillus plantarum*
LBRZ12	*Lactobacillus plantarum*
X2	*Lactobacillus plantarum*

Table 2. Amylolytic activity of the newly isolated *Lactobacillus* strains. d_{well} = 6mm. Lactobacilli concentration - 10^{12} - 10^{13}CFU/cm^3

Strain	Amylolytic activity, d_{zone}
L. buchneri LBRZ6	-
L. sanfranciscensis R	-
L. brevis LBRZ7	10,25±0,50
L. brevis LBRZ8	8,38±0,48
L. brevis X1	8,25±0,50
L. paracasei LBRC11	12,25±0,50
L. paracasei PX3	10,38±0,75
L. paracasei RN5	8,25±0,50
L. fermentum LBRH9	10,25±0,50
L. fermentum LBRH10	-
L. fermentum Z14	12,13±0,25
L. plantarum F3	12,25±0,50
L. plantarum LBRZ12	12,38±0,48
L. plantarum X2	10,13±0,25

Data show significant differences between the different strains of one and the same species when it comes to their ability to synthesize amylolytic enzymes. *L. buchneri* LBRZ6 and *L. sanfranciscensis* R did not exhibit any amylolytic activity. All strains of *L. brevis* possessed amylolytic activity; the amylolytic activity *L. brevis* LBRZ7 being the most pronounced. All three strains of *L. paracasei* demonstrated amylolytic activity, but the highest amylolytic activity was observed in *L. paracasei* LBRC11, followed by *L. paracasei* PX3 and *L.paracasei* RN5. Of the species *L. fermentum* amylolytic activity exhibited only *L.*

fermentum LBRH9 and *L. fermentum* Z14; the amylolytic activity of *L. fermentum* Z14 being higher. All three *L. plantarum* strains were characterized by amylolytic activity (Table 2).

Table 3. Proteolytic activity of the newly isolated *Lactobacillus* strains. d_{well} = 6mm. CL (culture liquid); ASN (acellular supernatant) and CSSS (cell suspension in saline solution). Lactobacilli concentration - 10^{12} - 10^{13}CFU/cm^3

Strain	Proteolytic activity, d_{zone}	
L. brevis LBRZ7	CL	9,75±0,50
	ASN	9,75±0,50
	CSSS	9,25±0,50
L. brevis LBRZ8	CL	11,63±0,75
	ASN	9,25±0,50
	CSSS	9,25±0,50
L. paracasei LBRC11	CL	13,38±0,48
	ASN	13,50±0,58
	CSSS	10,25±0,50
L. paracasei PX3	CL	10,25±0,50
	ASN	10,25±0,50
	CSSS	9,50±0,58
L. paracasei RN5	CL	9,13±0,25
	ASN	9,13±0,25
	CSSS	9,00±0,00
L. plantarum F3	CL	12,25±0,50
	ASN	12,13±0,25
	CSSS	9,50±0,58
L. plantarum LBRZ12	CL	14,50±0,58
	ASN	13,25±0,50
	CSSS	12,13±0,25
L. plantarum X2	CL	10,25±0,50
	ASN	10,13±0,25
	CSSS	8,00±0,00
L. fermentum LBRH9	CL	12,63±0,75
	ASN	12,13±0,25
	CSSS	1025±0,50
L. fermentum LBRH10	CL	18,00±0,00
	ASN	11,50±0,58
	CSSS	16,50±0,58
L. fermentum Z14	CL	10,0±0,00
	ASN	10,00±0,00
	CSSS	-
L. buchneri LBRZ6	CL	12,25±0,50
	ASN	12,13±0,2
	CSSS	9,00±0,00
L. sanfranciscensis R	CL	10,25±0,50
	ASN	9,13±0,50
	CSSS	8,00±0,00

2.2. Examination of the Proteolytic Activity of the Newly Isolated Lactobacillus Strains

Dough proteins determine the structural and mechanical properties of bread to a great extent. Thus the presence of proteolytic activity in the newly isolated strains of the genus *Lactobacillus* - *L. fermentum* LBRH9, *L. fermentum* LBRH10, *L. buchneri* LBRZ6, *L. brevis* LBRZ7, *L. brevis* LBRZ8, *L. plantarum* LBRZ12, *L. paracasei* LBRC11, *L. paracasei* PX3,

L. paracasei RN5, *L. brevis* X1, *L. plantarum* X2, *L. sanfranciscensis* R was examined by the well-diffusion method. The proteolytic activity was determined using culture liquid (CL), acellular supernatant (ASN) and cell suspension in saline solution (CSSS) of each of the lactobacilli strains (Table 3).

All representatives of the species *L. brevis, L. fermentum, L. paracasei, L.plantarum, L. buchneri* and *L. sanfranciscensis* included in the study were characterized by proteolytic activity (Table 3).

The data obtained for the diameters of the hydrolysis zones of the culture liquids (CL), acellular supernatants (ASN) and the cell suspensions in saline solution (CSSS) show that the observed proteolytic activity was due both to the production of inducible proteolytic enzymes by the lactobacilli cells, and to the acid hydrolysis by the lactic acid, acetic acid and other organic acids, produced by the lactobacilli strains.

2.3. Determination of the Sensitivity of Saprophytic Microorganisms and the Newly Isolated Lactobacillus Strains to Calcium Propionate and Potassium Sorbate

The most common preservatives in the production of bread and bakery products are calcium propionate (E 281) and potassium sorbate (E 202). Therefore, the action of these substances on the growth of the newly isolated lactobacilli strains and the most common bread saprophytic microorganisms was determined by the well-diffusion method (Table 4).

Table 4. Effect of calcium propionate and potassium sorbate on saprophytic microorganisms. pH = 5. The concentration of saprophytes in the medium was 10^5 CFU/cm^3. '-' – The preservative was not found to suppress the growth of the saprophytic microorganism. Incubation temperature - 30°C

Test-microrganism	Calcium propionate			
	C	0,1%	0,2%	0,3%
B. subtilis	10,00±0,00	10,25±0,50	12,00±0,00	14,50±0,58
S. cerevisiae	-	-	-	-
A. niger	-	-	-	-
Penicillium sp.	-	-	-	-
Rhizopus sp.	15,00±0,00	15,13±0,25	20,25±0,50	20,50±0,58
	Potassium sorbate			
	C	0,1%	0,2%	0,3%
B. subtilis	10,00±0,00	12,13±0,25	13,00±0,00	13,25±0,50
S. cerevisiae	-	-	-	-
A. niger	7,13±0,25	8,0±0,00	11,25±0,50	11,50±0,58
Penicillium sp.	7,00±0,00	8,13±0,25	8,25±0,50	8,25±0,50
Rhizopus sp.	8,13±0,25	15,25±0,50	20,13±0,25	20,50±0,58

The newly isolated lactobacilli strains were resistant to the presence of calcium propionate and potassium sorbate at concentrations of 0,1%; 0,2% and 0,3%, which allows their inclusion in the composition of sourdough starters simultaneously with the investigated preservatives, if necessary.

Calcium propionate and potassium sorbate did not inhibit the growth *Saccharomyces cerevisiae* (baker's yeasts), which is why they are the most commonly used preservatives in bread making. Both preservatives inhibited the growth of *B. subtilis* and *Rhizopus* sp., potassium sorbate also inhibited *A. niger* and *Penicillium* sp.; logically, the higher preservative concentrations exhibited higher inhibitory activity against the test-saprophytic microorganisms (Table 4).

2.4. Antimicrobial Activity of the Newly Isolated Lactobacillus Strains against Saprophytic Microorganisms

In a series of experiments was investigated the antimicrobial activity of the newly isolated *Lactobacillus* strains - *L. brevis* LBRZ7, *L. brevis* LBRZ8, *L. paracasei* LBRC11, *L. paracasei* PX3, *L. paracasei* RN5, *L.fermentum* LBRH9, *L. fermentum* LBRH10, *L. fermentum* Z14, *L. plantarum* F3, *L. plantarum* LBRZ12, *L. plantarum* X2, *L. buchneri* LBRZ6, *Lactobacillus sanfranciscensis* R, against saprophytes by the well-diffusion method (Table 5 and Table 6).

L. brevis LBRZ7 and *L. brevis* LBRZ8 had low antimicrobial activity against *Penicillium* sp. as opposed to the strong inhibitory action on the growth of *A. niger* and *Rhizopus* sp.; *L. brevis* LBRZ7 having higher activity. *L. brevis* LBRZ7 inhibited the growth of *B. subtilis* at 30°C but not at 37°C, while *L. brevis* LBRZ8 was active against *B. subtilis* at 37°C, but not at 30°C. Both strains had no effect on *S. cerevisiae* growth at 30°C and at 37°C.

L. paracasei LBRC11, isolated from homemade cheese, *L. paracasei* PX3 and *L. paracasei* RN5, isolated from spontaneously fermented sourdough, suppressed fungal growth of *Rhizopus* sp., *Penicillium* sp. and *A.niger*. The culture liquids of the three strains exhibited antimicrobial activity against *Saccharomyces cerevisiae* only at a temperature of 37°C. *L. paracasei* LBRC11 had no inhibitory activity against *B. subtilis* at 30°C and at 37°C. *L. paracasei* PX3 exhibited antimicrobial activity against *B. subtilis* at 30°C and at 37°C; at 30°C only in the culture liquid were observed single colonies in the inhibition zone, while at 37°C were observed pronounced inhibition zones.

L. paracasei RN5 possessed antimicrobial activity against *B. subtilis* both at 30°C and at 37°C. *L. paracasei* LBRC11 had the highest inhibitory activity against *Rhizopus* sp. and *Penicillium* sp. *L. paracasei* LBRC11 had the highest activity against *A. niger* at 37°C, followed by *L. paracasei* RN5 and *L.paracasei* PX3, while at 30°C the highest antimicrobial activity demonstrated *L. paracasei* PX3, followed by *L. paracasei* RN5 and *L. paracasei* LBRC11. With the highest inhibitory activity against *B. subtilis* was characterized *L. paracasei* RN5; against *A.niger, Rhizopus* sp. and *Penicillium* sp. - *L. paracasei* LBRC11, followed by *L.paracasei* RN5 and *L. paracasei* PX3.

L. fermentum LBRH10, *L. fermentum* LBRH9 and *L. fermentum* Z14 suppressed fungal growth of *Rhizopus* sp. and *A. niger*, as well as of bacteria *B. subtilis* at 30°C and at 37°C. *L. fermentum* Z14 exhibited antimicrobial activity against *Penicillium* sp. *L. fermentum* Z14 demonstrated antimicrobial activity against *S. cerevisiae* at 37°C - only in the culture liquid were observed single colonies in the inhibition zone. *L. fermentum* Z14 had the highest inhibitory activity against *Rhizopus* sp., *Penicillium* sp. and *A. niger*, followed by *L. fermentum* LBRH10 and *L. fermentum* LBRH9. The highest antimicrobial activity against *B. subtilis* was observed in *L. fermentum* LBRH10, followed by *L. fermentum* LBRH9 and *L. fermentum* Z14.

Table 5. Antimicrobial activity of the newly isolated *L. brevis, L. paracasei, L. fermentum* and *L. plantarum* strains. d_{well} = 6mm. Lactobacilli concentration - 10^{12} - 10^{13}CFU/cm^3. '-' – The lactobacilli were not found to suppress saprophytic growth

Saprophyte Concentration			*B. subtilis* 1,9x10^{5}CFU/cm^3		*S. cerevisiae* 9,2x10^{4}CFU/cm^3		*A. niger* 1,2x10^{5}CFU/cm^3		*Rhizopus sp.* 1,8x10^{5}CFU/cm^3	*Penicillium sp.* 5,2x10^{5}CFU/cm^3
Incubation temperature			30°C	37°C	30°C	37°C	30°C	37°C	30°C	30°C
L. brevis	**LBRZ7**	CL	11,00±0,00	-	-	-	10,25±0,50	13,50±0,58	14,50±0,58	8,00±0,00
		ASN	10,25±0,50	-	-	-	10,13±0,25	10,13±0,25	1225±0,50	8,00±0,00
		NASN	10,13±0,25	-	-	-	10,00±0,00	9,00±0,00	12,13±0,25	-
	LBRZ8	CL	-	10,25±0,50	-	-	9,50±0,58	11,25±0,50	10,50±0,58	8,13±0,25
		ASN	-	10,13±0,25	-	-	8,13±0,25	9,13±0,25	9,50±0,58	8,13±0,25
		NASN	-	9,00±0,00	-	-	-	-	-	-
L. paracasei	**LBRC11**	CL	-	-	-	15,00±0,00	10,25±0,50	16,00±0,00	14,50±0,58	10,25±0,50
		ASN	-	-	-	-	9,25±0,50	10,13±0,25	11,25±0,50	8,13±0,25
		NASN	-	-	-	-	9,13±0,25	-	8,00±0,00	8,00±0,00
	PX3	CL	+ single colonies in the inhibition zone	13,25±0,50	-	15,00±0,00	12,50±0,58	10,50±0,58	13,50±0,58	8,00±0,00
		ASN	-	11,00±0,00	-	-	10,25±0,50	9,13±0,25	10,13±0,25	-
		NASN	-		-	-	10,13±0,25	9,00±0,00	10,00±0,00	-
	RN5	CL	18,13±0,25	15,25±0,50	-	11,50±0,58	11,13±0,25	11,13±0,25	15,13±0,25	13,50±0,58
		ASN	13,25±0,50	13,13±0,25	-	10,13±0,25	9,13±0,25	10,25±0,50	10,50±0,58	8,13±0,25
		NASN	10,50±0,58	-	-	9,00±0,00	9,00±0,00	9,13±0,25	8,00±0,00	8,00±0,00
L. fermentum	**LBRH10**	CL	10,50±0,58	12,00±0,00	-	-	11,50±0,58	10,00±0,00	12,13±0,25	-
		ASN	-	10,25±0,50	-	-	10,13±0,25	-	9,50±0,58	-
		NASN	-	10,13±0,25	-	-	10,50±0,58	-	-	-
	LBRH9	CL	13,25±0,50	10,50±0,58	-	-	9,00±0,00	10,13±0,25	11,13±0,25	-
		ASN	-	10,13±0,25	-	-	9,00±0,00	9,13±0,25	11,00±0,00	-
		NASN	-	10,00±0,00	-	-	9,00±0,00	9,00±0,00	11,13±0,25	-
	Z14	CL	10,13±0,25	9,00±0,00	-	+ single colonies in the inhibition zone	12,50±0,58	10,13±0,25	13,50±0,58	10,00±0,00
		ASN	9,50±0,58	-	-	-	10,00±0,00	9,00±0,00	12,13±0,25	9,13±0,25
		NASN	8,00±0,00	-	-	-	10,13±0,25	-	9,00±0,00	8,25±0,50

Table 5. (Continued)

Saprophyte Concentration			*B. subtilis* 1,9x10^{5}CFU/cm^3		*S. cerevisiae* 9,2x10^{4}CFU/cm^3		*A. niger* 1,2x10^{5}CFU/cm^3		*Rhizopus sp.* 1,8x10^{5}CFU/cm^3	*Penicillium sp.* 5,2x10^{5}CFU/cm^3
Incubation temperature			30°C	37°C	30°C	37°C	30°C	37°C	30°C	30°C
L. plantarum	**LBRZ12**	CL	-	-	-	-	12,00±0,00	12,00±0,0	14,13±0,25	13,50±0,58
		ASN	-	-	-	-	10,13±0,25	12,00±0,00	10,50±0,58	11,25±0,58
		NASN	-	-	-	-	10,00±0,00	9,13±0,25	-	10,00±0,00
	F3	CL	13,50±0,58	10,50±0,58	-	20,13±0,2	9,00±0,00	14,50±0,58	14,50±0,58	10,00±0,00
		ASN	12,50±0,58	10,00±0,00	-	13,25±0,50	8,13±0,25	10,13±0,25	12,00±0,00	9,25±0,50
		NASN	-	-	-	-	8,13±0,25	9,13±0,25	10,13±0,25	8,13±0,25
	X2	CL	11,00±0,00	9,13±0,25	-	14,50±0,58	16,50±0,58	13,13±0,25	16,00±0,00	12,25±0,50
		ASN	-	-	-	10,00±0,00	12,25±0,50	9,13±0,25	12,13±0,25	10,00±0,00
		NASN	-	-	-	-	11,00±0,00	9,00±0,00	11,50±0,58	-

L. plantarum LBRZ12, *L. plantarum* F3 and *L. plantarum* X2 exhibited antimicrobial activity against *Rhizopus* sp., *Penicillium* sp. and *A.niger*. *L.plantarum* LBRZ12 did not show any inhibitory action against *S.cerevisiae* and *B. subtilis* neither at 30°C, nor at 37°C. *L.plantarum* F3 and *L. plantarum* X2 exhibited antimicrobial activity against *B. subtilis* both at 30°C and at 37°C, and against *S. cerevisiae*; *L. plantarum* X2 suppressed *S. cerevisiae* only at 37°C. *L. plantarum* X2 had the highest inhibitory activity against *Rhizopus* sp. and *Penicillium* sp. Against *A. niger* at 37°C the highest activity exhibited *L. plantarum* F3, followed by *L. plantarum* X2 and *L. plantarum* LBRZ12, while at 30°C the highest antimicrobial activity showed *L. plantarum* X2, followed by *L. plantarum* LBRZ12 and *L. plantarum* F3. *L.plantarum* F3 demonstrated higher antimicrobial activity than *L.plantarum* X2 against *S. cerevisiae*. The highest inhibitory activity against *B. subtilis* was observed in *L. plantarum* F3, followed by *L. plantarum* X2, both at 30°C and at 37°C. *L. plantarum* X2 was distinguished by the highest inhibitory activity against the fungi *A. niger*, *Rhizopus* sp. and *Penicillium* sp., followed by *L. plantarum* LBRZ12 and *L. plantarum* F3 (Table 5).

L. sanfranciscensis R and *L. buchneri* LBRZ6 inhibited the growth of *B.subtilis, A. niger, Rhizopus* sp. and *Penicillium* sp., but did not inhibit *S. cerevisiae* growth (Table 6).

Table 6. Antimicrobial activity of *L. sanfranciscensis* R and *L. buchneri* LBRZ6 against saprophytic microorganisms. The values are in mm. d_{well} = 6 mm. CL - culture liquid; ASN - acellular supernatant without pH adjustment and NASN - neutralized acellular supernatant (pH = 6.5). Lactobacilli concentration - 10^{12} - 10^{13}CFU/cm^3. Incubation temperature - 30°C

d inhibition zone, (mm)		*B. subtilis* 1,9x10^{5}CFU/cm^3	*S. cerevisiae* 9,2x10^{4}CFU/cm^3	*A. niger* 1,2x10^{5}CFU/cm^3	*Rhizopus sp.* 1,8x10^{5}CFU/cm^3	*Penicillium sp.* 5,2x10^{5}CFU/cm^3
***L. buchneri* LBRZ6**	CL	10, 00±0,00	-	13,25±0,50	11,50±0,58	9,00±0,00
	ASN	-	-	10,13±0,25	9,25±0,50	9,00±0,00
	NASN	-	-	10,00±0,00	8,13±0,25	9,00±0,00
***L.sanfranciscensis* R**	CL	10,25±0,50	-	14,50±0,58	11,50±0,58	9,25±0,50
	ASN	10,13±0,25	-	11,13±0,25	10,13±0,25	-
	NASN	10,00±0,00	-	11,00±0,00	10,13±0,25	-

The lactobacilli strains suppressed the saprophytic growth, not only because of the nutrition competition between them, but also because of the produced and accumulated lactic acid and other organic acids, resulting in a pH decrease, as well as other substances with antimicrobial activity.

The observed differences between the strains of the species *L. paracasei, L.plantarum, L. brevis, L. fermentum* confirm the need for mandatory evaluation of the antimicrobial activity of each strain intended for incorporation in sourdough starters as the antimicrobial activity is strain-specific, not species-specific.

3. Application of Lactobacilli Strains in Sourdough Starters for Wheat Bread

A proper selection of lactic acid bacteria strains with suitable properties, intended to be included in the composition of sourdough starters for bread and bakery products, needs to be conducted.

3.1. Selection of Lactobacilli Strains

During lactic acid bacteria growth are produced and accumulated lactic acid, acetic acid and other organic acids as well as other metabolites such as bacteriocins, through which lactic acid bacteria inhibit saprophytic growth. It is of paramount importance for the lactobacilli strains to quickly accumulate high concentrations of viable cells to carry out targeted fermentation process. Therefore the reproductive ability of *L. brevis* LBRZ7, *L. fermentum* LBRH9, *L. fermentum* LBRH10, *L. fermentum* Z14, *L. paracasei* LBRC11, *L. paracasei* RN5, *L. paracasei* PX3, *L. plantarum* LBRZ12, *L. plantarum* X2, *L. plantarum* F3, *L. buchneri* LBRZ6 and *L. sanfranciscensis* R to grow in flour/water environment and to reach high viable cell concentrations and to accumulate organic acids was investigated. Thus, single-strain sourdoughs were prepared with each *Lactobacillus* strain, the single-strain suspension being with a concentration of viable cells of 10^{12}CFU/cm^3. The changes in the concentration of living lactobacilli cells in the single-strain sourdoughs and the total titratable acidity at daily back-slopping for 96 hours at 30°C or 37°C were monitored.

In all studies was used flour containing 10^3CFU/g lactobacilli. In the control sample on the 24th hour the concentration reached values below 10^5CFU/g, the number of cells of "wild yeasts" exceeded 10^8CFU/g, which is why after the 24th hour only alcohol fermentation was observed. All lactobacilli strains grew well in the flour/water environment, reaching 10^{14}-10^{15}CFU/cm^3 viable cells on the 96th hour, and the acidity of the resulting sourdoughs increased to values above 10°N (Denkova et al., 2014c).

3.2. Development of Symbiotic Starters

Based on the results for *L. brevis* LBRZ7, *L. fermentum* LBRH9, *L. paracasei* LBRC11 and *L. plantarum* LBRZ12 were developed 2 sourdough combinations for wheat bread by mixing the strains in a certain ratio. The four-strain combination comprised the strains *L. plantarum* LBRZ12, *L. paracasei* LBRC11, *L. brevis* LBRZ7 and *L. fermentum* LBRH9 in a ratio of 2 : 1 : 1 : 1, respectively; and the two-strain combination - *L. brevis* LBRZ7 and *L. paracasei* LBRC11 in a ratio of 7 : 3, respectively. The *L. brevis* and *L. plantarum* strains were included in the composition of the two combinations, because it has been found that strains of these species have the most appropriate profiles of flavor and aroma components for incorporation in sourdough starters (Gobbetti et al., 1995). The two combinations were used for the preparation of multi-strain sourdough. The inoculum concentration of the four-strain combination was 5.0x10^9 CFU/cm^3, and of the two-strain combination - 9.5x10^8 CFU/cm^3. The changes in the accumulation of biomass and in the total titratable acidity of the sourdoughs in daily back-slopping for 96 hours were monitored. On the 48th hour to each of the two sourdoughs was added 0,1% baker's yeasts (*S. cerevisiae*). The results of the parallel studies of the sourdoughs with the two combinations for wheat bread are shown in Figure 1. The strains in the four-strain combination grew together with the accumulation of high concentrations of viable lactobaciili cells (over 10^{10} CFU/g) and the total titratable acidity increased from 2,5 °N (0 h) to 17,3 °N (96 h) (Figure 1b). In the sourdough with the two-

strain combination the number of viable lactobacilli cells reached 10^{14} CFU/g, while the total titratable acidity increased from 2,94 °N (0 h) to 16,4 °N (96 h) (Figure 1a). In determining the microbiological quality of both sourdoughs no molds were established. Furthermore, the metabolites produced by the lactobacilli in the composition of the two combinations inhibited wild yeasts that came from the flour (Figure 1a and Figure 1b). This fact is particularly important in sourdough fermentation for the continuous daily back-slopping of the two sourdoughs (6-9 months) (Denkova et al., 2014 c).

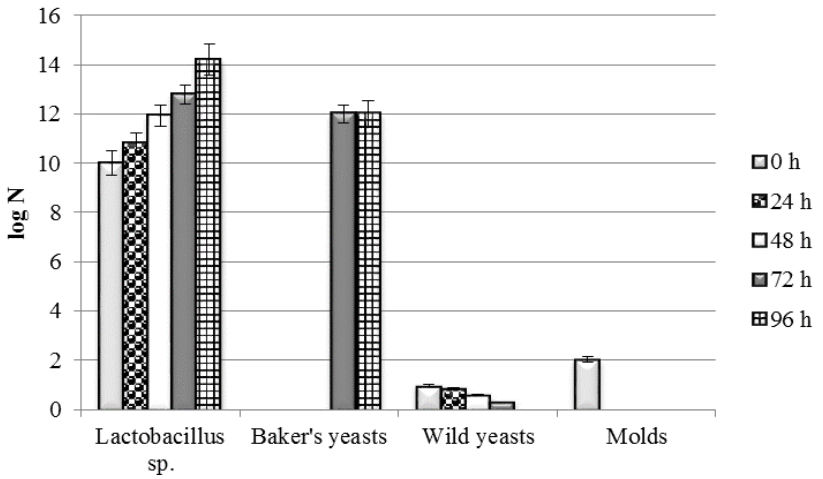

Figure 1a. Changes in the concentration of viable lactobacilli cells, the lateral microflora and the total titratable acidity of the two-strain sourdough at daily back-slopping for 96 hours.

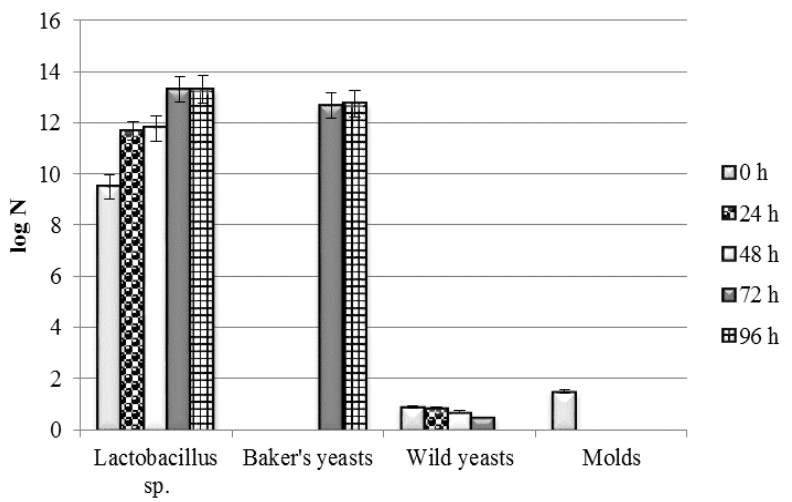

Figure 1b.Changes in the concentration of viable lactobacilli cells, the lateral microflora and the total titratable acidity of the four-strain sourdough at daily back-slopping for 96 hours.

For 48 to 72 hours of daily back-slopping the two sourdoughs with the two combinations reached normal sourdough consistency with a pleasant lactic acid aroma which was more pronounced in the sourdough with the two-strain combination (Denkova et al., 2014 c).

The antimicrobial activity of the two 96-hour sourdoughs against saprophytic microorganisms was determined (Denkova et al., 2014 c). Both sourdoughs did not inhibit *Saccharomyces cerevisiae* growth (baker's yeasts), but possessed antimicrobial activity against the other saprophytes included in the study – *B.subtilis, B.mesentericus, Aspergillus niger, Rhizopus* sp., *Penicillium* sp. The sourdough with the four-strain combination demonstrated higher inhibitory action than the sourdough with the two-strain combination. The observed inhibition was due to the metabolites produced by lactic acid bacteria in the composition of the combinations.

3.3. Approbation of Starter Combinations for Wheat Bread in Industrial Production

Wheat bread variants using different percentage of the two 96-hour sourdoughs were prepared. The two wheat bread variants with the two-strain starter sourdough included 5% and 7% of the two-strain sourdough, respectively. The three wheat bread variants with the four-strain starter sourdough included 5%, 7% and 10% of the four-strain sourdough. A control bread with no sourdough was prepared as well.

The baked bread variants were evaluated according to 6 criteria: aroma, flavor, crumb softness, crumb color, crust color and loaf volume (Figure 2a and Figure 2b). Experimental data suggested speeding up the fermentation process. The presence of *L. plantarum* in the composition of one of the starters increases the fermentation activity of the dough, which confirms the research of Coppola et al., 1998; Pepe et al., 2004. The resulting starter sourdoughs were stronger, more elastic, and the bread pieces were higher. Bread with better technological and organoleptic characteristics - higher volume, better flavor and aromatic complex and brighter and softer crumb was obtained with the use of 7% of the four-strain starter sourdough (Denkova et al., 2014c).

Prevention of fungal and bacterial spoilage of bread and bakery products can be achieved through the implementation of a number of physical methods, but the addition of sourdough is the only option that meets the informed consumer's demand for safe foods that are minimally processed, without preservatives. Thus experiments to determine the sourdough concentration to be added to the main dough to produce bread with extended shelf life without the addition of preservatives, but with preserved organoleptic characteristics using inseminated flour (concentration of microorganisms - over 10^5CFU/g) were conducted.

8 new four-strain sourdough combinations for wheat and rye bread were developed (Table 7). The combinations were passaged daily in MRS-broth for 96 hours and the changes in the titratable acidity and the concentration of viable lactobacilli cells were monitored (Table 7). In each of the eight combinations there was a symbiotic relationship between the included strains. The four strains in each combination grew well together with the accumulation of high concentrations of living cells (over 10^{11}CFU/cm^3 - from $1x10^{11}$CFU/cm^3 for Combination 3.1. to $6,1x10^{13}$CFU/cm^3 for Combination 1.2.) and the titratable acidity increased to more than 100 °T (from 102,94 °T for Combination 2.2. to 147,39 °T for Combination 1.1.) (Denkova et al., 2014b).

The antimicrobial activity of the eight combinations on the 96th hour of daily passaging against saprophytic microorganisms was determined. All combinations inhibited the growth of *Bacillus subtilis*, *Aspergillus niger*, *Rhizopus* sp. and *Penicillium* sp., but did not inhibit baker's yeasts *Saccharomyces cerevisiae* (Denkova et al., 2014b).

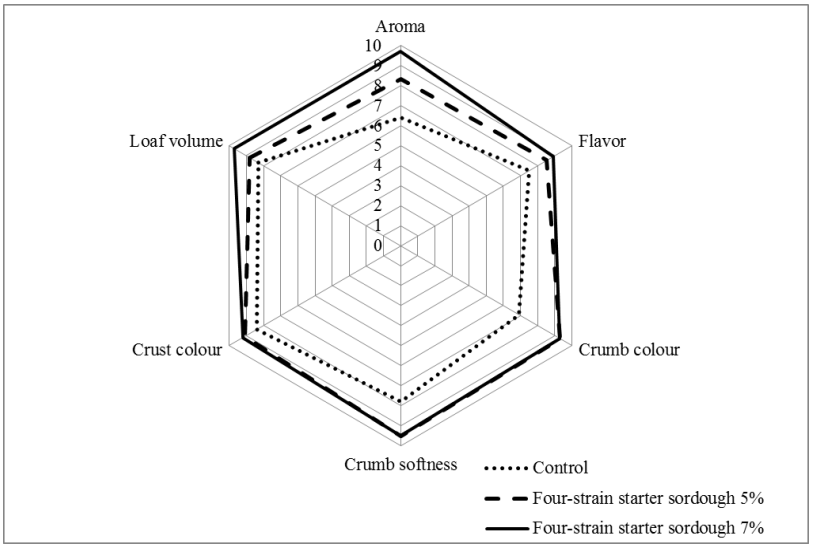

Figure 2a. Organoleptic evaluation of the wheat bread variants with the four-strain starter sourdough.

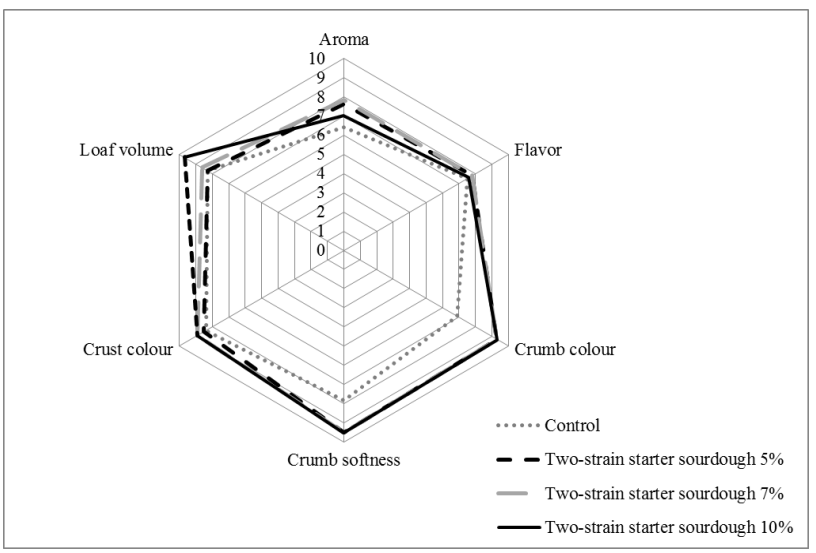

Figure 2b. Organoleptic evaluation of the wheat bread variants with the two-strain starter sourdough.

Combination 1.1., Combination 2.2. Combination 3.2. and Combination 4.2. were probated in industrial production. They were used for the preparation of multi-strain sourdoughs that were back-slopped daily for the duration of 96 hours. The changes in the total titratable acidity and the concentration of viable lactobacilli cells during daily back-slopping for 96 hours were monitored (Table 8) (Denkova et al., 2014b).

The antimicrobial activity of the 96-hour starter sourdoughs against saprophytic microorganisms was examined. All sourdoughs inhibited *B. subtilis*, *Penicillium* sp., *A. niger* and *Rhizopus* sp., but did not inhibit *S. cerevisiae* (baker's yeasts), as the highest antimicrobial activity was observed in sourdough with Combination 4.2., followed by

sourdough with Combination 3.2.; sourdough with Combination 1.1 and sourdough with Combination 2.2. (Denkova et al., 2014b).

Sourdough with Combination 4.2. exhibited the highest inhibitory activity. Thus, Combination 4.2. (*L.paracasei* RN5: *L.plantarum* X2: *L.brevis* LBRZ7: *L.fermentum* LBRH10 = 1: 2: 1: 1) was used for the development of four new combinations – two of them (Combination 7 and Combination 8) included *L. buchneri* LBRZ6 (for the preparation of wheat bread), and the other two (Combination 5 and Combination 6) included *L.sanfranciscensis* R (for the preparation of rye bread). Two of the combinations (Combination 6 and Combination 8) also included the probiotic strain *Pr. shermanii* NBIMCC 327 (Figure 3a, Figure 3b, Figure 3c and Figure 3d).

Table 7. Changes in the titratable acidity and the concentration of viable lactobacilli cells of the 8 new combinations in daily passaging for 96 hours

Combination		N (CFU/cm^3)		TA ($^\circ$T)	
		0 h	**96 h**	**0 h**	**96 h**
1.1.	***L.paracasei* LBRC11: *L.plantarum* F3 : *L.brevis* LBRZ7 : *L.fermentum* Z14 = 1 : 1 : 1 : 1**	**3x10^9**	**5.5x10^{12}**	**63.49**	**147.39**
1.2.	*L.paracasei* LBRC11: *L.plantarum* F3 : *L.brevis* LBRZ7 : *L.fermentum* Z14 = 1 : 2 : 1 : 1	1.1x10^9	6.1x10^{13}	58.96	140.59
2.1.	*L.paracasei* PX3: *L.plantarum* X2 : *L.brevis* LBRZ7 : *L.fermentum* LBRH10 = 1 : 1 : 1 : 1	3x10^8	2.2x10^{12}	51.7	103.85
2.2.	***L.paracasei* PX3: *L.plantarum* X2 : *L.brevis* LBRZ7 : *L.fermentum* LBRH10 = 1 : 2 : 1 : 1**	**9x10^8**	**2.8x10^{12}**	**50.79**	**102.94**
3.1.	*L.paracasei* RN5: *L.plantarum* F3 : *L.brevis* LBRZ7 : *L.fermentum* Z14 = 1 : 1 : 1 : 1	6x10^9	1x10^{11}	51.7	149.2
3.2.	***L.paracasei* RN5: *L.plantarum* F3 : *L.brevis* LBRZ7 : *L.fermentum* Z14 = 1 : 2 : 1 : 1**	**1.4x10^9**	**5x10^{12}**	**58.96**	**151.92**
4.1.	*L.paracasei* RN5: *L.plantarum* X2 : *L.brevis* LBRZ7 : *L.fermentum* LBRH10 = 1 : 1 : 1 : 1	2.7x10^9	5.7x10^{12}	49.89	117.46
4.2.	***L.paracasei* RN5: *L.plantarum* X2 : *L.brevis* LBRZ7 : *L.fermentum* LBRH10 = 1 : 2 : 1 : 1**	**5x10^8**	**2x10^{11}**	**54.42**	**117.91**

Table 8. Changes in the total titratable acidity (TTA, °N) and the concentration of viable lactobacilli cells in daily back-slopping of the new four-strain sourdoughs for 96 hours

Combination		N (CFU/cm^3)		TTA, (°N)	
		0 h	96 h	0 h	96 h
1.1.	*L.paracasei* LBRC11: *L.plantarum* F3 : *L.brevis* LBRZ7 : *L.fermentum* Z14 = 1 : 1 : 1 : 1	<10^7	1x10^{13}	13.8	14.6
2.2.	*L.paracasei* PX3: *L.plantarum* X2 : *L.brevis* LBRZ7 : *L.fermentum* LBRH10 = 1 : 2 : 1 : 1	8x10^7	2.6x10^{11}	10.4	15
3.2.	*L.paracasei* RN5: *L.plantarum* F3 : *L.brevis* LBRZ7 : *L.fermentum* Z14 = 1 : 2 : 1 : 1	7x10^7	4x10^{10}	13	14.4
4.2.	*L.paracasei* RN5: *L.plantarum* X2 : *L.brevis* LBRZ7 : *L.fermentum* LBRH10 = 1 : 2 : 1 : 1	4x10^7	1x10^{12}	10.6	14

The four new combinations were passaged daily in MRS-broth for 96 hours. The changes in the titratable acidity and the concentration of viable cells of lactobacilli and propionic acid bacteria in daily passaging were monitored (Figure 3a, Figure 3b, Figure 3c and Figure 3d). The experimental results clearly prove the existence of a symbiotic relationship between the strains in the four new combinations (Denkova et al., 2014b).

The four combinations were used for the preparation of multi-strain sourdoughs that were back-slopped daily in industrial production for 96 hours. The changes in the total titratable acidity and the concentration of viable cells of lactobacilli and propionic acid bacteria were monitored (Table 9) (Denkova et al., 2014b).

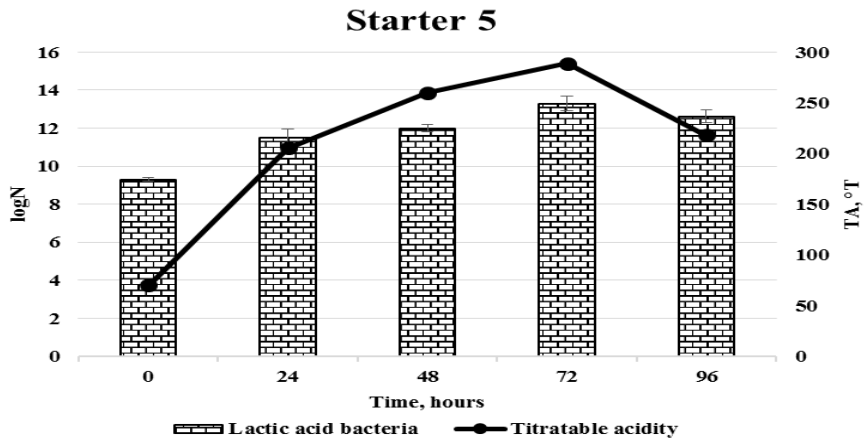

Figure 3a. Changes in the concentrations of viable lactic acid bacteria cells and the titratable acidity during daily passaging of Starter 5 (Composition - (*L. paracasei* RN5: *L. plantarum* X2 : *L. brevis* LBRZ7: *L. fermentum* LBRH10 = 1 : 2 : 1 : 1) : *L.sanfranciscensis* R = 2 : 1) for 96 hours.

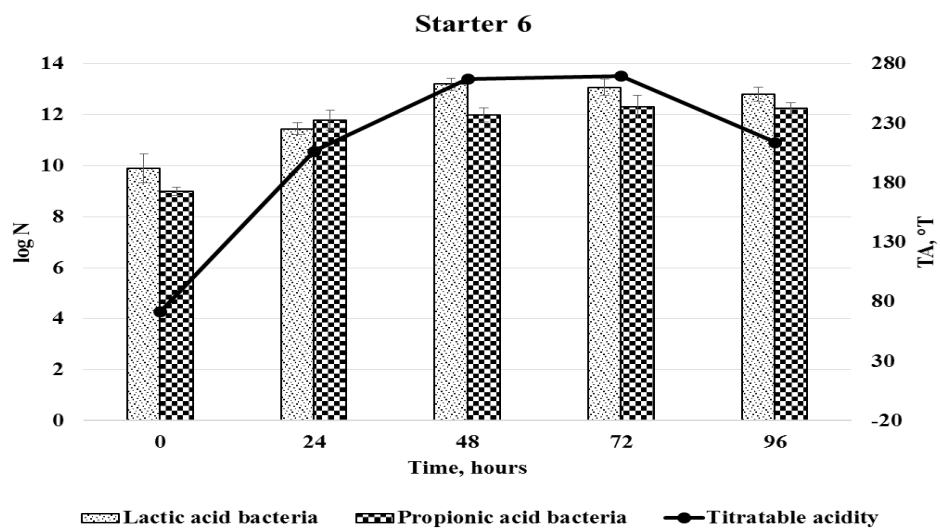

Figure 3b. Changes in the concentrations of viable lactic acid bacteria and propionic acid bacteria cells and the titratable acidity during daily passaging of Starter 6 (Composition - (*L. paracasei* RN5 : *L. plantarum* X2 : *L. brevis* LBRZ7: *L. fermentum* LBRH10 = 1 : 2 : 1 : 1): *L.sanfranciscensis* R : *Propionibacterium freudenreichii* ssp. *shermanii* NBIMCC 327 = 2 : 1 : 1) for 96 hours.

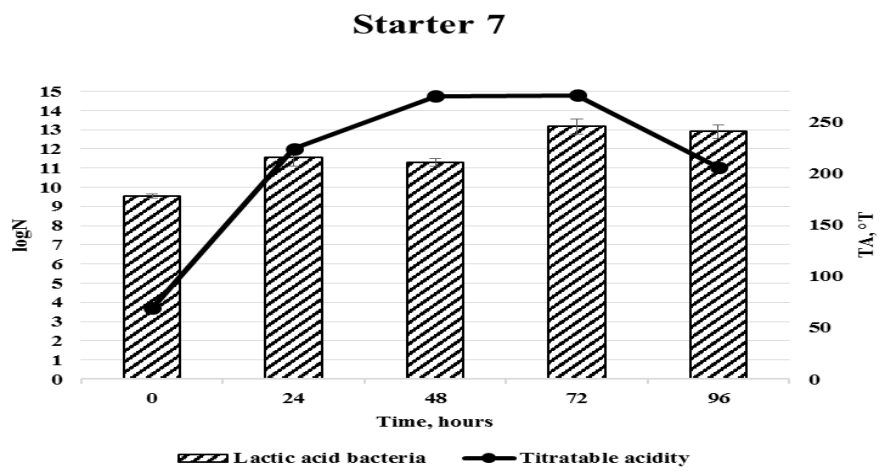

Figure 3c. Changes in the concentrations of viable lactic acid bacteria cells and the titratable acidity during daily passaging of Starter 7 (Composition – (*L. paracasei* RN5: *L. plantarum* X2: *L. brevis* LBRZ7: *L. fermentum* LBRH10 = 1 : 2 : 1 : 1): *L.buchneri* LBRZ6 = 2 : 1) for 96 hours.

For 96 hours of daily back-slopping the four sourdoughs with the four new starters harmonized their aroma and were stabilized with respect to their total titratable acidity. The greatest change in the concentration of viable cells was recorded for sourdough with Combination 7 (5 logN), followed by sourdough with Combination 6 (4 logN), sourdough with Combination 8 (3 logN) and sourdough with Combination 5 (2 logN) (Denkova et al., 2014b).

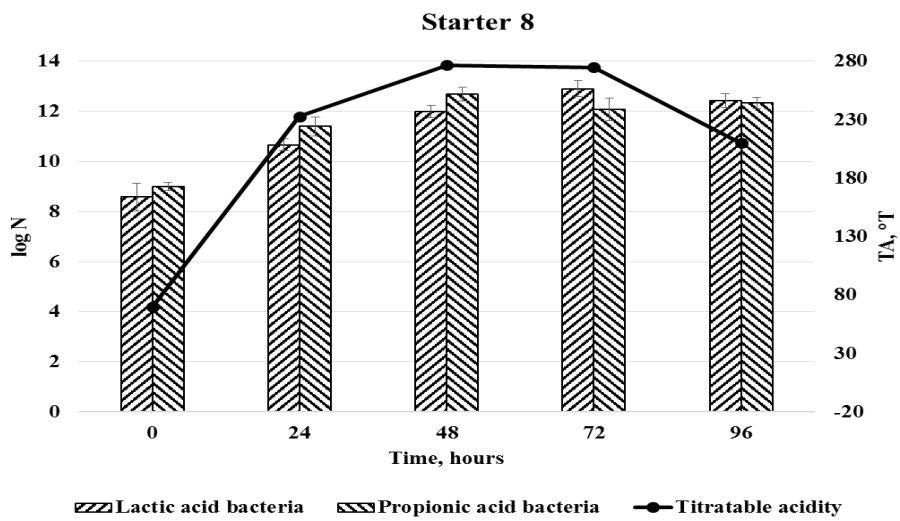

Figure 3d. Changes in the concentrations of viable lactic acid bacteria and propionic acid bacteria cells and the titratable acidity during daily passaging of Starter 8 (Composition - (*L. paracasei* RN5 : *L. plantarum* X2: *L. brevis* LBRZ7: *L. fermentum* LBRH10 = 1 : 2 : 1 : 1): *L.buchneri* LBRZ6 : *Propionibacterium freudenreichii* ssp. *shermanii* NBIMCC 327 = 2 : 1 : 1) for 96 hours.

Table 9. Changes in the total titratable acidity (TTA), the aroma and the concentration of viable cells in daily back-slopping of the new multi-strain sourdoughs. LAB – lactic acid bacteria; PAB – propionic acid bacteria

Starter		N (CFU/cm^3)		TTA, (°N)	
		0 h	96 h	0 h	96 h
5	LAB	$4.4x10^8$	$3x10^{10}$	3,2	15,4
6	LAB	$4.6x10^7$	$1.1x10^{11}$	4,4	13,6
	PAB	$5x10^6$	$9x10^{10}$		
7	LAB	$3.9x10^7$	$6x10^{13}$	3	14,8
8	LAB	$5.5x10^8$	$7x10^{11}$	4,8	15
	PAB	$3x10^8$	$2x10^{11}$		

Table 10. Bacterial spoilage caused by *Bacillus* sp. during storage of the baked bread variants at 37°C and at room temperature (RT). DS – degree of spoilage

Baked bread variant	Temperature	24 h		48 h		72 h		96 h	
		DS	Odour	DS	Odour	DS	Odour	DS	Odour
Control	37°C	-	No	I	Yes	II	Yes	III	Yes
	RT	-	No	-	No	I	Yes	II	Yes
Sourdough with Rye Starter 5; 10%	37°C	-	No	-	No	I	Yes	II	Yes
	RT	-	No	-	No	I	Yes	II	Yes
Sourdough with Rye Starter 5; 15%	37°C	-	No	-	No	-	No	I	Yes
	RT	-	No	-	No	-	No	I	Yes
Sourdough with Rye Starter 5; 20%	37°C	-	No	-	No	-	No	I	Yes
	RT	-	No	-	No	-	No	-	No
Sourdough with Rye Starter 6; 10%	37°C	-	No	-	No	I	Yes	II	Yes
	RT	-	No	-	No	-	No	-	No
Sourdough with Rye Starter 6; 15%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No
Sourdough with Rye Starter 6; 20%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No
Baked bread variant	Temperature	24 h		48 h		72 h		96 h	
		DS	Odour	DS	Odour	DS	Odour	DS	Odour
Sourdough with Wheat Starter 7; 10%	37°C	-	No	-	No	I	Yes	II	Yes
	RT	-	No	-	No	I	Yes	II	Yes
Sourdough with Wheat Starter 7; 15%	37°C	-	No	-	No	-	No	I	Yes
	RT	-	No	-	No	-	No	I	Yes
Sourdough with Wheat Starter 7; 20%	37°C	-	No	-	No	-	No	I	Yes
	RT	-	No	-	No	-	No	I	Yes
Sourdough with Wheat Starter 8; 10%	37°C	-	No	-	No	I	Yes	II	Yes
	RT	-	No	-	No	-	No	-	No
Sourdough with Wheat Starter 8; 15%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No
Sourdough with Wheat Starter 8; 20%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No

Degree of Spoilage (DS): I barely noticeable (pleasant fruity odour); II weak (change in the odour - distinct); III medium (moisty, sticky crumb, sharp odour); IV strong (unpleasant odour, brown-yellow crumb)

The antimicrobial activity of the 96-hour sourdoughs with the four new combinations against saprophytic microorganisms was determined. All sourdoughs had inhibitory activity against *B. subtilis, A. niger, Rhizopus* sp. and *Penicillium* sp. Neither of the sourdoughs inhibited *S. cerevisiae*. The comparison between the sourdoughs with the combinations with *L. buchneri* LBRZ6 (Combination 7 and Combination 8) showed that the sourdough with Combination 8 (featuring *Pr. shermanii* NBIMCC 327) had higher antimicrobial activity against the saprophytes included in the study. Similar conclusions were drawn from the comparison of the sourdoughs with the two combinations with *L. sanfranciscensis* R (Combination 5 and Combination 6), which was clear evidence of the inhibitory action of *Pr. shermanii* NBIMCC 327 against the saprophytic microorganisms (Denkova et al., 2014b).

Bread variants using 10%, 15% and 20% of the four new 96-hour multi-strain starter sourdoughs were prepared (the percentage was determined according to the amount of the flour). The aim was to establish the best multi-strain sourdough starter for wheat and rye bread and the optimal percentage of inclusion of the best multi-strain starter sourdough for the prevention of bacterial (bread roping) and mold spoilage of wheat and rye bread. The baked bread variants were stored at room temperature and at 37°C for 96 hours for the determination of bacterial spoilage (Table 10); and at room temperature and at 30°C for 120 hours to determine the appearance of mold spoilage (Table 11). The earliest signs of bacterial spoilage occurred in the control bread – on the 48th hour at 37°C and on the 72nd hour at room temperature. According to food standards no signs of bacterial spoilage should be observed at the 48th hour. The control bread did not meet the standard requirements for microbial safety of bakery products. In the bread variant with 10% sourdough signs of bacterial spoilage were established on the 72nd hour both at 37°C and at room temperature for the bread variants prepared with sourdough with Starter 5 or Starter 7. In the bread variants with the inclusion of 10% sourdough with Starter 6 or Starter 8, bacterial spoilage was observed on the 72nd hour at 37°C. When the percentage of inclusion of sourdough rose to 15%, in the bread variants prepared with sourdough with Starter 5 or Starter 7 bacterial spoilage became visible on the 96th hour, while in the bread variants with sourdough with Starter 6 or Starter 8, no signs of bacterial spoilage were observed on the 96th hour both at 37°C and at room temperature. In the bread variants with 20% sourdough no bacterial spoilage was observed except for the bread variants prepared with 20% sourdough with Starter 5 or Starter 7 during storage at 37°C, in which bacterial spoilage was evident on the 96th hour (Table 10) (Denkova et al., 2014b).

The developed multi-strain starter sourdoughs were used in amounts of 10%, 15% or 20% under non-sterile conditions and the baked bread variants were stored at different temperatures under non-sterile conditions, resembling the conditions of home storage, in contrast to the experiments described by Mentes et al., 2007, which are carried out under aseptic conditions.

The earliest signs of mold spoilage were observed in the control bread – on the 72nd hour both at 30°C and at room temperature. Upon addition of 10% sourdough signs of fungal spoilage were established on the 96th hour, the degree of spoilage being the lowest in the bread variants prepared with the inclusion of sourdough with Starter 6 or Starter 8 both at 30°C and at room temperature. When the percentage of sourdough inclusion was increased to 15%, fungal spoilage became visible on the 120th hour for all bread variants at 30°C and at room temperature, except for those prepared with sourdough with Starter 6 or Starter 8, stored at room temperature in which mold spoilage was not noticeable even on the 120th hour. In the

bread variants with addition of 20% sourdough, no mold spoilage was established up to the 120th hour both at 30°C and at room temperature (Table 11) (Denkova et al., 2014b).

Based on the experimental results, the best starter for rye bread was Starter 6, while for wheat bread it was Starter 8. The optimum concentration of inclusion of sourdough with Starter 6 for rye bread and Starter 8 for wheat bread without negatively affecting the volume, the flavor and the aroma of the resulting bread was 10-15% for the prevention of bacterial spoilage and 15-20% for the prevention of mold spoilage.

Table 11. Mold spoilage during storage of the baked bread variants at 30°C and at room temperature (RT)

Baked bread variant	Temperature	24 h	48 h	72 h	96 h	120 h
Control	30°C	No	No	Yes	Yes	Yes
	RT	No	No	Yes	Yes	Yes
Sourdough with Rye Starter 5; 10%	30°C	No	No	No	Yes	Yes
	RT	No	No	No	Yes	Yes
Sourdough with Rye Starter 5; 15%	30°C	No	No	No	No	Yes
	RT	No	No	No	No	Yes
Sourdough with Rye Starter 5; 20%	30°C	No	No	No	No	No
	RT	No	No	No	No	No
Sourdough with Rye Starter 6; 10%	30°C	No	No	No	Yes / No – single colonies	Yes
	RT	No	No	No	Yes / No – single colonies	Yes
Sourdough with Rye Starter 6; 15%	30°C	No	No	No	No	Yes
	RT	No	No	No	No	No
Sourdough with Rye Starter 6; 20%	30°C	No	No	No	No	No
	RT	No	No	No	No	No
Sourdough with Wheat Starter 7; 10%	30°C	No	No	No	No	No
	RT	No	No	No	Yes	Yes
Sourdough with Wheat Starter 7; 15%	30°C	No	No	No	No	Yes
	RT	No	No	No	No	Yes
Sourdough with Wheat Starter 7; 20%	30°C	No	No	No	No	No
	RT	No	No	No	No	No
Baked bread variant	Temperature	24 h	48 h	72 h	96 h	120 h
Sourdough with Wheat Starter 8; 10%	30°C	No	No	No	Yes / No – single colonies	Yes
	RT	No	No	No	Yes / No – single colonies	Yes
Sourdough with Wheat Starter 8; 15%	30°C	No	No	No	No	Yes
	RT	No	No	No	No	No
Sourdough with Wheat Starter 8; 20%	30°C	No	No	No	No	No
	RT	No	No	No	No	No

The results confirmed the findings of other research teams. The inclusion of 15% or more sourdough during kneading of the main dough inhibited the growth of bacteria and mold spores and provided longer shelf life of the baked products (Mentes et al., 2007), and although acidification is necessary for the optimal bread baking, the control of the enzyme

activities, the crumb elasticity and the long shelf life (Pepe et al., 2003; Spicher, 1983), excessive acidification had an adverse effect on some rheological parameters (Collar et al., 1994; Pepe et al., 2004).

Sourdoughs with Starter 6 (for rye bread) or Starter 8 (for wheat bread) were back-slopped daily in industrial production in a ratio of ¼: ¾ = 24-hour starter sourdough : freshflour/water mixture for six months and bread variants with different percentages of inclusion of the corresponding sourdough were baked and evaluated periodically. This opened up possibilities for manufacturers to maintain starter sourdough themselves in industrial production for the production of rye and wheat bread.

3.4. Frozen Sourdough

Stabilized sourdoughs with Starter 6 for rye bread or Starter 8 for wheat bread were stored at -20°C for 15, 30 and 45 days, after which the frozen sourdoughs were reconstituted according to the scheme ¼ frozen sourdough: ¾ fresh flour/water mixture. Then the sourdoughs were back-slopped daily in accordance with the same proportion for 96 hours. The changes in the total titratable acidity, the concentration of viable lactic acid bacteria and propionic acid bacteria cells and the odour of the starter sourdoughs in the process of daily back-slopping were monitored. Sourdoughs stored at -20°C, were recovered successfully and could be applied in the production of rye (sourdough with Starter 6), and wheat (sourdough with Starter 8) bread.

With the reconstituted after 45 days of frozen storage at -20°C sourdoughs were prepared and baked bread variants with different percentage of reconstituted sourdough - 10%, 15% and 20%. The appearance of bacterial spoilage during storage of the baked bread variants at 37°C and at room temperature (Table 12) and mold spoilage during storage of the baked bread variants at 30°C and at room temperature (Table 13) were examined.

Table 12. Bacterial spoilage caused by *Bacillus* sp. during storage of the baked bread variants at 37°C and at room temperature (RT). DS – degree of spoilage

Baked bread variant	Temperature	24 h		48 h		72 h		96 h	
		DS	Odour	DS	Odour	DS	Odour	DS	Odour
Control	37°C	-	No	I	Yes	II	Yes	III	Yes
	RT	-	No	-	No	I	Yes	II	Yes
Sourdough with Rye Starter 6; 10%	37°C	-	No	-	No	I	Yes	II	Yes
	RT	-	No	-	No	-	No	-	No
Sourdough with Rye Starter 6; 15%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No
Sourdough with Rye Starter 6; 20%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No
Sourdough with Wheat Starter 8; 10%	37°C	-	No	-	No	I	Yes	II	Yes
	RT	-	No	-	No	-	No	-	No
Sourdough with Wheat Starter 8; 15%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No
Sourdough with Wheat Starter 8; 20%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No

Degree of Spoilage (DS): I barely noticeable (pleasant fruity odour); II weak (change in the odour - distinct); III medium (moisty, sticky crumb, sharp odour); IV strong (unpleasant odour, brown-yellow crumb)

Table 13. Mold spoilage during storage of the baked bread variants at 30°C and at room temperature (RT)

Baked bread variant	Temperature	24 h	48 h	72 h	96 h	120 h
Control	30°C	No	No	Yes	Yes	Yes
	RT	No	No	Yes	Yes	Yes
Sourdough with Rye Starter 6; 10%	30°C	No	No	No	Yes / No – single colonies	Yes
	RT	No	No	No	Yes / No – single colonies	Yes
Sourdough with Rye Starter 6; 15%	30°C	No	No	No	No	Yes
	RT	No	No	No	No	No
Sourdough with Rye Starter 6; 20%	30°C	No	No	No	No	No
	RT	No	No	No	No	No
Sourdough with Wheat Starter 8; 10%	30°C	No	No	No	Yes / No – single colonies	Yes
	RT	No	No	No	Yes / No – single colonies	Yes
Sourdough with Wheat Starter 8; 15%	30°C	No	No	No	No	Yes
	RT	No	No	No	No	No
Sourdough with Wheat Starter 8; 20%	30°C	No	No	No	No	No
	RT	No	No	No	No	No

The earliest signs of bacterial spoilage occured in the control bread – on the 48th hour at 37°C and on the 72nd hour at room temperature. Therefore, the control bread did not meet the standard requirements for microbial safety of bakery products. Upon addition of 10% sourdough the first signs of bacterial spoilage were established on the 72nd hour at 37°C. When the percentage of sourdough inclusion was increased to 15%, no signs of bacterial spoilage were observed even on the 96th hour both at 37°C and at room temperature. In the addition of 20% sourdough no apparent bacterial spoilage was detected (Table 12) (Denkova et al., 2014b).

The earliest appearance of mold spoilage was observed in the control bread – on the 72nd hour both at 30°C and at room temperature. Upon addition of 10% sourdough signs of fungal spoilage were established on the 96th hour both at 30°C and at room temperature. When the percentage of sourdough inclusion rose to 15%, mold spoilage became visible on the 120th hour in all bread variants, stored at 30°C, but in those stored at room temperature, mold spoilage was not noticeable even on the 120th hour. When adding 20% sourdough no mold spoilage was established up to the 120th hour both at 30°C and at room temperature (Table 13).

The results of these studies confirmed that the optimal concentration of inclusion of sourdough with Starter 6 for rye bread or sourdough with Starter 8 for wheat bread without negatively influencing the volume, the flavor and the aroma of the resulting bread, was 10 - 15% for the prevention of bacterial spoilage and 15 - 20% for the prevention of mold spoilage.

3.5. Freeze-Dried Starter Concentrates

Starter 6 and Starter 8 were cultured in a bioreactor with a working volume of 1.5 dm^3, agitation rate of 150 rpm at 30°C. The resulting cell suspensions were immobilized and freeze-dried in the presence of high-ester apple pectin and sodium alginate, and the resulting freeze-dried starter concentrates were stored at room temperature (20°C - 22°C) for 18 months. The changes in the concentration of viable lactic acid bacteria and propionic acid bacteria cells during storage were monitored (Figure 4, Figure 5).

A. Microbiological Status of the Freeze-Dried Starter Concentrates

The microbiological status of the freeze-dried starter concentrates was examined according to standard methods and absence of insemination with pathogenic microflora was established (Table 14). The freeze-dried starter concentrates did not contain pathogenic microflora and met the standard requirements for microbial purity of food. The absence of insemination with pathogens proved that the whole process of freeze-drying was carried out in accordance with the sanitary standards and requirements (Denkova et al., 2014a).

The hydrocolloids used for mechanical immobilization and as cryoprotectants during lyophilization had high efficiency and the survival rate of the strains in the composition of the two sourdough starters was high. The high survival rate of lactobacilli and propionic acid bacteria in the freeze-dried starter concentrates were also due to the optimal programming of the entire process - conditions of freezing, use of a suitable cryoprotectant medium, regime parameters of freeze drying, properly specified duration of the cycle, which provided low residual moisture content in the final concentrates, therefore resistance to thermal processes and extension of the shelf life of the freeze-dried starter concentrates. The technology of freeze-drying prevents the growth of pathogenic microflora. The greater the degree of dehydration is, the smaller the survival rate of pathogenic microflora is. On the other hand, the applied storage conditions of the freeze-dried starter concentrates (room temperature and relative humidity of up to 35%) were favorable for their long-term storage, in which the two freeze-dried starter concentrates retained high concentrations of viable lactic acid bacteria and propionic acid bacteria cells (Figure 4, Figure 5).

Table 14. Microbiological status of the freeze-dried starter concentrates immediately after freeze-drying

Parameter	Norm according to the Standard	Analysis
1. General number of mesophilic aerobic microorganisms, CFU/g	No more than 800	220 – 360
2. *Escherichia coli* in 0.l g of the product	Not to be found	Not found
3. Sulfite reducing clostridia in 0.l g of the product	Not to be found	Not found
4. *Salmonella* sp. in 25.0 g of the product	Not to be found	Not found
5. *Staphylococcus aureus* in 1.0 g of the product	Not to be found	Not found
6. Spores of microscopic molds, CFU/g	No more than 100	25 – 35
7. Yeasts, CFU/g	No more than 100	38 - 50

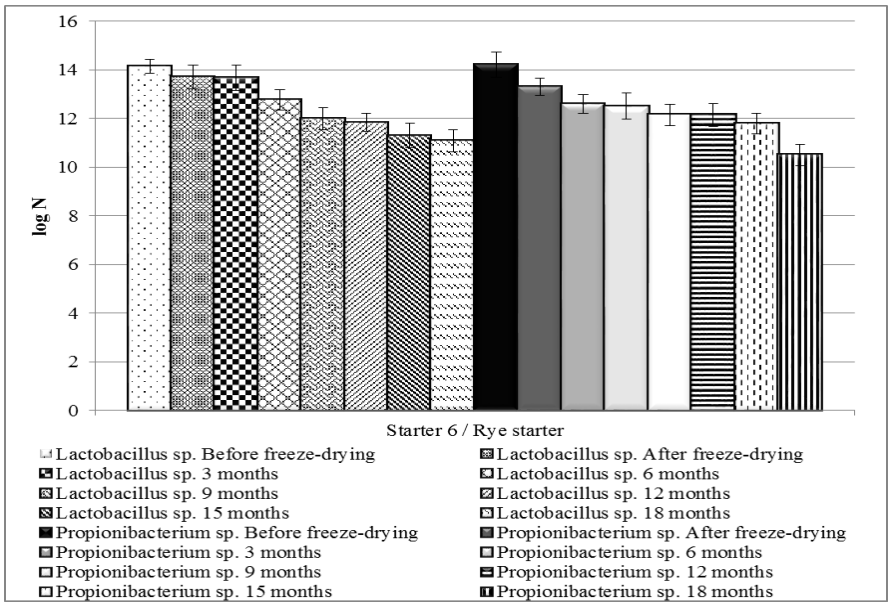

Figure 4. Survival of *Lactobacillus* sp. and *Propionibacterium* sp. in the composition of the freeze-dried starter 6 concentrate during freeze-drying and storage at 20°C - 22°C for 18 months.

In the course of 18-month storage at room temperature the content of viable cells in both starter concentrates was greater than 10^9CFU/g (Figure 4, Figure 5). The obtained results showed that the two lyophilized starter concentrates were characterized by high survival rate of beneficial microorganisms after freeze-drying - the number of lactobacilli cells before freeze-drying was over 10^{13}CFU/cm^3 - 10^{14}CFU/cm^3, and after lyophilization - over 10^{13}CFU/cm^3; the concentration of propionic acid bacteria cells before freeze-drying was over 10^{14}CFU/cm^3, and after lyophilization - over 10^{13}CFU/cm^3. These results showed the efficiency of the hydrocolloids used (the combined hydrocolloid matrix consisting of sodium alginate and high-ester apple pectin) as a carrier for the immobilization and as a cryoprotectant medium.

The absence of pathogens and the low residual moisture content of the freeze-dried starter concentrates were the key factors for their prolonged shelf life - in the 18-month period no adverse changes in the quality indicators were observed and minor changes in the survival rates of lactic acid bacteria and propionic acid bacteria occurred (Figure 4, Figure 5) (Denkova et al., 2014a).

After 8 months of storage of the freeze-dried starter concentrates at room temperature, they were reconstituted by 48-hour culturing in MRS-broth at 30°C and then were used for sourdough preparation. In a parallel experiment sourdough was prepared with direct application of the freeze-dried starter concentrates.

Both starters were fully recovered in the preparation of starter sourdough and its daily back-slopping regardless of whether they were previously reconstituted by culturing in MRS-broth at 30°C, or were incorporated directly in the preparation of the starter sourdough (Denkova et al., 2014a).

Using 96-hour starter sourdoughs (sourdough with reconstituted freeze-dried starter 6 concentrate; sourdough with reconstituted freeze-dried starter 8 concentrate) were prepared baked bread variants with different percentage of sourdough inclusion - 10%, 15% and 20%.

The baked bread variants were stored at room temperature and at 37°C for 96 hours for determination of bacterial spoilage (Table 15) and at room temperature and at 30°C for 120 hours to determine the appearance of mold spoilage (Table 16).

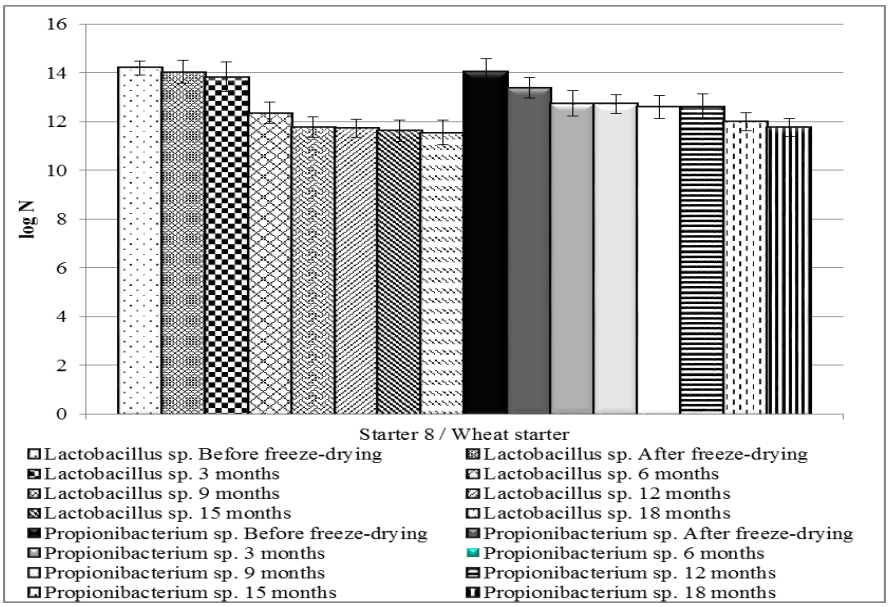

Figure 5. Survival of *Lactobacillus* sp. and *Propionibacterium* sp. in the composition of the freeze-dried starter 8 concentrate during freeze-drying and storage at 20°C - 22°C for 18 months.

Table 15. Bacterial spoilage caused by *Bacillus* sp. during storage of the baked bread variants at 37°C and at room temperature (RT). DS – degree of spoilage

Baked bread variant	Temperature	24 h		48 h		72 h		96 h	
		DS	Odour	DS	Odour	DS	Odour	DS	Odour
Control	37°C	-	No	I	Yes	II	Yes	III	Yes
	RT	-	No	-	No	I	Yes	II	Yes
Sourdough with Rye Starter 6; 10%	37°C	-	No	-	No	I	Yes	II	Yes
	RT	-	No	-	No	-	No	-	No
Sourdough with Rye Starter 6; 15%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No
Sourdough with Rye Starter 6; 20%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No
Sourdough with Wheat Starter 8; 10%	37°C	-	No	-	No	I	Yes	II	Yes
	RT	-	No	-	No	-	No	-	No
Sourdough with Wheat Starter 8; 15%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No
Sourdough with Wheat Starter 8; 20%	37°C	-	No	-	No	-	No	-	No
	RT	-	No	-	No	-	No	-	No

Degree of Spoilage (DS): I barely noticeable (pleasant fruity odour); II weak (change in the odour - distinct); III medium (moisty, sticky crumb, sharp odour); IV strong (unpleasant odour, brown-yellow crumb)

Table 16. Mold spoilage of the baked bread variants during storage at 30°C and at room temperature (RT)

Baked bread variant	Temperature	24 h	48 h	72 h	96 h	120 h
Control	30°C	No	No	Yes	Yes	Yes
	RT	No	No	Yes	Yes	Yes
Sourdough with Rye Starter 6; 10%	30°C	No	No	No	Yes/No – single colonies	Yes
	RT	No	No	No	Yes/No – single colonies	Yes
Sourdough with Rye Starter 6; 15%	30°C	No	No	No	No	Yes
	RT	No	No	No	No	No
Sourdough with Rye Starter 6; 20%	30°C	No	No	No	No	No
	RT	No	No	No	No	No
Sourdough with Wheat Starter 8; 10%	30°C	No	No	No	Yes/No – single colonies	Yes
	RT	No	No	No	Yes/No – single colonies	Yes
Sourdough with Wheat Starter 8; 15%	30°C	No	No	No	No	Yes
	RT	No	No	No	No	No
Sourdough with Wheat Starter 8; 20%	30°C	No	No	No	No	No
	RT	No	No	No	No	No

The earliest signs of bacterial spoilage occured in the control bread – on the 48th hour at 37°C and on the 72nd hour at room temperature, i.e., the control bread did not meet the standard requirements for microbial safety of bakery products. In the bread variants with the inclusion of 10% rye or wheat starter sourdough bacterial spoilage was detected on the 72nd hour only at 37°C. When the percentage of sourdough inclusion was increased to 15%, no signs of bacterial spoilage were detected on the 96th hour neither at 37°C, nor at room temperature (Table 15) (Denkova et al., 2014a).

The earliest appearance of mold spoilage was in the control bread – on the 72nd hour both at 30°C and at room temperature. When adding 10% starter sourdough signs of mold spoilage were established on the 96th hour. When the percentage of starter sourdough inclusion was increased to 15%, fungal spoilage became visible on the 120th hour in the bread variants stored at 30°C, while in those kept at room temperature, there was no visible mold spoilage even on the 120th hour. Upon addition of 20% starter sourdough no mold spoilage was observed even on the 120th hour both at 30°C and at room temperature (Table 16) (Denkova et al., 2014a).

The results of the studies with the freeze-dried starter concentrates and the sourdoughs prepared with them confirmed the findings of the research on liquid and frozen sourdough: the optimal concentration of inclusion of rye starter sourdough (sourdough with Starter 6) and wheat starter sourdough (sourdough with Starter 8) without negatively affecting the volume, the flavor and the aroma of the resulting bread was 10-15% for the prevention of bacterial spoilage and 15-20% for the prevention of fungal spoilage, while the addition of over 20% starter sourdough led to a contraction in bread volume.

B. Development of "LB-Acidifier" for Rye Bread and "LB-Acidifier" for Wheat Bread

Starter 6 for rye bread and Starter 8 for wheat bread were used for the development of the technological scheme for the industrial production of "LB-acidifiers" ("LB-acidifier" for rye bread and "LB-acidifier" for wheat bread, respectively). The biotechnological scheme included several stages that are described in detail in the "Materials and methods" section of the present chapter.

The resulting two products - "LB-acidifier" for wheat bread and "LB-acidifier" for rye bread met the standard requirements for microbiological safety of food. They had high total titratable acidity (22.6 - 47,5ºN) and no lateral microflora was established in them in the examination of their microbiological status immediately after production and after 6 months of storage at room temperature at relative humidity below 65% (Table 17).

Right after production the "LB-acidifier" for rye bread and the "LB-acidifier" for wheat bread carried significant amount of useful microflora - lactobacilli and propionic acid bacteria (10^7CFU/g), ensuring the proper course of fermentation and the preservation of bread and bakery products from bacterial and mold spoilage, along with substrates of plant origin, which have beneficial physiological effects on the human body (Table 17). The results from the examination of their microbiological status after 6 months of storage at room temperature at relative humidity below 65% showed that the "LB-acidifier" for rye bread and "LB-acidifier" for wheat bread can be stored at room temperature for 6 months, while maintaining the amount of active microflora with a slight increase in their total titratable acidity (Table 17).

Table 17. Microbial status of "LB-acidifier" for rye bread and "LB-acidifier" for wheat bread immediately after production and after 6 months of storage

Parameter	"LB-acidifier" for wheat bread		"LB-acidifier" for rye bread	
	1st day	6th month	1st day	6th month
General number of mesophilic aerobic microorganisms, CFU/g	$3,2x10^3$	$4.9x10^2$	$1,6x10^3$	$1.2x10^3$
Pathogenic microorganisms, CFU/g including *Salmonella* sp., *E.coli* and *Staphylococcus aureus*	Not found	Not found	Not found	Not found
Molds and yeasts, CFU/g	Below 10	Below 10	Below 10	Below 10
Lactic acid bacteria, CFU/g	$2,0x10^7$	$3.0x10^7$	$1,0x10^7$	$2.0x10^6$
Propionic acid bacteria, CFU/g	$1,2x10^7$	$1.0x10^7$	$3,4x10^7$	$1.3x10^7$
Total titratable acidity, °N	22,6	23.8	47,5	49.5

Together with the firm "Prosperity," Pavlikeni, Bulgaria were developed and marketed "LB-acidifier" for rye bread and "LB-acidifier" for wheat bread as well as a variety of flour mixes with included "LB-acidifier" for rye bread and "LB-acidifier" for wheat bread to allow the production of bread and bakery products with extended shelf life without the use of preservatives both in industrial production and at home. The optimal percentage of "LB-acidifier" for rye bread and "LB-acidifier" for wheat bread was determined to be 3-5%.

The acidifiers currently marketed are obtained by mixing chemically pure lactic acid and acetic acid. They lower the pH, but the fermentation that takes place is alcohol fermentation as a result of the inoculated baker's yeasts. The application of starter sourdough or "LB-

acidifier" for rye bread and "LB-acidifier" for wheat bread significantly increase bread shelf life, its nutritional value and improved bread flavor and aroma without the application of preservatives. The increase in the shelf life is due to the higher levels of acidity and higher attainable concentration of organic acids in comparison to the bread produced with baker's yeasts only. The improvement in the organoleptic characteristics is due to the presence of significant volatile and non-volatile compounds which improve bread flavor and bread aroma.

The availability of the developed sourdough starters and the consumer's awareness of their qualities will enable the enrichment of the diet of contemporary man with products with additional health benefits without preservatives, but with improved quality and extended shelf life.

CONCLUSION

Homo- and heterofermentative lactobacilli strains with amylolytic and proteolytic activity, antimicrobial activity against saprophytes, resistance to the preservatives most commonly used in bread production (potassium sorbate and calcium propionate) and ability to grow in flour/water environment accumulating high concentrations of viable cells were selected. Fourteen sourdough starters for rye and wheat bread were developed using the selected lactobacilli strains with the inclusion of a probiotic propionic acid bacteria strain. The sourdough starters were probated in industrial production. Stabilization of the sourdough in terms of aroma, texture and acidity was obtained by daily back-slopping for 96 hours which opened up possibilities for the maintenance and application of starter sourdough for a long period of time by daily back-slopping. The importation of starter sourdough could be carried out in three ways: as liquid starter sourdough (Type I sourdough), as frozen starter sourdough or as a freeze-dried starter concentrate. The addition of 7% four-strain starter sourdough provided obtaining bread with improved organoleptic characteristics.

Flour with average quality carrying spores of *B. subtilis* and molds of the genera *Mucor, Rhizopus, Aspergillus* and *Penicillium* was used in the further studies. To prevent bacterial and mold spoilage of the baked bread it was necessary to develop and select new sourdough starters and to increase the amount of imported starter sourdough. Thus, it was determined that the best rye starter was Starter 6 (including *L.paracasei* RN5, *L.plantarum* X2, *L.brevis* LBRZ7, *L.fermentum* LBRH10, *L.sanfranciscensis* R and *Propionibacterium freudenreichii* ssp. *shermanii* NBIMCC 327) and the best wheat starter was Starter 8 (including *L.paracasei* RN5, *L.plantarum* X2, *L.brevis* LBRZ7, *L.fermentum* LBRH10, *L.buchneri* LBRZ6 and *Propionibacterium freudenreichii* ssp. *shermanii* NBIMCC 327). 10-15% of the corresponding starter sourdough prevented the growth of bacterial spores and 15-20% of starter sourdough prevented fungal spoilage. Upon addition of 20% and above of starter sourdough, a decrease in bread volume was observed.

A new biotechnological scheme for the production of dried starter sourdoughs (Type III sourdoughs - "LB-acidifier" for rye bread and "LB-acidifier" for wheat bread) for the production of wheat and rye bread with increased shelf-life without the addition of preservatives was developed. The optimal percentage of "LB-acidifier" for rye bread and "LB-acidifier" for wheat bread was determined to be 3-5%. With the introduction of lactobacilli and propionic acid bacteria included in the composition of starter sourdough or

"LB-acidifier" for rye bread and "LB-acidifier" for wheat bread in the main dough would be ensured the carrying out of targeted fermentation process that results in obtaining safe food with longer shelf life.

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Publications Last 3 Years:

1. Goranov B., D. Blazheva, G. Kostov, Z. Denkova, Y. Germanova, *Lactic acid fermentation with encapsulated Lactobacillus casei ssp. rhamnosus ATCC 11979 (NBIMCC 1013) in alginate/chitosan matrices*, Bulgarian Journal of Agricultural Science, 19 (2) 2013, 101–104, IF=0.19
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Chapter 4

BIOACTIVE COMPOUNDS TO FORTIFY CHILDREN'S CHOCOLATE MILK: HEALTH IMPROVEMENTS AND FUTURE PERSPECTIVES

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1. ABSTRACT

Functional Food contains known biologically-active compounds which, in defined quantitative and qualitative amounts, provide a clinically confirmed and documented health benefit, and thus, providing an important source in the prevention, management and treatment of chronic nowadays diseases. Child nutrition is a worldwide concern. In South America, according to World Health Organization database (WHO), an average of 3% of children under 5 years old are underweight. The problem is particularly severe in countries such as Bolivia and Peru (http://www.who.int/nutrition/databases/infantfeeding/en/). The numbers are even higher in other continents such us Africa and conflict zones like Afghanistan where 30% of children under 5 years old are underweight.

This chapter deals with the design and the structural and functional characterization of bioactive compounds (BC) as functional food ingredients for an industrial application in drinkable food such as chocolate milk. The BC is better if added through natural vehicles like liposomes and more suitable if made of natural products such as soy lecithin. Why liposomes? They can contain BC (PUFAs and vitamin E) and other vitamins like folic acid (FA) or vitamin C (VC) for the fortification of chocolate milk and ions such as calcium increasing nutritional value of drinkable foods. The chocolate milk has the advantage over traditional milk to provide a pleasant taste and smell for most of consumers, especially for the children's sector.

When new functional foods are designed, the characterization, stability and sensorial evaluation is a necessary step. Characterization of liposomes includes oxidative stability, flavour and appearance by using wide variety of methodologies. Oxidative stability (thiobarbituric acid and ORAC methods) followed by characterization studies is a

necessary step to be performed in food-model systems in order to reach the market under local regulations and to cover health prevention. It was found that vehicles like liposomes showed significant oxidative stability and a protective effect over different hydrophobic and hydrophilic compounds. We will specifically touch basis on thermolabile vitamins like Folic Acid and Vitamin C.

Moreover, functional foods to enrich different levels of high-risk population (children and elderly), sensory evaluations must be performed in all final products, and they should demonstrate acceptability in general positive effects. One way is to perform sensory evaluation using commercial milk and adjoining liposomes with BC.

Currently, the application of functional foods and consumption has been gaining increasing interest among consumers and researchers because studies have been associated with a positive direct effect on human health. Indeed, the incorporation of bioactive compounds into drinkable foods can become an alternative to protect and enhance health care of two main stages in human life: children and elderly.

Therefore, the aim of this review is to describe the potential use of natural lipids as stabilizing matrices holding BC, counteracting the disadvantages of direct application and enhancing the functional properties of drinkable foods through the incorporation of BC.

Future perspectives are discussed based on the requirements to focus on studies on this subject in order to develop new functional foods applications with improved functionality and high sensory performance with enhanced shelf life and nutritional quality and with increased consumer acceptance.

2. INTRODUCTION

Bioactive substances present as natural constituents in food provide health benefits beyond the basic nutritional value of the product (Biesalski et al., 2009). They are extranutritional constituents that typically appear in small quantities in foods and they are being intensively studied to evaluate their effects on health (Kris-Etherton et al., 2002). Examples of these compounds are lignans, antioxidants, phenolic acids, phytosterols, minerals, tocotrienols, and other vitamins (Liukkonen et al., 2006).

Particularly, in the case of vitamins, they have important functions in certain metabolic processes in the human body. Vitamin E (VE) or α-tocopherol is the major fat soluble antioxidant in the body. It protects the lipids against oxidative damage (Atkinson et al., 2008), reduces the formation of hydroperoxides and delays the initial phase of the oxidative process (Ordóñez et al., 1998). This action protects cell membranes and other lipid disorders caused by severe peroxidation (Atkinson et al., 2008, Primo Yúfera, 1998). Many scientific papers have been published related to the antioxidant action of VE in animal tissues, their ability to capture and peroxide radicals, thereby delaying cell aging. There are several lines of research about peroxidation associated with cellular aging and, in this regard, the role of VE becomes important. Furthermore, several clinical studies describe all the beneficial effects of VE, alone or combined with other vitamins, in some types of tumors such as prostate, gastric and lung. All of this is based on experimental studies that place emphasis on the role of free radicals as a key factor associated with the development of cancer, and it is precisely the effectiveness of antioxidants from the diet as tocopherols that have an important role in preventing development and progression of this disease (López-Rodríguez, 2006). Also, it is related to the decrease in blood cholesterol, having a positive effect on the incidence of atherosclerosis and cardio-circulatory system (Primo Yúfera, 1998).

With respect to VE deficiency, according to Primo Yúfera (1998), some related retinopathies, haemolytic anaemias, fragility of the cell membrane, and various diseases in the newborn are found. The main manifestation of the deficiency is peripheral neuropathy, in which various alterations are present like ataxia, areflexia, abnormal photoperception, weakness or muscle hypertrophy (Lopez and Suárez, 2003).

Other antioxidant vitamins, which are hydrosoluble, are the folic acid (FA) or vitamin B9 and the vitamin C (VC) or ascorbic acid. The FA acts as a cofactor in carbon transfer reactions (formyl, hydroxymethyl, and methyl) nucleotide biosynthesis (purine bases and pyrimidine), amino acid metabolism (methionine, histidine) and metabolism neurotransmitters (serine, choline) (Bekaert et al., 2008). Deficiency of FA is related to neural tube defects, heart disease and megaloblastic anemia (López and Suárez, 2003).

During pregnancy, increased cell division is associated with the rapid growth of the fetus and placenta and the increasing number of maternal cells. These events are associated with a marked acceleration in the transfer reactions of mono-carbon units, including those required for nucleotide synthesis and cell division, which is basic for the folate increment requirements during this period, since these function as coenzymes in the transfer reactions of one-carbon in nucleotide metabolism and amino acids. Because of this increase demand of folates, pregnant women are more likely to develop folate deficiency than non-pregnant women, hence the importance of proper intake or supplement FA to maintain the concentration of this vitamin (Olivares Martinez et al., 2005). In addition, more recent studies link low folate intake with neurocognitive dysfunction. Thus, folates play an important role in the development of the central nervous system and the metabolism of some neurotransmitters; low levels of folate may also be associated with dementia and decreased cognitive function (Olivares Martinez et al., 2005).

Besides, an important discovery in recent years is that there are studies linking FA intake with a decreased risk of heart disease and cancer (Bailey et al., 2003; Malinow et al., 1998). Epidemiological studies have shown that folate supplements can significantly reduce the risk of pancreatic cancer and breast cancer (Joshi et al., 2001). In addition, there are studies linking intake of FA with a decrease in colon cancer and neurological diseases such as Alzheimer's (Younis et al., 2009). The protective effect of FA in certain cardiovascular diseases, haematological and neurological diseases and cancer has been associated with the antioxidant activity of this vitamin (Atteia et al., 2009). Furthermore, other authors have also demonstrated the existence of an antioxidant activity of this vitamin (Joshi et al., 2001).

VC is a water-soluble antioxidant vitamin, as an electron donor or reductant, involved in various reactions and in the reduction of superoxide radicals and other reactive oxidants that can cause DNA or low density lipoproteins damage (Primo Yúfera, 1998). Furthermore, it attributed the role of peroxide radical scavenger cells, which is important because these radicals degrade membranes and contribute to cellular aging (Maia et al., 2007). Other functions of the VC are inhibiting the formation of nitrosamines and intervening iron metabolism and histamine (Maia et al., 2007). It is also a cofactor for the activity of eight enzymes involved in different reactions: the hydroxylation of proline and lysine both fundamental in the chain of collagen, which explains the bleeding and capillary fragility symptoms, softening and swelling of tissues in patients deficient VC; hydroxylation of dopamine to norepinephrine; carnitine biosynthesis and oxidation of phenylalanine and tyrosine (Lopez and Suarez, 2003).

Hypovitaminosis C is manifested by symptoms of fatigue, weakness, swelling in the legs, neurosis, swollen gums and tooth loss, bleeding due to capillary fragility, skin diseases and difficulty in wound healing, among others. Severe deficiency causes scurvy and death (Primo Yúfera, 1998).

The antioxidant activity of certain compounds has been related with many biological functions, such as antimutagenic and anticarcinogenic activity of an anti-aging, among others (Holasova et al., 2002). For that reason, a type of antioxidant vitamins like vitamin C, vitamin E and folic acid can apply as bioactive compounds.

Another type of bioactives compounds (BC) are the polyunsaturated fatty acids (PUFAs). There are two families of PUFAs: the n-6 and n-3 family. Family PUFAs n-6 derived from linoleic acid, with two doubles bonds, and it is characterized by its first double bond at carbon chain number 6 (Carrero et al., 2005). The family of n-3 PUFAs derived from α-linolenic acid (with three double bonds), whose fatty acids have their first double bond in the carbon chain number 3. Different numbers and positions of the double bonds in the fatty acids chain confer different physiological properties derived from their metabolism, which makes the relationship between fatty acids n-3 and n-6 very important in children diet. Linoleic acid is metabolized to arachidonic acid and α-linolenic leads to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). They all use the same metabolic pathways and compete for the same enzymes elongases and desaturases (Carrero et al., 2005).

Importantly, the benefit of the essential contribution of fatty acids is that man needs them for normal functioning, that is why they are known as essential (Lopez and Suarez, 2003). Both the α-linoleic linolenic and fatty acids can't be synthesized by the body and therefore, they must be supplied in the diet (Carrero et al., 2005). It has been established, by several authors, that the essential fatty acids are very beneficial in preventing cardiovascular disease (Earnest et al., 2009; Leaf and Weber, 1998; Lee and Lip, 2003), schizophrenia (Sivrioglu et al., 2007) and cancer (Jenski et al., 1995), among others. Furthermore, fatty acids have vasodilators, antihypertensive, anti-inflammatory, and anti-atherothrombotic properties associated (Baguma-Nibasheka et al., 1999).

In addition, certain PUFAs such as linolenic acid (ω-3) and linoleic (ω-6) represented by 18: 2 and 18: 1, respectively, are contained in soy lecithin or phosphatidylcholine (SPC) and are also essential fatty acids (Carrero et al., 2005). SPC is a naturally occurring lipid, which has been used in the preparation of liposomes to encapsulate, protect and transport efficiently both hydrophobic and hydrophilic compounds (Fabani et al., 2002 Hiesh et al., 2002 Monroig, 2007; O'Neill and Leopold, 1982; Pérez Molina et al., 2011;. Soto-Arriaza et al., 2008).

3. LIPOSOME'S BUILDING BLOCKS IN THE GENERATION OF FUNCTIONAL FOODS

Most of the bioactive compounds: e.g., fatty acids, carotenoids, tocopherols, flavonoids, polyphenols, phytosterols, oil soluble vitamins have hydrophobic nature (Kris-Etherton et al., 2002). Besides, it is not easy to add vitamins to aqueous foods while retaining their activity. For instance, VE is easily oxidized in the air (Ordóñez et al., 1998), while FA is thermolabile and is degraded after thermal treatments like pasteurization and light exposure (Fennema,

2000). And VC is easily oxidized in the presence of oxygen and, above all, is thermolabile and it is dramatically reduced by different thermal treatment process (Abioye et al., 2007).

The addition of these vitamins to aqueous foods using liposomes appears to be a promising solution. Liposomes are microscopic spherical vesicles composed of polar lipids like phospholipids, which enclose liquid compartments within their structure (consisting of lipid bilayers) and enable the encapsulation of both hydrophilic and lipophilic materials (Keller, 2001).

With respect to VE, it was demonstrated that this vitamin mixes perfectly well with the phosphatidylcholine of the bilayer (Wang and Quinn, 2000). With respect to hydrosoluble vitamins, liposomes promote the protection and activity of vitamins like vitamin C (Marsanasco et al., 2011; Marsanasco et al., 2016). Liposomes could be made of SPC allowing the incorporation of bioactive compounds like PUFAs, VE, VC and FA in food like milk, generating a functional food.

If we consider the definition of a functional food as any food natural or processed, in addition to its nutritional components contains additional components that promote health, physical ability and mental state of a person (Alvídrez-Morales et al., 2002).

This means that these foods should necessarily contain any of the components called functional ingredients, among which the following may be mentioned as examples:

- Vitamins: organic compounds are nutritionally essential for the organism, as they regulate metabolic processes and cannot be synthesized by the body. The best known are A, C, D and B.
- Antioxidants are components that prevent the attack of free radicals into cells, e.g., vitamin A (carotenes), C and E, selenium and coenzyme Q 10.
- Minerals: calcium, iron, phosphorus, magnesium, selenium, boron, chromium, copper, nickel and zinc.
- Dietary fibers: improves nutrient absorption, promotes gastrointestinal transit and can help reduce the risk of cardiovascular disease, among others.

SPC based liposomes, containing essential fatty acids and with the addition of vitamins E, C and/or folic acid, generate a functional food. They are not given as a capsule or a pill but products are part of a normal diet regime (Moreno, 2014).

Microencapsulation is a technique used in specific areas such as biologicals, pharmaceuticals, food, cosmetic and chemicals (Keller, 2001). Through this procedure, the solid or liquid compounds can be protected and its release is controlled in desired conditions (Hsieh et al., 2002).

One type of structure used in microencapsulation technique is the liposome. Liposomes are microscopic spherical vesicles formed by lipids that contain liquid compartments in structure (Hsieh et al., 2002) and enables the encapsulation of molecules either lipo or hydrosoluble. They can be formulated with phospholipids, which are polar lipids characterized by hydrophilic and lipophilic groups in the same molecule (Keller, 2001). These spherical vesicles (liposomes) are formed when certain conditions phospholipids are hydrated and are organized in lipid bilayers (Moh'd Atrouse, 2002). In a cross section of a liposome, as shown in Figure 1, one can see the location of phospholipids aligned next to each other with the hydrophilic heads facing the aqueous compartment while lipophilic tails are located away from water to the centre of the vesicle, thereby forming a lipid bilayer (Keller, 2001). These

lipid bilayers called lamellae, then join to form the phospholipid sphere enclosing water (Moh'd Atrouse, 2002). Liposomes can have one or multiple concentric lamellae (Keller, 2001).

Because of the structure and composition of the liposomes, they can encapsulate both hydrophilic and lipophilic materials. Hydrophilic compounds are localized in the aqueous compartment while the lipophilic compounds are located in the lipid bilayer. These vesicles can be prepared with natural or synthetic phospholipids, and can also be spiked with bilayer stabilizers such as stearic acid (Hsieh et al., 2002; Keller, 2001; Marsanasco et al., 2011).

To obtain a large production scale of liposomes based on a new high-technology biotechnological discipline, the transference from the laboratory bench to an industrial level is a crucial step for liposomes. Liposomes can be obtained directly from dry SPC films giving rise to large multilamellar vesicles (LMV).

The process of formation of large multilamellar vesicles (MLVs) comprises mixing lipids in ethanol, which is then removed by evaporation. Subsequently, the dry lipid film is hydrated, while maintaining the temperature above the phase transition temperature of the lipid mixture (Keller, 2001). So that these liposomes are formed spontaneously when the dry lipid film is hydrated with water or buffered (Lasic, 1988). Normally, the size distribution is ranging from 0.1 mμ to a maximum value which can be up to 500 mμ in diameter and it contains hundreds of concentric lamellae (Keller, 2001; Lasic, 1988).

These LMV liposomes can be converted into smaller, unilamellar liposomes by several methods but, for large-scale applications, extrusion and homogenization are basically the only applicable techniques (Keller, 2001; Lasic, 1988).

In addition to a liquid suspension, lyophilized formulations with liposomes can also be delivered either in water concentrated solution or as a finely dispersed powder. The lyophilized powder can transform into liposomes upon hydration at the end of the commercial product line (Lasic, 1998).

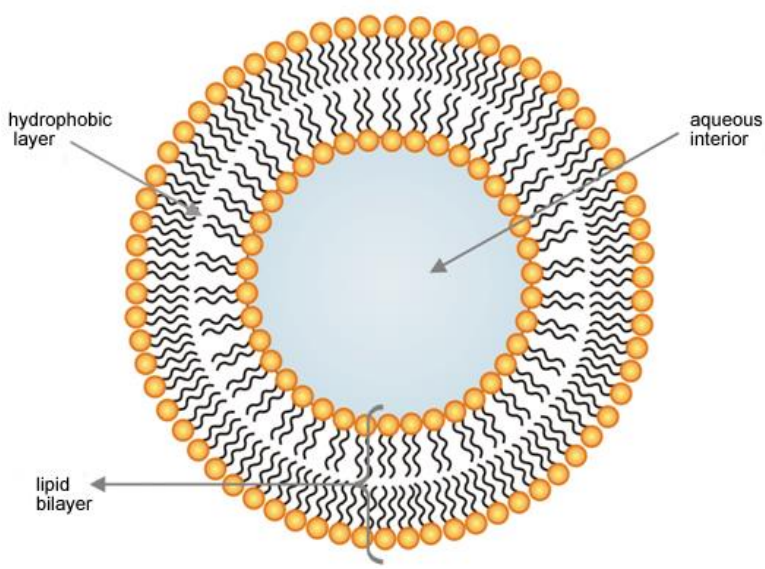

Figure 1. Schematic representation of a liposome and its main differential parts.

4. STABILIZING MATRICES FOR BIOACTIVE COMPOUNDS

In the food industry, several matrices are being applied for encapsulation or association of BC. They include liposomes, nanoemulsions, microemulsions, solid lipid nanoparticles (SLNs), and polymeric nanoparticles. The dispensability, stability and bioavailability of many bioactive compounds can be improved by encapsulation into these nanocarrier particles (Singh, 2016).

For example, micelles are submicron spherical particles, typically 5–100 nm in diameter, that are formed spontaneously upon dissolution of surfactants in water at concentrations that exceed a critical level, known as the "critical micelle concentration" (CMC). In this process, the interactions of the hydrophobic tail group of surfactants with water are minimized while interactions of the hydrophilic surfactant head groups with water are maximized. This type of system has the ability to encapsulate non-polar molecules such as lipids, flavourings, antimicrobials, antioxidants, vitamins, limonene, lycopene, lutein and omega-3 fatty acids (Chen et al., 2006). Another type of carrier are the SLNs with 50~1,000 nm in diameter that are made from lipids that solidify at room temperature to form a crystalline or amorphous under-cooled matrix in which the bioactive compound is incorporated. The particle is stabilized by a surfactant layer, which typically consists of a mixture of surfactants. To prepare SLNs, the bioactive compound is first solubilised in melted lipid, forming a 'melt', and nanoparticles are prepared from the melt in a number of different ways. The most common approach involves dispersion of the melt in a hot aqueous solution of surfactant followed by homogenization at temperatures above the melting point of the lipid phase. They have the potential to provide greater stability to labile hydrophobic compounds by trapping them in a structured solid matrix and to provide controlled release of these compounds by designing systems with different melting characteristics. SLNs have been used to deliver and protect fat-soluble vitamins (A, D, and K) (Singh, 2016).

Nanoemulsions are simply very fine oil-in-water (o/w) emulsions with mean droplet diameter of 50–200 nm which are defined as a mixture of two completely or partially immiscible liquids, such as oil and water, with one liquid being dispersed in the other in the form of droplets. Nanoemulsions and macroemulsions can be manufactured in a similar fashion using high-pressure homogenizers. Because of their small size, nanoparticles have excellent penetration properties to ensure rapid delivery of high concentrations of active ingredients. Especially in the case of lipophilic active ingredients the bioavailability can be substantially improved by delivery in nanoemulsions (Chen et al., 2006).

Biopolymeric nanoparticles consist of a matrix of biopolymers that may be linked through intermolecular attractive forces or through chemical covalent bonds to form solid particles. Because of their versatility in terms of compounds, they can be encapsulated. Polylactic acid is a component of many biodegradable nanoparticles and today, a wide variety of natural and synthetic polymers have been used to encapsulate and deliver compounds. Among these are chitosan, a natural antimicrobial and antioxidativ polymer obtained from crustacean shells and the synthetic polymers L-, D-, and D,L-polylactic acid (PLA), polyglycolic acid (PGA), and polycaprolactic acid (PCL) (Chen et al., 2006).

From all the above matrices, liposomes were preferred based on the easy preparation, scaling up and characterization. These spherical structures, with diameters ranging from 20 nm to several microns, formed through the self-assembly of amphiphilic molecules, are

usually phospholipids (Singh, 2016). Liposomes have wide application in the food industry based on that they have the advantage of encapsulating water or liposoluble compounds either separately or jointly. Applications include efficient encapsulation of proteins since the interior of the liposome provides a microenvironment that maintains protein functionality. For example, in accelerated ripening of cheese, the vesicles provide a uniform hydrophilic enzymes (Keller, 2001) distribution. As well as encapsulation of flavours, acidulants (citric acid, ascorbic acid, alkali and buffer), enzymes, antioxidants, dyes, essential oils, vitamins and minerals. In addition, such systems are used to encapsulate lactoferrin, one bacteriostactic glycoprotein as well as nisine, an antimicrobial polypeptide that increases the average shelf life of dairy products. Liposomal systems are also used to capture phosvitin (antioxidant) which inhibits lipid oxidation in a variety of dairy products and ground pork (Taylor et al., 2005).

Regarding the use of vitamins in food application, it was shown that vitamin C encapsulated in the liposomes retained 50% activity after 50 days in refrigerated storage, while non-encapsulated vitamin C lost its activity after 19 days (Taylor et al., 2005).

The nanometric version of a liposome is usually referred to as nanoliposome and has a similar structural, physical and thermodynamic properties that of a liposome. Because of the smaller size of nanoliposomes, they have a larger interfacial surface contact area with biological tissues, possibly providing greater bioavailability to encapsulated compounds. Release of the entrapped materials can be a gradual process, either resulting from diffusion through the membranes or following membrane disruption caused by changes in pH or temperature (Singh, 2016).

In addition to everything mentioned above, liposomes are one of the matrices most applied in the encapsulation of various compounds as a bioactive in the food industry in consideration of the low cost of the natural raw material used for manufacturing (lecithin) and the ease of obtaining large very quantities with easy to follow lab protocols (production). We preferred to design different natural lipids in the production of liposomes as soy phosphatidylcholine (SPC), a two-component formulation like soy phosphatidylcholine and stearic acid in 1.00:0.25 molar ratio (SPC:SA), or soy phosphatidylcholine and calcium stearate in 1.00:0.25 molar ratio (SPC:CaS) (Marsanasco et al., 2011, Marsanasco et al., 2015; Marsanasco et al., 2016).

Considering the importance that morphology has when thinking that something has to be added to food, liposomes obtained must be characterized. They usually show a great variety of sizes with spherical and/or spheroidal form. Parameters that influence the morphology are those related to the preparation method and to the composition of the lipids involved as seen in Figure 2 microscopes (Marsanasco et al., 2011; Marsanasco et al., 2015). Also, the microscope field reveals single or aggregated liposomes that can be checked before or after pasteurization. These results coincide with those obtained by other authors (De la Maza et al., 1998), where single bilayer liposomes (unilamellar vesicles) made of egg lecithin at pH 7.2 showed aggregation. Nacka et al. (2000) also showed the same result for phosphatidylcholine and phosphatidylethanolamine liposomes at various pH´s. This aggregation process is a physicochemical mechanism that takes place in certain conditions as a result of pH, thermal treatment, external charges and presence of cations mainly (Marsanasco et al., 2011; Marsanasco et al., 2015; Nacka et al., 2000).

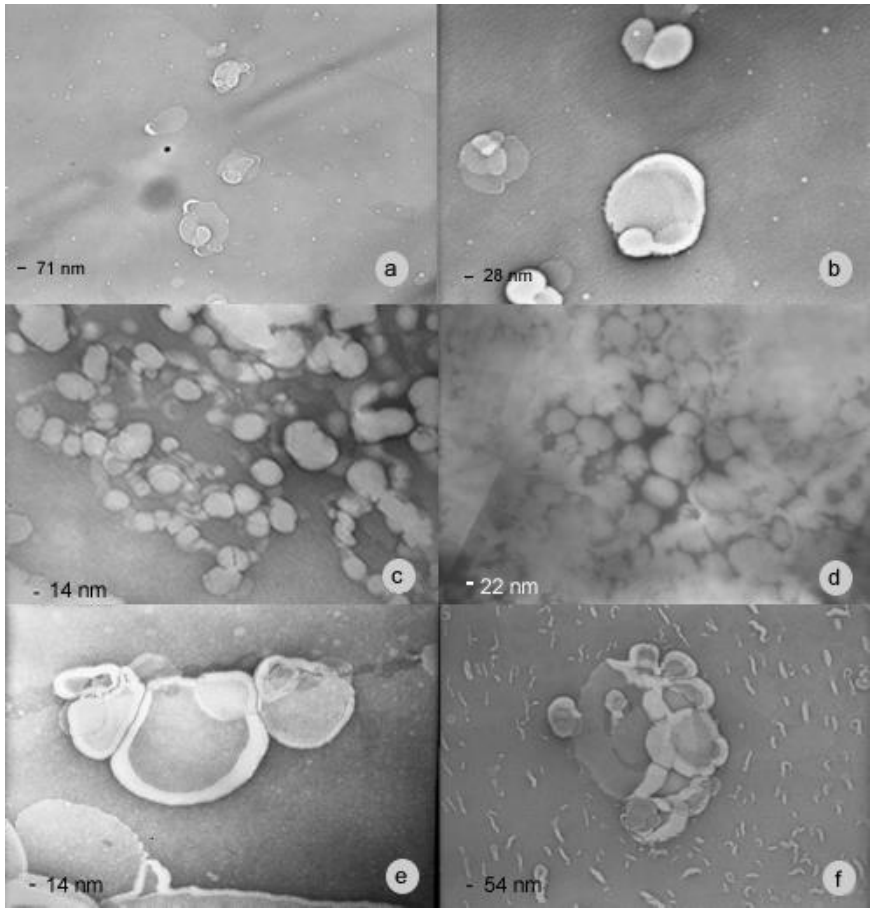

Figure 2. "Negative stained TEM of the liposomal formulations without vitamins in distilled water before and after pasteurization (PAST). Data correspond to stained TEM: a:SPC, b:SPC PAST c:SPC:SA, d:SPC:SA PAST, e:SPC:CaS, f:SPC:CaS PAST".

Furthermore, the most important point is that the systems under study do not vary with thermal treatments or within adequate shelf life, meaning morphology and aggregation conserved after pasteurization.

5. Oxidative Stability and the Effect of Vitamins

For a particular industrial application, liposomal stability is measured from the following factors:

- Oxidative degradation and chemical stability of lipids and other components.
- Stability of liposome size distribution and encapsulation efficiency through time of the active component (Keller, 2001).

Furthermore, the stability of liposomes includes various aspects such as morphology, structure, viscosity and surface charge even against a thermal treatment (Hsieh et al., 2002; Keller, 2001; Kikuchi et al., 1991; Xia and Xu, 2005).

Lipid peroxidation is a complex process that involves the formation and propagation of lipid radicals, generating three species: malondialdehyde (MDA), lipid hydroperoxide and conjugated dienes containing lipids (Allen and Hamilton, 1994). The MDA is an aldehyde formed by the breakdown of polyunsaturated fatty acids and is used as an index to determine the extent of the peroxidation reaction. This compound can be detected easily with the thiobarbituric acid (TBA) method (Lester and Fleischer, 1997). The TBA test is often used to monitor lipid peroxidation in vitro because of their sensitivity and simplicity. The variation in the production of MDA may be a reflection of the lipid composition and susceptibility to peroxidation. Furthermore, there is no possibility that the results fluctuate by decomposition of the measured product, as the MDA being a final product of the oxidation, it is not disintegrated as is the case of hydroperoxides and conjugated dienes (Lester and Fleischer, 1997).

The method of oxygen radical absorbance capacity (ORAC) is becoming a widely used method for evaluating the antioxidant capacity in biological and food samples (Prior et al., 2003). The final measure of ORAC is known as the total antioxidant capacity and is the result of the sum of the lipophilic and hydrophilic fractions reported as total equivalents to Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) per litre or per Kg depending on the product (Prior et al., 2003; Stockham et al., 2011).

Oxidative stability of liposomal formulations can be determined using TBA or ORAC methods. These methods are proposed here as the most adequate in food industry within those suggested within legal food regulations. Lipid peroxidation followed by the Tiobarbituric acid (TBA) method is described by Lester and Fleischer (1997). In the same manner, ORAC method is described by Atala and collaborators (2009).

Sotomayor and collaborators (2008) studied relationships between membrane structure and lipid oxidation. Membrane structure was analyzed with the fluorescent probe Laurdan and lipid oxidation was analyzed by the TBA method and oxygen uptake. Authors conclude that rigidification of the inner part of the surface of the bilayer leads to a lower rate of water permeation. Decreased water permeation decreases the oxidation rate.

Lúcio and co-workers (2007) used ORAC method to study peroxidation in liposome systems. Also, other authors used different probes such as diphenylhexatriene propionic acid (DPH-PA) in order to analyze membrane structure. Authors conclude that membrane lipoperoxidation is related not only to the scavenging characteristics of the antioxidants but also to their ability to interact with the lipid bilayers.

Results obtained with our formulations varied around 0.10 μM with the TBA method. Systems without vitamins for different SPC based formulations (Table 1A) revealed low TBARS values, meaning high oxidative stability. This result agrees with those published by Monroig and collaborators (2007), which showed that SPC presented a low tendency in peroxidation with the TBA method. The same result was obtained with the ORAC method (Table 1B). Data allowed inferring that the oxidative stability was enhanced by saturated fatty acids, such as stearic acid or calcium stearate that increase the stability thereof against peroxidation. According to other studies (Soto-Arriaza et al., 2008), the incorporation of saturated fatty acids in the bilayer increases rigidity causing a decrease in the mobility of alkyl chains of the bilayer and in the water flow. Considering that the reactions are favoured

by the presence of water in the bilayer, this reduced flow causes a decrease in velocity of oxidative reactions (Marsanasco et al., 2016, and references therein).

According to bibliography, certain vitamins such as VE and VC may also have a pro-oxidant or antioxidant action. This action depends on several factors, such as the induction of lipid peroxidation, the composition of the liposomes and the way of introducing these antioxidants to the liposomes. For example, in the case of the VE, the addition of ethanol to form dipalmitoyl-phosphatidylcholine liposomes generated a pro-oxidant effect in the presence of Cu^{2+}. VE in aqueous media has been attributed the pro-oxidant effect of tocopheryl radicals formed and their interaction in the aqueous zone to reduce Cu^{2+} to Cu^{+} (Schnitzer et al., 2007). Our results revealed that the presence of VE can generate a pro-oxidant effect reducing Fe^{+3} to Fe^{+2} only in systems containing SPC and calcium ions (SPC:CaS) (Marsanasco et al., 2016).

Table 1. Peroxidation assay performed to liposomal formulations after pasteurization in distilled water with TBA (A) and ORAC (B) methods

A: TBA method			
Liposomal formulation	SPC	SPC:SA	SPC:CaS
Control	0.10 ± 0.01	0.13 ± 0.01	0.10 ± 0.02
VE	0.09 ± 0.01	0.15 ± 0.05	0.17 ± 0.03*
VC	0.48 ± 0.09***	0.41 ± 0.05***	0.39 ± 0.02***
VE and VC	0.28 ± 0.04**	0.54 ± 0.04***	0.49 ± 0.02***

B: ORAC method			
Liposomal formulation	SPC	SPC:SA	SPC:CaS
Control	96.38 ± 18.80	86.15 ± 8.40	88.75 ± 11.85
VE	114.0 ± 32.76	89.10 ± 4.34	95.83 ± 13.36
VC	186.70 ± 27.85***	188.50 ± 13.79***	205.70 ± 45.85***
VE and VC	183.7 ± 35.20***	177.60 ± 13.37***	202.10 ± 34.45***

Data correspond to Soy phosphatidylcholine (SPC), Soy phosphatidylcholine and stearic acid in 1:0.25 molar relation (SPC:SA), Soy phosphatidylcholine and calcium stearate in 1.0.25 molar relation (SPC:CaS) without vitamins (control), with 5 mM of VE, 90 mM of VC and both vitamins (5 mM for VE and 90 mM for VC). Each value represents the mean ± SD of three and four independent assays for TBA and ORAC methods, respectively. A statistical comparison was made: Among the formulations without vitamins with respect to SPC (control) with the Dunnett test. The systems did not present significant differences. Between each system with vitamin/s with respect to the same system without vitamins (control) through the Dunnett test. Significant differences from the control are shown as *$p < 0.5$, **$p < 0.01$, ***$p < 0.001$

Another interesting result was that formulations with VC or VC with VE showed a significantly higher TBARS in relation to their respective controls (Table 1A). This result can be related to the pro-oxidant effect of VC, which depends on several factors, as mentioned above (Schnitzer et al., 2007). Even also in the case of the VC, other studies (Liebler et al., 1986) demonstrated that SPC liposomes suspended in neutral pH and VC added in the presence of Fe^{2+} are able to generate a pro-oxidant effect. VC can promote lipid oxidation as it can catalytically reduce metals such as Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+}, respectively (Decker

et al., 1990). This result is related to the thermal degradation of VC due to the pasteurization or as an antioxidant effect of VE, both counteracting the pro-oxidant effect of VC (Marsanasco et al., 2016).

The ORAC results showed that systems with VC or combined with VE presented a significantly higher value compared to controls (Table 1B) meaning that VC is active and exerting its antioxidant activity under these conditions. What is striking about these results is the observed activity of the VC after pasteurization. The effect of heat treatment, including the LTLT, dramatically reduced this vitamin stability (Abioye et al., 2013). Then, on the basis of these results, we inferred the existence of a protective effect of liposomal systems over this thermolabile vitamin (Marsanasco et al., 2016).

Several studies have revealed that FA has an antioxidant action that lies on the elimination of free radicals (Joshi et al., 2001) or a pro-oxidant action (Gupta et al., 2012). This action depends on several factors, such as the induction of lipid peroxidation and its concentration, the composition of the liposomes and the way of introducing these antioxidants to the liposomes and its concentration (Schnitzer et al., 2007). Also, in the case of formulations studied by us with FA (Table 2), they have the same behaviour as VC, showing significantly higher values of TBARS in comparison with controls (before and after pasteurization). The FA can promote lipid oxidation as it can reduce catalytic metals such as Fe^{3+} (using in the TBA method), regenerating Fe^{2+} that are involved in oxidation. These results are in concordance with the study made by Gupta et al. (2012) that showed a pro-oxidant action of FA with the TBA method (Marsanasco et al., 2015).

Regarding the systems with FA and VE (with or without pasteurization), only the SPC system presented a significantly higher TBARS value. Also, in the presence of VE, formulations with FA had a good oxidative stability, demonstrating the ease of application in commercial chocolate milk (Marsanasco et al., 2015).

Table 2. Peroxidation assay performed to liposomal formulations before and after pasteurization (PAST) in distilled water with TBA method

Liposomal formulation	SPC	SPC:SA	SPC:CaS
Control	0.07 ± 0.01	0.07 ± 0.03	0.09 ± 0.05
FA	0.19 ± 0.06*	0.42 ± 0.01***	0.44 ± 0.07***
VE and FA	0,31 ± 0,01***	0,05 ± 0,04	0,12 ± 0,02
Control PAST	0.10 ± 0.01	0.13 ± 0.01	0.10 ± 0.02
FA PAST	0.40 ± 0.01***	1.04 ± 0.13***	0.30 ± 0.10*
VE and FA PAST	0.24 ± 0.02***	0.20 ± 0.06	0.18 ± 0.06

Data correspond to Soy phosphatidylcholine (SPC), Soy phosphatidylcholine and stearic acid in 1:0.25 molar relation (SPC:SA), Soy phosphatidylcholine and calcium stearate in 1.0.25 molar relation (SPC:CaS) without vitamins (control), with 0.136 mM of FA and with 5 mM VE and 0.136 mM of FA. Each value represents the mean ± SD of three independent assays before and after pasteurization (PAST) in distilled water. Statistical comparison was made: Between each system with vitamin/s with respect to the same system without vitamins (control) through the Dunnett test, before and after pasteurization. Significant differences from control are shown as *$p < 0.05$, ***$p < 0.001$. Respect to SPC in systems without vitamins before and after pasteurization with the Dunnett test. No significant differences were observed

6. FLAVOURS THROUGH SENSORIAL ANALYSIS

Sensory analysis allows knowing the organoleptic properties of food evaluated through the senses. Sensory evaluation is innate in man from the starting point of when a product is tested, a judgment about it is done, whether you like or dislike, and describes and recognizes its characteristics of taste, smell, and texture, etc. (Anzaldúa-Morales, 1994).

When a food market requires so, a certain product must meet requirements for nutrition, hygiene, safety, quality and sensory aspects, to be accepted by the consumer. It is from all such properties that sensory analysis of foods is an effective tool for quality control. In such a way that sensory evaluation gives always the same global sensory characteristics and acceptability of a food (Anzaldúa-Morales, 1994). There are different sensory methods of evaluations. In general, they can be descriptive, discriminative and acceptable and preferable. Sensory evaluation gives determinant information about (consumers') consumer´s perception of edible products properties due to different ingredients and processing. Professional sensory evaluation gives more certainty in the product acceptance by consumers and for the investors. The better the sensory evaluation obtained, the higher the quality of final products.

For an industrial application and production line of a new food, it is very important to assess its acceptability and ease of production. No one would be willing to invest in the development and production on a larger scale of a food that is not acceptable for potential consumers (Marsanasco et al., 2015).

These self-swelling mixtures can be delivered in large quantities to be added directly to the final commercial product. In this way, our liposomes with BC (PUFAs, VE, VC or FA) were added to commercial chocolate milk (1/100 ratio) for a sensory evaluation. Commercial milk was used with the purpose of analyzing standardized food that always has the same physicochemical, microbiological and sensory characteristics. So, the only variation in the flavour, if it exists, would be induced by the addition of liposomes (Marsanasco et al., 2015; Marsanasco et al., 2016).

Two different tests were used to study the addition of liposomes with BC in the commercial chocolate milk. The first essay applied was the discriminative test, which was performed to compare the difference between commercial chocolate milk alone and the same sample with liposomes. As the main goal of this assay is to analyze similarities (if any) between samples, 78 evaluators were used, being the accepted number of evaluators between 50 and 100 (Marsanasco et al., 2015; Marsanasco et al., 2016).

For the triangular test, evaluators should be familiar with the test and evaluate the product. That is why 78 consumers of commercial chocolate milk (men and women over 18 years old) were selected and instructed in the test (Santa Cruz et al., 2005). In each test, two samples were the same and the third one was different (product with and without liposomes). The samples were given to each evaluator in disposable cups of 200 mL; each cup had 30 mL of product. The milk was at room temperature and the randomness of the samples was ensured throughout the test. Thus, each evaluator performed the triangular test for the three formulations, testing a total of 9 samples each. Between samples from the same triangle, evaluators were requested to drink water for neutralization and, prior to move from one triangle to the next, they were requested to eat a cracker and drink water to avoid sensory fatigue. Finally, each evaluator filled a card with their evaluation (Marsanasco et al., 2015; Meilgaard et al., 1999).

The other test employed was the overall acceptability. For effective tests, like overall acceptability, it was necessary to have a minimum of 30 untrained judges. So, the panel was composed of 40 potential consumers, men and women over 18 years old, which were habitual or potential consumers of commercial milk (Anzaldúa-Morales, 1994). The evaluators were called and were presented two pairs of samples. In the first pair, they evaluated the acceptability without knowing what they were judging. In contrast, in the second pair of samples, they evaluated knowing which the functional commercial milk was and which the commercial milk was. The samples were given to each evaluator in disposable cups of 200 mL; each cup had 30 mL of chocolate milk with or without liposomes. The milk was at room temperature and the randomness of the samples was ensured throughout the test. Hedonic rating scales associated with score was used and detailed as follows: 1) I really dislike it; 3) I dislike it; 5) I neither dislike nor like it; 7) I like it; 9) I really like it. The evaluators could use these values or intermediate ones (Meilgaard et al., 1999). Between samples, evaluators were instructed to drink water and, between pairs, they were asked to eat unsalted crackers and drink water in order to avoid sensory fatigue, allowing a span of 5 minutes. (Marsanasco et al., 2015; Marsanasco et al., 2016).

In the triangular test of the liposomes with FA and VE in the commercial chocolate milk the numbers of the correct answers in triangular test for liposomes with vitamins were 28 for SPC, 42 for SPC:SA and 35 for SPC:CaS. The correct answer means that the evaluator could find the difference between commercial chocolate milk samples with or without liposomes (Marsanasco et al., 2015).

In the case of chocolate milk with liposomes with VC and VE, the product with SPC, SPC:SA or SPC:CaS presented 39, 32 y 42 correct answers, respectively.

Applying the statistical table for the triangular test in the chocolate milk with liposomes and BC, with a significant level of 0.10 and 78 reviewers, the minimum number of correct answers from which the samples showed significant differences is 32 (Meilgaard et al., 1999). From the above, it is concluded that SPC with FA and VE did not change sensory perception in chocolate milk (Marsanasco et al., 2015). Instead, the addition of SPC:SA or SPC:CaS formulations with any combination of vitamins and SPC with VE and VC in commercial chocolate milk presented significant differences with respect to the same product without liposomes. So it was necessary to analyze if this difference was negative or positive with the test of acceptability.

Results of the acceptability test of liposomes with VE and FA in chocolate milk are shown in Table 3. In the case of commercial chocolate milk with SPC and vitamins, it did not show changes in the acceptability with respect to the commercial product without liposomes. This result was maintained with or without knowledge of what was being evaluated. This result agrees with the triangular test which showed that commercial chocolate milk with this liposomal formulation did not provide sensory changes with respect to the commercial milk without liposomes (Marsanasco et al., 2015).

In chocolate milk with SPC:SA with VE and FA, although there were significant differences presented in the triangular test, in this evaluation it was found that this difference could modify acceptability. Thus, commercial milk with this liposomal formulation had significantly lower acceptability with respect to the commercial milk without liposomes, when the panellists did not know what was being evaluated. Although it should be noted that milk with a nutritional promise seems to generate a positive effect based on the fact that when

the evaluators knew what they were judging, there were no significant differences in milk samples with or without liposomes (Marsanasco et al., 2015).

Finally, in milk with SPC:CaS and vitamins, although the triangular test showed differences, this liposomal formulation would not be affecting the acceptability because both milk samples with or without liposomes were not significantly different in trials with panellists that did not know what they were tasting. Again, as in the case of milk with SPC:SA, knowledge of the nutritional promise produced a positive effect with a score significantly higher with respect to the milk without liposomes (Marsanasco et al., 2015).

From the obtained results, it can be concluded that the addition of liposomes in milk not only adds nutritional value but also, in the case of SPC, does not alter its acceptability or differs from commercial milk. Moreover, in the case of SPC:CaS, it does not alter the acceptability but it is increased when the nutritional promise is known. Finally, the SPC:SA system, although it has differences and showed lower acceptability, knowledge of the nutritional promise generated a positive effect on the acceptability of milk. Our results demonstrated an excellent acceptability of potential consumers when knowing that they were drinking the functional milk, which meant an advantage in the sale and consumption of the product (Marsanasco et al., 2015).

Table 3. Total assay acceptability of samples of chocolate milk (CM) knowing or without knowing that contained liposomal formulations

A: Test without knowing the samples					
CM with SPC	CM	CM with SPC:SA	CM	CM with SPC:CaS	CM
7.3 ± 1.11	7.10 ± 1.68	6.18 ± 1.78	7.20 ± 1.32**	6.83 ± 1.41	7.13 ± 1.31

B: Test knowing the samples					
CM with SPC	CM	CM with SPC:SA	CM	CM with SPC:CaS	CM
7.38 ± 1.19	7.30 ± 1.29	7.15 ± 1.39	7.48 ± 1.36	7.65 ± 1.21*	7.13 ± 1.29

Data correspond to Soy phosphatidylcholine (SPC), Soy phosphatidylcholine and stearic acid in 1:0.25 molar relation (SPC:SA), Soy phosphatidylcholine and calcium stearate in 1.0.25 molar relation (SPC:CaS). Qualifications of 40 panellists in commercial chocolate milk (CM) without liposomes or with 5 mM of VE and 0.136 mM of FA for each type of liposomal formulation. Statistics were performed using the test for paired samples between each milk sample with and without liposomes. The results with significant differences are shown as *$p < 0.05$, **$p < 0.01$

In Table 4, the acceptability test results of liposomes with VC and VE in chocolate milk are shown. Commercial chocolate milks with SPC and SPC:CaS showed lower acceptability. In contrast, the chocolate milk with the SPC:SA system did not change the acceptability with respect to the commercial milk and did not change the sensory perception considering that the evaluators knew what they were judging. These results allowed inferring that the additive made of SPC:SA was the most suitable to fortify milk with vitamins E and C (Marsanasco et al., 2016).

From the results it can be inferred that in samples of fortified chocolate milk, even though when the product with the liposomes has differences, it will not produce any negative effect at the sensory level (as was the case of SPC:SA or SPC:CaS with VE and FA) the knowledge of

what was being evaluated increased acceptability. When the addition of the liposomes produces a change in the commercial product and would then possibly generate a negative sensorial effect, as much they know about the benefits of the product, it will prevail the negative characteristics of the samples and their acceptability will not increase as it happened with SPC and SPC:CaS with VE-VC in both cases. While in the case of SPC with VE and FA, they did not generate differences in the final product, a fact that remained in overall acceptability tests.

Table 4. Total assay acceptability knowing that the sample of chocolate milk (CM) contained liposomal formulations

Test knowing the samples					
CM with SPC	CM	CM with SPC:SA	CM	CM with SPC:CaS	CM
6,30 ± 1,91	7,20 ± 1,20**	7,38 ± 1,17	7,63 ± 0,782	6,00 ± 1,71	7,03 ± 1,44***

Data correspond to Soy phosphatidylcholine (SPC), Soy phosphatidylcholine and stearic acid in 1:0,25 molar relation (SPC:SA), Soy phosphatidylcholine and calcium stearate in 1.0,25 molar relation (SPC:CaS) Qualifications of 40 panellists for commercial chocolate milk (CM) with or without liposomes with 5 mM of VE and 90 mM of VC, for each type of formulation. Statistics were performed using the test for paired samples between each commercial chocolate milk sample with and without the additive. The results with significant differences are shown as **$p < 0.01$, ***$p < 0.001$

7. HEALTH IMPROVEMENT AND FUTURE PERSPECTIVES

In addition to the existing role of liposomes as a vehicle of vitamins, they offer excellent opportunities for the incorporation of functional bioactive ingredients. In fact, they are already used as such. Both fat-soluble and water-soluble functional ingredients can be applied in edibles, which are, for some ingredients, a real technological challenge. Many positive effects of the applicable functional and bioactive ingredients have been described within the chapter.

The three systems with FA showed a protective effect over this thermolabile vitamin that remained active after pasteurization. Also the liposomes with FA and VE remained stable after pasteurization, as demonstrated by oxidative stability.

With respect to the liposomal formulations with VC and VE, they maintained oxidative stability even after the heat treatment. And in the case of VC, the pro-oxidant or antioxidant activity was persistent after pasteurization. This dependence was reflected by the protection of the liposomes even for the thermolabile VC. These results allowed us to infer that all are excellent candidates to be used in aqueous pasteurized food like milk; they enabled vitamins food activity and at the same time the addition of liposoluble VE.

In the sensory evaluation, the incorporation of SPC or SPC:CaS with FA and VE did not change the acceptability of commercial milk. Also, when the potential consumer beforehand knew the addition of nutritional bioactive compounds, it generated a positive effect on acceptability for all the formulations.

In the case of commercial chocolate milk with liposomes with VE and VC, the addition of SPC:SA did not affect the sensory characteristics of the final product.

From all the above discussed, it can be concluded that in the case of liposomes with VE and FA, SPC and SPC:CaS are suitable for food industry application, incorporating BC and generating functional chocolate milk. And in the liposomal formulations with VC and VE, SPC:SA system showed the best applicability for an industrial level application in chocolate milk and presented great stability after pasteurization.

Nowadays, the development of health-promoting functional foods is promoted by the global interest, which gives rise to an opportunity to improve milk containing bioactive components.

Food consumption habits are changing continuously all around the world. Encapsulation of ingredients is a useful tool in order to fulfil these requirements. As mentioned and discussed in the present chapter, ingredients can be encapsulated into functional foods. These will satisfy the consumer's demand for more convenient, nutritious, safe to eat, natural and energy-providing foods.

The focus of this work is based on the application of liposomes, divided in two strategies: a short-term and a long-term. The short-term strategy is based on liposomal usage as transporters with established techniques and raw materials that comply with national and international legislation on nutrition, ensuring coverage of the sectors of the population with high nutritional requirements. This chapter covers one side of the characterization but the major objective covers an extended characterization study of these transporters (liposomes) with bioactive compounds involving membrane surface rigidity and charge, morphology, size distribution, rheological behaviour and encapsulation efficiency of vitamins. These studies were carried out before and after pasteurization to evaluate the thermal stability in these areas; alongside those of oxidative stability and sensory analysis detailed in this chapter, the formulations have been evaluated over a period of post pasteurization storage. In this way, it was possible to assess the stability of the liposomes with bioactive compounds in thermal-treating and storage-mimicking conditions that will take place when they are offered to the population in the form of a commercial and functional chocolate milk. This will ensure that the final product meets the nutritional requirements, safety and quality according to the institution control (National Food Institute or National Administration of Medicines, Food and Medical Technology, Argentina) and international (FDA; USA).

The long-term perspective will be the implementation of chocolate milk/liposomes in a production line, the evaluation studies and economical risk analysis made to show that it can be produced industrially. This will result in the positioning of the functional chocolate milk in a particular niche market according to the needs of consumers, which will be economically profitable to produce and bring it to market. A deep local market analysis has been covered in order to calculate the volume of production of this type of product, establishing the lay-out of the production line. These analyses are part of a comprehensive economic study that has been made under various considerations such as initial investment, total operating costs, financial budgets and economic, income statement and balance point. The analysis is culminated with the economic evaluation of investment and the determination of the economic profitability of the project using economic indicators.

So the future challenge is to produce other more complex functional products by microencapsulation in matrices containing ingredients that enhance nutritional value. Moreover, to achieve a most successful production of BC enriched food that will get to the

supermarket shelf. Alongside this, in a near future, it should be obtained an improved scale production with little human effort or additional cost on the production line and ease of accessibility, that could finally reach the low-income population at low cost.

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Chapter 5

PROBIOTICS, PREBIOTICS AND SYNBIOTICS

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ABSTRACT

Probiotics are living microorganisms which, when ingested in certain amounts, have a positive impact on human health, mainly due to their roles in improving the balance of the intestinal microflora.

On the other hand, the prebiotic are food ingredients that may also have a positive impact in the improvement of the intestinal flora. These components, which fall into the category of fibers, are not digested in the upper gastrointestinal tract, and therefore reach the colon where they stimulate the growth and/or the activity of some types of bacteria. The term synbiotic is used for products that contain both probiotics and prebiotics, thus taking advantage of both the addition of beneficial bacteria and the encouragement of the growth of resident beneficial bacteria.

The present chapter aims to review the scientific literature related to prebiotics, probiotics and synbiotics, including their identification, properties and health benefits.

Keywords: human health, nanotechnology, prebiotic, probiotic, symbiotic

1. PROBIOTICS

Probiotics are living microorganisms (mono or multiple culture), which include *Lactobacillus* species, *Bifidobacterium* species and yeasts, and when administered in

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sufficient amounts, have a positive impact on human health, besides the nutritional effects, by improving the balance of the intestinal microflora of their host (Khani et al. 2012, Panwar et al. 2013, Samah et al. 2016).

For many centuries, folklore advocated that fermented dairy products containing live active cultures were healthful, and the dietary use of live microorganisms has a long history, as stated in the Bible and the sacred books of Hinduism, which already mentioned the use of cultured dairy products. One of the oldest methods used to produce and preserve food is fermentation, carried out by microorganisms. Much before the knowledge about the existence of microorganisms, soured milks and cultured dairy products, such as kefir, koumiss, leben and dahi, were frequently used for therapeutic purposes. Recent controlled scientific investigation supports these traditional views, suggesting that probiotics are a valuable part of a healthy diet (Guiné et al. 2010).

Back in the end of the 19th Century Élie Metchnikoff in his book *The Prolongation of Life: Optimistic Studies* spoke highly about the possible health benefits of the lactic acid-bacteria (LAB) *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Metchnikoff 2004). He affirmed that consuming yogurt with live bacteria was beneficial for the human wellbeing in general and the gastrointestinal health, in particular, assuring longevity. His beliefs inspired the Japanese scientist Minoru Shirota, from the Kyoto Imperial University´s School of Medicine, to start investigating the causal relationship between bacteria and good intestinal health. Convinced that a healthy balance of intestinal bacteria held the key to man's general wellbeing, Shirota dedicated his life and work to isolating a strain of LAB which would pass into the intestines, positively contributing to the balance of gut flora. In 1935 Dr. Shrirota cultivated a bacterium named *Lactobacillus casei* strain Shrirota, which was then incorporated in a drink, originating the first yogurt-like probiotic drink, sold in 1935 in Japan (Guiné et al. 2010).

Some research suggests that certain live microorganisms may have various health benefits, including immunomodulatory and anticarcinogenic effects (Drago and Toscano 2016, Maleki et al. 2016, Shida et al. 2015). Furthermore, the emergence of some new public health risks suggests an important role for effective probiotics in the mitigation of many illnesses. For example, the ability of probiotic bacteria to support the immune system could be important most particularly to the elderly or other people with compromised immune function (Wachholz et al. 2016)**.**

Many research studies have focused on the development of target-specific probiotics containing well-characterized bacteria, selected due to their health-enhancing characteristics. These new probiotics are commercialized in the form of nutritional supplements and functional foods (Illanes and Guerrero 2016). Recently, there has been an interest in the application of probiotics and prebiotics as bioactive compounds in food nanotechnology, in which the nanocarrier is produced from milk proteins (Oluk and Karaca 2016).

Human beings, like all animals, are hosts of a wide variety of microbes in a large scale, so that it has been suggested that there are even more microorganisms associated with the human body (about 10^{14} bacterial cells) than human cells (about 10^{13}). Besides, it has been estimated that more than 400 different species or types of bacteria colonize the human body (Macfarlane et al. 2005, Todar 2002, X. Wang et al. 2003). Taking this into consideration, it is not surprising that microorganisms have been found to play an important role in human health. Most of these bacteria are beneficial, contributing positively to the normal growth and development of humans. However, some of these bacteria can have negative influences, being

therefore important to maintain a balance that favours the beneficial bacteria over the potentially harmful ones (Guiné et al. 2010).

1.1. Role of Probiotics in the Gastrointestinal Functions

The digestive process begins as soon as food enters the mouth, while it is chewed so as to reduce the food to small pieces, promoting the action of the digestive enzymes, including those from saliva, and facilitating the movement throughout the gastrointestinal system. In the stomach, food is mixed with gastric juices, containing digestive enzymes and hydrochloric acid, producing the chyme. This goes to the small intestine, where more enzymes and bile are joined, to complete the breakdown of dietary proteins, fats and sugars. Most nutrients are absorbed in the small intestine and what is left of the food passes into the large intestine, or colon, where water and electrolytes are absorbed and faecal matter is stored until it passes out through the rectum (Guiné et al. 2010).

Probiotics are live microorganisms sensitive to heat, moisture, oxygen and acidic environments, but their sensitivities to surrounding environments are variable according to the strain. The microbes present in the gastrointestinal tract may have a positive, negative or neutral effect. Probiotic microorganisms must be maintained alive until they reach the site of action in the body to exert their beneficial effect on the host. Probiotic products need to contain more than 10 million colony forming units (CFU) per gram to effectively provide a claimed health benefit. Therefore, it is crucial to maintain cell viability and probiotic functions in the final product (Ying et al. 2016). In the stomach or upper small intestine the concentrations of microbes are not very high due to unfavourable conditions. However, towards the lower small intestine, they begin to attain higher populations and in the colon the bacterial concentration ranges from 10^{11} to 10^{12} colony-forming units (CFU) per milliliter (Goldin 1996).

The gastrointestinal tract (GIT) represents a complex ecosystem, with a delicate balance between the intestinal microflora and the host. The microflora are mostly comprised of obligate anaerobes (≈ 95%), including *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Fusobacterium*, *Peptococcus*, *Peptostreptococcus* and *Bacteroides*, and facultative anaerobes (1-10%), including *Lactobacillus*, *Escherichia coli*, *Klebsiella*, *Streptococcus*, *Staphylococcus* and *Bacillus*. Aerobic organisms are not present in the intestinal tract of healthy individuals with the exception of *Pseudomonas*, which is present in very small amounts (Goldin 1996).

In the large intestine, microbes will complete the digestion process of any food components that were not digested in the small intestine. This happens for example to lactose in lactose intolerant people and some fibres which resist the enzymes in the small intestine. The intestinal microflora has important roles on the maturation of the immune system, the development of normal intestinal morphology and the maintenance of a chronic and immunological balanced inflammatory response. On the other hand, the microflora reinforces the barrier function of the intestinal mucosa, thus preventing the attachment of pathogenic microorganisms and the entrance of allergens. Besides, some microorganisms may contribute to the body's requirements for certain vitamins, such as biotin, pantothenic acid and vitamin B_{12}, by stimulating the production of vitamins and fatty acids. Hence, alteration of the microbial flora of the intestine, such as those associated with antibiotic use, disease and

aging, can negatively affect its beneficial effects (Mattia Pia Arena et al. 2016, Guiné et al. 2010).

The probiotics, sometimes also called colonic foods, are commercialized in functional foods or as nutritional supplements. Most of the presently available probiotics are bacteria, like for example *L. acidophilus* or *Bacillus cereus*; however, *Saccharomyces boulardii* is an example of a probiotic live yeast and another is *Saccharomyces cerevisiae* (Guiné et al. 2010, Munir et al. 2016).

Probiotic bacteria are typically, but not exclusively, chosen from the safe bacteria that normally inhabit the gastrointestinal system of humans (Rutella et al. 2016). These bacteria are purified, grown to large numbers, concentrated to high doses and preserved. The three ways of providing them are: 1) as a culture concentrate added to a food (usually a dairy product) at medium levels, with little or no opportunity for culture growth; 2) inoculated into a milk-based food (or dietary supplement) and allowed to grow to achieve high levels in a fermented food, or 3) as concentrated and dried cells packaged as dietary supplements such as powders, capsules, or tablets (Guiné et al. 2010).

Probiotic bacteria have since early times been associated with dairy products, because these can provide a desirable delivery vehicle owing to many factors: a) dairy foods can protect probiotic bacteria by buffering the stomach acid and increasing the chances of survival until reaching the intestine; b) refrigerated storage of dairy products helps promote probiotic stability; c) live cultures in dairy foods carry a positive image; and d) the healthful properties of probiotic bacteria blend with the healthful properties of milk products (Guiné et al. 2010). Undoubtedly that one of the most popular fermented milk products for the delivery of probiotics cultures is yogurt (Rutella et al. 2016). Yogurt starter cultures may be considered probiotic since they may help to lessen the symptoms of lactose intolerance thanks to the release of lactose-hydrolyzing enzymes. However, yogurt starter cultures are not bile-resistant or acid-tolerant and thus cannot survive under the gastrointestinal tract (GIT) conditions. According to Saxelin et al. (2010) yogurts were as effective as capsules for the administration of probiotic bacteria, emphasizing the importance of such matrices as functional foods.

1.2. Examples of Probiotics

Probiotic cultures include: *Bifidobacterium*, *Lactobacillus*, *Lactococcus*, *Saccharomyces*, *Streptococcus thermophilus* and *Enterococcus*.

Bifidobacteria are anaerobic microorganisms, normal inhabitants of the human and animal colon, whose population in the colon is relatively stable until advanced age, and only then starts to decline. Notably, bifidobacteria comprise up to 25% of the cultivable gut microflora, with *Bifidobacterium longum* and *Bifidobacterium adolescentis* predominating in adults (Arboleya et al. 2012, Martínez-López et al. 2016). The population of bifidobacteria is influenced by numerous factors, including diet, antibiotics and stress. Bifidobacteria are gram-positive anaerobes, non-motile, non-spore forming and catalase-negative. They are saccharolytic organisms that produce acetic and lactic acids without generation of CO_2, except during degradation of gluconate. They are also classified as non-spore-forming, gram-positive, lactic acid producing bacteria (Vlasova et al. 2016). Bifidobacteria are important producers of short chain fatty acids (SCFA) (Tojo et al. 2014). To date, 30 species of

bifidobacteria have been isolated. Bifidobacteria used as probiotics include *Bifidobacterium adolescentis*, *Bifidobacterium bifidum*, *Bifidobacterium animalis*, *Bifidobacterium thermophilum*, *Bifidobacterium breve*, *Bifidobacterium longum*, *Bifidobacterium infantis* and *Bifidobacterium lactis* (Guiné et al. 2010).

Lactobacilli are a part of the autochthonous microbiota of the human intestine and vagina. Lactobacilli are gram-positive facultative anaerobes, non-spore forming and non-flagellated rod or coccobacilli. They are either aerotolerant or anaerobic and strictly fermentative. In the homofermentative case, glucose is fermented predominantly to lactic acid, and therefore Lactobacilli are also classified as lactic acid bacteria (LAB). To date, 56 species of the genus Lactobacillus have been identified. Lactobacilli used as probiotics include *Lactobacillus acidophilus*, *Lactobacillus brevis*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus cellobiosus*, *Lactobacillus crispatus*, *Lactobacillus curvatus*, *Lactobacillus fermentum*, *Lactobacillus GG* (*Lactobacillus rhamnosus* or *Lactobacillus casei* subspecies *rhamnosus*), *Lactobacillus gasseri*, *Lactobacillus johnsonii*, *Lactobacillus plantarum* and *Lactobacillus salivarus.* Lactobacilli are the most commonly used probiotic organisms (Guiné et al. 2010, Guo et al. 2015).

Lactococci are gram-positive facultative anaerobes, also classified as LAB (Ma et al. 2011). They are ubiquitous in foods and they are widely present in dairy products because of their technological properties (Tabanelli et al. 2014). Lactococci can have an important role as protective cultures in food preservation, since they can exert important antimicrobial actions by synthesizing a variety of bacteriocins, such as nisins, lacticins and lactococcins (Ghanbari et al. 2013). *Lactococcus lactis* (formerly known as *Streptococcus lactis*) is found in dairy products and is commonly responsible for the souring of milk.

Saccharomyces belong to the yeast family. The principal probiotic yeast is a non-pathogenic yeast, *Saccharomyces boulardii*, and has been used to treat diarrhoea associated with antibiotic use (Szajewska and Kołodziej 2015, Tan et al. 2015). In addition, it has beneficial health effects like all other probiotic bacteria strains, that include the production of chelate degradation enzymes like phytase and short chain fatty acids, degradation of pathogenic toxins and support to the immune system (Arslan et al. 2015).

Streptococcus thermophilus is a gram-positive facultative anaerobe. It is a cytochrome-, oxidase- and catalase-negative organism that is non-motile, non-spore forming and homofermentative, with restricted natural habitats in the bovine mammary mucosa and raw milk (Pega et al. 2016). *Streptococcus thermophilus* is an alpha-hemolytic species of the viridans group. It is also classified as a LAB. *Steptococcus thermophilus* is one of the most important dairy starter species, being used in defined starter cultures for the production of a variety of cheeses and fermented milks, including yoghurt (Guiné et al. 2010, Ricciardi et al. 2016).

Enterococci are gram-positive, facultative anaerobic cocci of the *Streptococcaceae* family, and are catalase-negative, non-spore forming and usually nonmotile. Enterococci are ubiquitous in a variety of foods and dairy products, water surfaces and plants, and are a natural part of the intestinal microflora of humans and animals. Also classified as lactic acid bacteria (LAB), enterococci, as a prebiotic, have been used in the management of diarrheal illnesses besides other beneficial effects, like for example, restoration of microbiota balance of GIT with antibiotic-induced dysbiosis, antiviral activity, antitumor protective effect, ability to lower serum cholesterol level, and immune regulation (Guiné et al. 2010, Zhang et al. 2016).

2. PREBIOTICS

Originally, a prebiotic has been defined as 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 improving host health (Valcheva and Dieleman 2016). The term *prebiotic* was introduced by Gibson and Roberfroid in 1995 who exchanged "pro" for "pre," which means "before" or "for." It is, therefore, applied to food ingredients that may have a positive impact in the host by the improvement of the intestinal flora. Based on this description, only few compounds of the carbohydrate group could be considered as prebiotics, most notably short- and long chain β-fructans [fructo-oligosaccharides (FOS), and inulin], galacto-oligosaccharides (GOS) and lactulose. However, more recently a new definition was proposed for "dietary prebiotics" as being "selectively fermented ingredients that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson et al. 2010). According to this definition, prebiotics may exert benefits not only in the colon, but in other parts of the human body, like for example in the oral cavity, the urogenital tract or on the skin (Valcheva and Dieleman 2016).

Prebiotics are nondigestible, i.e., they must escape digestion in the upper gastrointestinal tract, reaching the colon where they stimulate the growth and/or the activity of a limited number beneficial of bacteria comprising the colonic microflora (Roberfroid et al. 2010, Rodrigues et al. 2016). This description is somehow similar to the definition of dietary fibre, but differs in the way that prebiotics show a selectivity for certain species, like bifidobacteria, which may be promoted by the ingestion of certain substances (Guiné et al. 2010).

Naturally occurring prebiotic carbohydrates include dietary fibre (DF) and sugar alcohols. DF is comprised of non-digestible starch polysaccharides including resistant starch (RS) and non-starch polysaccharides, while sugar alcohols such as sorbitol or mannitol are derived from simple sugars (Roberfroid 2007).

2.1. Role of Prebiotics in Enhancing Human Wellbeing

The fermentation process that is associated with prebiotic molecules results in various beneficial effects, so that prebiotic-rich diets promote the development of intestinal microbial diversity, stimulate the immune system, enhance mineral micronutrient absorption, increase the cellularity and number of crypts that result from the production of short-chain fatty acids, decrease the risk of developing colon cancer, reduce excessive triglyceride and cholesterol levels, modulate hyperglycemia (excessive blood glucose), and improve insulin sensitivity (Nishimura et al. 2015, Thavarajah et al. 2016). Besides, the positive effects of prebiotics include antimicrobial, anticarcinogenic, hypolipidemic, glucose-modulatory and anti-osteoporotic activities. They may be used for the treatment of constipation, hepatic encephalopathy and inflammatory bowel disease. They can protect against some intestinal pathogens and may exert favourable lipid effects as well as have some benefit in diabetes mellitus. Moreover, prebiotics also have a very important role in improving mineral absorption and balance, for instance, they may enhance the colonic absorption of calcium and magnesium in foods (Guiné et al. 2010).

The prebiotic soluble fibres are increasingly used as dietary supplements in different food products and infant formulae owing to scientific evidences that support their several positive health actions and immunomodulatory effects (Valcheva and Dieleman 2016).

2.2. Examples of Prebiotics

Fructans are a group of non-digestible carbohydrates that have a linear structure comprising (2 → 1)-linked β-d-fructofuranosyl units, mostly ending with a glucose residue. These molecules are a mixture of oligomer and polymer chains with a variable number of fructose molecules. Short-chain fructans (with 3 to 8 polymeric units) are called fructo-oligosaccharides (FOS) while inulin corresponds to fructan molecules with DP > 10 (Lopes et al. 2016). The β-configuration of anomeric carbon C2 in these carbohydrate molecules makes them non-digestible in the upper portion of the human intestinal tract, but they can be fermented in the colon by some beneficial colonic bacteria that selectively influence the growth and/or activity of microbiota (Morris and Morris 2012).

Prebiotics are mostly oligosaccharides, which stimulate selectively the growth of bifidobacteria, being therefore referred to as bifidogenic factors.

Prebiotic fibres like inulin, fructooligosaccharides (FOS) or galactooligosaccharides (GOS) have been used to enrich many different types of commonly consumed foods. All these prebiotic fibres have been evaluated by the Food and Drug Administration (FDA) and were considered as safe for human consumption (Brownawell et al. 2012). Hence, they can be used without restrictions in foods like yogurts, drinks, energy bars, cookies, beverages, cereals and bakery products. Therefore, these polysaccharides can be considered as functional ingredients, fulfilling the features of functional food. These functional ingredients can be used in the food industry both for their benefits to health and well-being (prebiotic activity) and also for their nutritional and technological effect in the food matrix, as they enhance the organoleptic characteristics, they may increase the creaminess of the final product and other rheological properties, and they contribute to caloric content reduction when replacing fat (Ishwarya and Prabhasankar 2014, Karimi et al. 2015).

Prebiotic fibres that have been included as ingredients in diverse food products include mostly inulin and FOS, and at a lesser extent GOS, lactulose, lactosucrose, xylooligosaccharides, isomaltooligosaccharides and transgalactooligosaccharides (Fernández et al. 2016, Sarao and Arora 2015).

Beta-glucans are non-digestible polysaccharides derived from different food sources and have proven different health promoting effects, also showing potential as a novel source of prebiotics (Lam and Cheung 2013). Other non-digestible oligosaccharides and sugar alcohols such as mannitol, maltodextrin, raffinose, and sorbitol have also demonstrated prebiotic properties and health benefits (Vamanu and Vamanu 2010, Yeo and Liong 2010).

2.2.1. Beta-Glucans

Beta-glucans, which are glucose polymers produced by plants, bacteria and fungi, have shown health-promoting properties and are attracting particular attention as potential prebiotics (Mattia Pia Arena et al. 2016). Preclinical investigations (*in vitro* and *in vivo* essays) and clinical trials have established that the dietary consumption of β-glucans provides

antitumor properties, immunostimulatory effects and blood cholesterol and blood glucose lowering capacity (Mattia Pia Arena et al. 2016, Lam and Cheung 2013, Mirjana et al. 2013).

Owing to the presence of β-linked sugar units, β-glucans are non-digestible to humans. However, numerous bacteria possess the enzymes needed to break the bonds and these long chain carbohydrates, which reach the colon undigested, may provide fermentable substrates to resident microbiota of the colon (Barsanti et al. 2011).

β-Glucans, especially those from fungi, have been ascribed various immune modulating activities (Volman et al. 2008). On the other hand, cereal β-glucans demonstrated to influence cytokine secretion, expression of inflammation-related genes, phagocytic activity and complement activation (Chanput et al. 2012, Rieder and Samuelsen 2012). Oat and barley β-glucans were reported to enhance some performances in probiotic lactobacilli, including growth rate, recovery from *in vitro* simulated digestion and adherence to enterocyte-like cells (Arena et al. 2014, Kedia et al. 2008).

According to FDA oat health claim, products containing a minimum of 0.75 g β-glucan/serving size may be characterized as functional, with the effective daily intake for cholesterol-lowering effect being estimated at 3 g β-glucan/day (Mitsou et al. 2010).

2.2.2. Fructo-Oligosaccharides

Fructo-oligosaccharides (FOS) (also called neosugar and short-chain FOS) are molecules comprised of D-fructose and D-glucose, containing from three to five monosaccharide units (Bouhnik et al. 2006). They are soluble naturally sweet compounds (35% sweetness compared with sugar) due to the short chain length and the high content of fructose (Fernández et al. 2016). FOS are valued as functional food ingredients due to their prebiotic properties, i.e., their ability to stimulate the growth and activity of lactobacilli and bifidobacteria in the digestive tract (Spohner and Czermak 2016). They are not digested in the upper gastrointestinal tract and act in the large intestine stimulating the growth of *Bifidobacterium* species (Sharma et al. 2016). FOS show a low glycaemic index because they are not hydrolysed by pancreatic enzymes or gastric juices, and therefore they are used to produce low caloric food, as well as to balance the sweetness and mask the residual flavour of the high-intensity sweeteners used in food preparations (Fernández et al. 2016).

FOS may have anticarcinogenic, antimicrobial, hypolipidemic and hypoglycemic actions. They may also help improve mineral absorption and balance, and may have anti-osteoporotic and anti-osteopenic activities. They can be used to treat breast cancer and reduce colon cancer risk, and its effects are favourable in diabetes mellitus. FOS also present additional beneficial effects, which include reduction of constipation and decrease of the levels of serum cholesterol (van den Heuvel et al. 2000, Sabater-Molina et al. 2009, Da Wang et al. 2016).

2.2.3. Galacto-Oligosaccharides

Galacto-oligosaccharides (GOS) are composed of a variable number of galactose units linked to a terminal glucose, with a number of galactose units usually varying from 2 to 5. The galactose units are linked to each other by β-(1–3), β-(1–6) and β-(1–4) bonds, while galactose-glucose linkage is in most cases of the type β-(1–4). The prebiotic effect of GOS is mostly associated to the trisaccharides (GOS-3), particularly 4'- and 6'-galactosyl-lactose, and to the tetrasaccharides (GOS-4), specially 6'-digalactosyl-lactose (Aburto et al. 2016).

GOS are powerful prebiotics which stand out for their ability to replicate the bifidogenic effect of human milk. They are particularly attractive for being derived from surplus

industrial by-products (whey or whey permeate) and having prominent properties as human milk oligosaccharide replacers (Córdova et al. 2016, Venema 2012).

Food supplemented with GOS are known to improve gut health and cancer preventive effects by supporting the maintenance of the gut homeostasis, modulating the intestinal microbiome, protecting the intestinal barrier integrity and stimulating gut associated immunity (Bruno-Barcena and Azcarate-Peril 2015, Jeurink et al. 2013, Varasteh et al. 2015).

2.2.4. Human Milk Oligosaccharides

Human milk contains, apart from lactose (70 g/L) and galacto-oligosaccharides (5 g/L), other carbohydrates called human milk oligosaccharides (HMO) (10 g/L). HMO are a diverse family of more than 200 types of combinations of 5 monosaccharides: glucose, galactose, fucose, N-acetylglucosamine, and N-acetylneuraminic acid. They are polysaccharides not digested by human enzymes, and include mainly 2′-fucosyl-lactose, lacto-N-tetraose, and lacto-N-neotetraose. HMO are responsible for the large numbers of *Bifidobacterium* present in the faeces of infants (Fernández et al. 2016, Ruiz-Moyano et al. 2013).

HMO have been associated with many beneficial effects. They are antiadhesive antimicrobials that serve as soluble decoy receptors, prevent pathogen attachment to infant mucosal surfaces and lower the risk for viral, bacterial and protozoan parasite infections. In addition, HMO may modulate epithelial and immune cell responses, reduce excessive mucosal leukocyte infiltration and activation, lower the risk for necrotizing enterocolitis and provide the infant with sialic acid as a potentially essential nutrient for brain development and cognition (Bode 2012, Kulinich and Liu 2016, Wickramasinghe et al. 2015).

2.2.5. Inulin

Inulin corresponds to a group of naturally-occurring oligosaccharides containing fructose, and belongs to the class of carbohydrates known as fructans. Inulin is colourless, odourless and slightly sweet (10% sweetness compared with sugar), combining well with other ingredients without modifying delicate flavours. It is moderately soluble in water and provides a low viscosity. Besides, one of its many advantages is that it may be successfully used as a fat replacer. When fat is removed from certain foods (like cheese or chocolate), some rheological, sensorial and textural problems can occur such as a rubbery texture, lack of taste, bitterness or an undesirable colour, and these problems can be solved by using a fat replacer like inulin. This is why inulin is used to produce foods with low fat content such as table spreads, butter-like products, dairy spreads, cream cheeses and processed cheeses. When inulin is added to yogurts and dairy drinks, it improves smoothness and creaminess (Ishwarya and Prabhasankar 2014, Karimi et al. 2015, Sangwan et al. 2011).

Inulin is only slightly digested in the small intestine and stimulates the growth of *Bifidobacterium* species in the large intestine. Inulin shifts the metabolic activity of the human colonic microbiota towards the production of less potentially toxic metabolites (van Nuenen et al. 2003). Inulin may have antitumor, antimicrobial, hypolipidemic and hypoglycemic actions, and may also help to improve mineral absorption and balance as well as have antiosteoporotic activity (Kaur and Gupta 2002).

2.2.6. Isomalto-Oligosaccharides

Isomalto-oligosaccharides (IMO) (also known as branched-oligosaccharides) comprise a mixture of oligosaccharides containing both α-1,4 and α-1,6 glucosidic linkages, which act to stimulate the growth of *Bifidobacterium* and *Lactobacillus* species in the large intestine (Nakakuki 2005). IMO such as isomaltose, panose, isomaltotriose, isomaltotetraose, isomaltopentaose, nigerose and kojibiose, naturally exist in fermented foods, and they constitute one of the emerging classes of prebiotics (Bharti et al. 2015, Ojha et al. 2015, Yen et al. 2011).

They prevent tooth decay when used together with sucrose. Its consumption improves bowel movement, stool output and microbial fermentation in the colon without adverse effects and may be used in relieving constipation in the elderly population (Yen et al. 2011). Besides, IMO have also proven beneficial for the management of diabetes (Bharti et al. 2015).

2.2.7. Lactilol

Lactilol is a disaccharide like lactulose, which can be used either to treat constipation and hepatic encephalopathy (Mas et al. 2003) or as a food sweetener. It is resistant to digestion in the upper gastrointestinal tract and, because it is fermented by a limited number of colonic bacteria, results in an increase in the biomass of *Bifidobacterium* and *Lactobacillus* in the colon (Guiné et al. 2010).

2.2.8. Lactosucrose

Lactosucrose (also known as 4G-β-D-galactosylsucrose or lactosylfructoside) is a trisaccharide comprised of D-galactose, D-glucose and D-fructose. It is produced enzymatically and is resistant to digestion in the stomach and small intestine. It is selectively utilized by intestinal *Bifidobacterium* species, resulting in significant induction of growth of these bacteria in the colon, and helps to maintain the balance of the intestinal environment. It has also been reported that lactosucrose digestion is useful for improving the clinical symptoms in patients with inflammatory bowel disease (Silvério et al. 2015, Xiaoli Zhou et al. 2013, Yan Zhou et al. 2015).

Various physiological benefits have been associated with the consumption of lactosucrose, resulting essentially from its resistance to digestion and ability to be fermented by intestinal microbiota (Silvério et al. 2015). Lactosucrose presents a higher laxative threshold when compared to other lactose-based prebiotics, such as lactulose and GOS, which represents an important advantage since diarrhoea is often referred as a side effect of prebiotic intake (Vrese and Marteau 2007). Besides, microbial fermentation of lactosucrose generates interesting end products such as short-chain fatty acids (SCFAs), which have recognized beneficial effect to the host by reducing the pH and enhancing mineral bioavailability (Roberfroid et al. 2010). Other beneficial effects of lactosucrose include prevention of obesity, improvement of the immune response, prevention of allergic diseases and prevention of abdominal symptoms of lactose intolerance (Chu et al. 2013, Mizote et al. 2009, Silvério et al. 2015).

2.2.9. Lactulose

Lactulose is a semisynthetic disaccharide constituted by the sugars D-lactose and D-fructose joined by a beta-glycosidic linkage. It is industrially obtained usually by isomerization of the lactose present in cheese whey permeate (Felicio et al. 2016). Since it is not absorbed in the small intestine, it has the potential to function as a prebiotic. It is resistant to hydrolysis by human digestive enzymes and, since it is fermented by a limited number of colonic bacteria, it favours the growth of *Bifidobacterium* and *Lactobacillus* species (Chae et al. 2015, Oliveira et al. 2011).

Lactulose is widely used in pharmaceutical industry, due to its effectiveness as a drug against diseases like acute and chronic constipation, besides the promising applications reported also in the domains of the nutraceutical and food industries (Oliveira et al. 2011). Moreover, it can be used for the treatment of constipation, intestinal infection and hepatic encephalopathy (Fritz et al. 2005).

Tests have shown that prebiotics were able to protect mammalian cells (HT-29 cells) against cytotoxicity and reduce the genotoxicity of known mutagens in bacterial mutation assays, thus exerting a protective effect against colon adenocarcinoma (Adebola et al. 2013).

2.2.10. Mannitol

Mannitol is a sugar used as an osmotic diuretic. It works by increasing the amount of fluid excreted by the kidneys and helps the body to decrease pressure in the brain and eyes. Medically it is used to treat increased intracranial pressure, constituting an osmotherapy exerting its cerebral effects via two mechanisms, an immediate effect because of plasma expansion and slightly delayed effect through its osmotic action (Kassim and Esmat 2016).

Mannitol has traditionally been administered before partial nephrectomy to reduce ischemic renal damage as an intravascular volume expander with antioxidant properties, as well as being an osmotic diuretic. It reduces oxidant-derived injury in kidneys, heart, and lungs. Besides, it also showed a protective effect against ischemia–reperfusion injury in testicular torsion (Kurt et al. 2016).

2.2.11. Pyrodextrins

Pyrodextrins comprise a mixture of glucose-containing oligosaccharides that is derived from the hydrolysis of starch. They are resistant to digestion in the upper gastrointestinal tract and promote the proliferation of Bifidobacterium species in the large intestine. They are used as nutritional supplements (Guiné et al. 2010).

2.2.12. Raffinose Family Oligosaccharides

Legumes are a good source of oligosaccharides, known as α-galactosides or raffinose family oligosaccharides (RFO) which are utilized by bifidobacteria. They are soluble, non-structural and non-reducing oligosaccharides essential in some physiological processes. However, in humans, a diet rich in RFO causes stomach discomfort, flatulence and diarrhoea. Nonetheless, when consumed in lower concentration RFO are considered as prebiotic, supporting the growth of beneficial intestinal microflora (Gangola et al. 2016, Martínez-Villaluenga and Gómez 2007).

2.2.13. Sorbitol

Sorbitol is a naturally occurring polyalcohol compound produced from catalytic hydrogenation of starch hydrolysate. It is widely used in the food industry as a sweetener, humectant and texturizing agent (Sarmiento-Rubiano et al. 2007).

When ingested, sorbitol has demonstrated to affect intestinal microbiota because it is used by some species of *Lactobacillus* and *Bifidobacterium* in the human intestine, thus indicating that sorbitol may act as a prebiotic by positively altering the gut microbiota (Perina et al. 2014).

2.2.14. Soybean Oligosaccharides

Soybean oligosaccharides (SBOS) are those found mainly in soybeans, but can also be found in other beans and peas. They are approved by the FDA as Generally Recognized as Safe (GRAS). The two principal soy oligosaccharides are the trisaccharide raffinose and the tetrasaccharide stachyose. Raffinose is constituted of one molecule of galactose, one of glucose and one of fructose, while stachyose is composed of two molecules of galactose, one molecule of glucose and one molecule of fructose (Fei et al. 2014).

Soy oligosaccharides also stimulate the growth of *Bifidobacterium* species in the large intestine. They are used as dietary supplements and in functional foods. Like other prebiotics, soy oligosaccharides may have antitumor, antimicrobial, hypolipidemic and hypoglycemic actions, and may also help improve mineral absorption and balance (Fei et al. 2014, Guiné et al. 2010). SBOS have demonstrated promising effects for the prevention of many chronic diseases such as diabetes, cancer, osteoporosis, atherosclerosis and menopausal disorders (Chen et al. 2010, Fei et al. 2014).

2.2.15. Transgalacto-Oligosaccharides

Transgalacto-oligosaccharides (TOS) are a mixture of oligosaccharides consisting of D-glucose and D-galactose. TOS are produced from lactose by the action of β-galactosidases having transgalactosylation activity (Mikkelsen et al. 2004). They are resistant to digestion in the upper gastrointestinal tract and stimulate the growth of bifidobacteria in the large intestine (Gibson et al. 2004). TOS have demonstrated positive effects on calcium absorption (van den Heuvel et al. 2000) and prevention of bone loss. In preliminary studies, TOS have shown some ability to lower triglycerides. Like other prebiotics, TOS are also marketed as dietary supplements and used in functional foods.

2.2.16. Xylo-Oligosaccharides

Xylo-oligosaccharides (XOS) are comprised of oligosaccharides containing beta-linked xylose residues. Since XOS resist digestion in the upper gastrointestinal tract, they are able to function in the large intestine to increase the growth of *Bifidobacterium* species, thus improving gastric function (Manisseri and Gudipati 2010). Apart from their prebiotic effect, XOS are believed to alleviate disease symptoms such as arteriosclerosis and colon cancer (Hsu et al. 2004, Swennen et al. 2006).

According to preliminary research, xylo-oligosaccharides have the potential to improve blood sugar levels and fat metabolism, restore normal intestinal flora following antibiotic, chemo, or radiation therapies, increase mineral absorption and vitamin B production, and

reduce intestinal putrification (Guiné et al. 2010, Manisseri and Gudipati 2010, Moura et al. 2008).

3. SYNBIOTICS

The concept of "synbiotics" was defined as "a mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract, by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria, and thus improving host welfare" (Kojima et al. 2016). Therefore, the term synbiotic is used for products that contain both probiotics and prebiotics. Such products take advantage of both the addition of beneficial bacteria and the encouragement of the growth of resident beneficial bacteria. Because the word suggests synergism, this term should be reserved only for products in which the probiotic bacteria are favoured by the present prebiotic compound. Hence, for example, a product containing oligofructose and probiotic bifidobacteria would fulfil the definition, whereas a product containing oligofructose and a probiotic *Lactobacillus casei* strain would not, for the above mentioned reasons (Cencic and Chingwaru 2010, Guiné et al. 2010).

3.1. Characteristics

The probiotic strains used in synbiotic formulations include *Lacbobacilli*, *Bifidobacteria spp*, *S. boulardii*, *B. coagulans*, among others, while the major prebiotics used comprise of oligosaccharides like FOS, GOS and XOS, inulin and, in a lesser extent, other prebioticsfrom natural sources (Pandey et al. 2015). The best synbiotic combinations currently available include bifidobacteria and FOS, *Lactobacillus GG* and inulin, and bifidobacteria and lactobacilli with FOS or inulin. The main advantage of using a synbiotic is that a true probiotic, without its prebiotic food, does not survive well in the digestive system. Therefore, to enhance viability, the product must provide a much greater growth rate of the healthy bacteria in order to minimize the growth of harmful bacteria. Moreover, without the adequate food source, the probiotic will show a greater intolerance for oxygen, low pH and temperature. In addition, the probiotic will have to compete against other bacteria that will take over if its specific food source is not available (Guiné et al. 2010).

Synbiotics were developed to overcome possible survival difficulties for probiotics. Apparently, the justification to use synbiotics is based on observations showing the improvement of survival of the probiotic bacteria during the passage through the upper intestinal tract. A more efficient establishment in the colon as well as a stimulating effect of the growth of probiotics and ubiquitous bacteria seem to contribute to maintain the intestinal homeostasis and an improved healthy state (Peña 2007). Therefore, synbiotic products result in a better choice, since concomitant intake of probiotics and prebiotics may enhance the possible effectiveness of both. Amongst the several positives effects of synbiotics, stand the antimicrobial, anticarcinogenic, antidiarrheal and antialergenic properties, the prevention of

osteoporosis, the reduction of serum fats and blood sugars, the regulation of the immune system and the treatment of the liver-related brain dysfunction (Pandey et al. 2015).

The synbiotics help to heal and regulate the intestinal flora, particularly after the destruction of microorganisms caused by antibiotic, chemotherapy, or radiation therapies. Besides, the beneficial organisms enhance proper digestion, absorption, and/or manufacture of nutrients throughout the digestive system. On the other hand, a synbiotic will also suppress the development of putrefactive processes in the stomach and intestines, thus preventing the occurrence of a number of serious diseases: food allergies, ulcerous colitis, constipation, diarrhea, cancers, gastrointestinal infections, among others. The health benefits claimed by synbiotics consumption by humans include: 1) Increased levels of lactobacilli and bifidobacteria and balanced gut microbiota, 2) Improvement of liver function in cirrhotic patients, 3) Improvement of immunomodulating ability, 4) Prevention of bacterial translocation and reduced incidences of nosocomial infections in surgical patients (Pandey et al. 2015).

3.2. Role of Synbiotics on the Human Body

The antimicrobial qualities of synbiotics are primarily due to the probiotic when compared to the prebiotic. The beneficial bacteria fight the pathogenic organisms, helping to prevent their attachment to the intestinal walls, thus lessening the risk of disease (Furrie et al. 2005). Moreover, probiotic bacteria also stimulate antigen specific and nonspecific immune responses. Still, these effects benefit from the presence of the prebiotic, which acts as a stimulator of the bacterial growth.

The synbiotics have shown anticarcinogenic effect, which appears to derive from substances that may inhibit the growth of carcinogenic cells, and which are produced by the probiotic bacteria as a result of sugar fermentation. Furthermore, probiotic bacteria have the ability to bind to some carcinogens, inactivating them, thus inhibiting the growth of some tumors, as well as any bacteria that may convert matter into carcinogens. Conversely, prebiotic sugars help to increase concentrations of minerals such as calcium and magnesium in the colon, exerting a positive effect in controlling the rate of cell turnover and the formation of insoluble bile or fatty acid salts, which can have a damaging effect (Le Leu et al. 2005, Rafter et al. 2007). Motoori et al. (n.d.) showed that synbiotics reduced the occurrence of adverse events of chemotherapy during neoadjuvant chemotherapy in esophageal cancer patients, through adjustments to the intestinal microbiota.

Prevention of osteoporosis can happen as a result of an improvement in mineral absorption and balance (Scholz-Ahrens et al. 2007). This can be favoured by the action of the oligosaccharides, which bind such minerals as calcium and magnesium in the small intestine, releasing them later in the large intestine, where they can be better absorbed. Besides, the absorption of these minerals is assisted by the fatty acids that result from fermentation processes.

The antidiarrheal qualities of synbiotics are due to the crowding out of pathogens known to cause diarrhoea, but, on the other hand, the strengthening of the intestinal wall also contributes to prevent this pathology.

Synbiotics and fermentable fibers have a beneficial effect on the liver as well as on a brain dysfunction that affects many who have liver disease, which results in changes in behaviour, intelligence, consciousness, and neuromuscular function (Liu et al. 2004).

The reinforcement of the barrier function of the intestinal wall by probiotics helps preventing the absorption of some antigens, thus conferring some antiallergenic properties to some synbiotics that may be helpful in such cases as food allergies.

Prebiotics demonstrated to diminish the triglycerides, as well as total cholesterol and low density lipoproteins (LDL) levels (Liong and Shah 2005). As for ability of probiotics and synbiotics to normalize serum glucose levels, prebiotics may help by delaying gastric emptying and/or shorten the transit time through the small intestine. Moreover, the propionate (a substance released through fermentation) seems to make better use of the glucose molecule conversion.

Since probiotic bacteria increase the levels of circulating immunoglobulin A (IgA), they help regulating the immune system. In addition, they enhance non-specific immune mechanisms, such as increasing phagocyte activity.

Kojima et al. (2016) showed that synbiotics can potentially be used against oral pathogens in the future.

The effectiveness of a synbiotic product depends on the ability of the various probiotic bacteria to survive digestive juices as well as the alkaline environment of the duodenum, as well as to their ability to adhere to the intestinal wall to colonize the colon. Since the prebiotics are not digested, they travel through the digestive tract to help feed the bacteria in the intestines. These sugars are then fermented by bifidobacteria, lactobacilli, and others in the colon to produce some beneficial by-products such as short-chain fatty acids acetate, propionate and butyrate; hydrogen, hydrogen sulfide, carbon dioxide, and methane gases; lactate, pyruvate, succinate, and formate.

4. Effects on Human Health

In the past decades, with most emphasis on the latest years, the field of probiotics has originated a huge interest among the medical and scientific communities, as well as the food and pharmaceutical industries. An enormous quantity of studies involving probiotics have been developed, with the objective of identifying and proving their claimed health benefits.

When considering the health effects of probiotics, it is important to recognize that different strains, species and genera of bacteria may have different effects, and therefore, the health benefits claimed for a certain probiotic may be dependent on the specific conditions tested. However, it seems fairly proved that certain strains consumed at adequate levels positively influence human health, namely concerning diarrhoea, antibiotics, irritable bowel syndrome, inflammatory bowel disease, hypertension, cancer, immune system stimulation, among others.

Nevertheless, it is also important to notice that some probiotics may cause side effects, such as systemic infections, deleterious metabolic activities, excessive immune stimulation or gene exchange. Prebiotics may cause side effects as well. Because of fermentation in the large intestine, the ingestion of higher quantities of prebiotics may lead to flatulence, abdominal disorders and diarrhoea.

4.1. Allergies, Asthma and Eczema

An allergy is a hypersensitivity reaction initiated by immunological mechanisms. The incidence of allergic diseases has greatly increased over the past decades, most particularly in industrialized nations. Probiotics may have a beneficial effect in modulating allergic reactions, by improving mucosal barrier function and microbial stimulation of the immune system (Ruszczyński and Feleszko 2016, Tang et al. 2015).

Probiotic bacteria act as regulator of inflammation associated with hypersensitivity reactions in patients with atopic eczema and food allergy (Pohjavuori et al. 2004). Probiotics may also be helpful in alleviating some of the symptoms of food allergies such as those associated with milk protein, possibly by degrading these proteins to smaller peptides and amino acids, which, added to the diet of infants on a hydrolysed whey formula, decreased the symptoms of atopic dermatitis. Probiotics had a superior efficacy in prevention rather than treatment of the condition. Probiotics may affect the improvement of immune tolerance during the 1st year of life, thus accounting for their potential effect in children with atopic dermatitis (Joohee Lee et al. 2008).

Asthma is a concern worldwide, constituting a major public health problem in industrialized nations. The disease is characterized by a state of chronic inflammation and has been associated with genetic predisposition, altered lung mechanics, resistance to treatment with inhaled corticosteroids, diet and vitamin D deficiency (Ly et al. 2011). The gut contains a large and diverse population of microbes that are most important for the stimulation of the immune system. Therefore, the gut composition in early life can have a significant influence in the development of the immune system with consequences for the possible establishment of asthma (Denise Kelly et al. 2007).

Recent findings indicate that probiotic intake may improve the quality of life in patients with allergic rhinitis, thus suggesting that probiotics may be useful in allergic rhinitis, although the findings were not sufficient to allow establishing treatment recommendations (Das et al. 2010, Gui Yang et al. 2013).

4.2. Antibiotics

Antibiotics are largely used with the purpose of killing harmful bacteria. However, and unfortunately, in many cases they also kill normal bacteria, often resulting in disruption of the bacterial flora, leading to diarrhoea and other intestinal disturbances. The use of antibiotics can lead to potential clinical complications, such as the emergence of antibiotic-resistant microorganisms, poor compliance, and increasing incidence of diseases related to antibiotic use. Antibiotic-associated diarrhoea (AAD) is the most common intestinal complication of antibiotic use, and occurs when antibiotic-mediated disturbance results in altered functioning of the microbiota. The elderly have an increased risk of AAD because the incidence and prevalence of acute and chronic illnesses, drug consumption, and polypharmacy all increase with age. Moreover, age may be associated with changes to the microbiota of the gut, thus making older patients particularly susceptible to the effects of antibiotics. Therefore, the impact of probiotics in people on antibiotic therapy has been significantly studied, and replenishing the flora with normal bacteria during and after antibiotic therapy seems to minimize disruptive effects of antibiotic use (Ouwehand and Tennilä 2016, Xie et al. 2015).

4.3. Anxiety and Depressive Symptoms

Recent investigations suggest that the gastrointestinal microbiota have decisive roles in regulating key aspects of the central nervous system (CNS) physiology, mood, and behaviour (Heijtz et al. 2011, Pirbaglou et al. 2016). Probiotics, with their influence on the gut microbiota have shown to be beneficial in fighting the symptoms of anxiety and depression (Crumeyrolle-Arias et al. 2014, Pirbaglou et al. 2016, Savignac et al. 2015). Probiotic bacteria that have demonstrated this effect include: primarily the Bifidobacteria (e.g., *Bifidobacterium longum* 1714, *Bifidobacterium breve* 1205) and Lactobacilli strains (e.g., *Lactobacillus rhamnosus* JB-1) (Bravo et al. 2011, Savignac et al. 2014, 2015).

4.4. Cancer

Cancer is a class of diseases characterized by out-of-control cell growth. There are over 200 different types of cancer, and each is classified by the type of cell that is initially affected. In general, cancer is caused by mutation or activation of abnormal genes that control cell growth and division. However, most of these abnormal cells do not result in cancer, since the immune system recognizes and destroys most abnormal cells, and also the normal cells usually out-compete the abnormal ones. The factors that may increase the occurrence of abnormal cells are much diversified, and some precautions that minimize the exposure to those factors will decrease the risk of developing cancer. One of the many potentially risky exposures is that to carcinogens, which are cancer-causing chemicals that can be ingested or generated by metabolic activity of microbes living in the gastrointestinal system (Cole and Kramer 2016, Pitt et al. 2016).

Probiotics have a very important role in decreasing the exposure to chemical carcinogens by a combination of actions: (a) detoxifying ingested carcinogens, (b) altering the environment of the intestine and thereby decreasing populations or metabolic activities of bacteria that may generate carcinogenic compounds, (c) producing metabolic products (e.g., butyrate) which improve the cell's ability to die when it should die (a process known as apoptosis or programmed cell death), (d) producing compounds that inhibit the growth of tumor cells, or (e) stimulating the immune system to better defend against cancer cell proliferation. Another hypothesis relies on the preventive effect of probiotics on carcinogenesis, since they might suppress the growth of bacteria that convert procarcinogens into carcinogens, thus reducing the amount of carcinogens in the intestine. The activity of the enzymes that convert procarcinogens into carcinogens is often used as an indicator of the effect of probiotics on the intestinal microflora and experimental results suggested that consumption of *L. casei* might delay the recurrence of bladder tumors. One hypothesis for the prevention or delay of tumor development by lactobacilli is that they might bind to mutagenic compounds in the intestine, thereby decreasing the absorption of these mutagens (Guiné et al. 2010, Isolauri 2004).

Clinical research suggests that the consumption of probiotic cultures may positively influence the gastrointestinal (GI) environment to decrease the risk of cancer (Desrouillères et al. 2015, Na-Kyoung Lee et al. 2015, Lunder 2015a). Most studies have been focused on Gastric cancer (GC) and (especially) colorectal cancer (CRC). The effects of microbiota on other segments of the GI tract (oesophagus, small bowel) are less studied (Serban 2014).

The use of probiotics for prevention of CRC has been established through *in-vitro*, *in-vivo* and gut microbiome studies, which provided ample evidence of the CRC preventive effects of probiotics (Ambalam et al. 2016, Dubey et al. 2016, Faghfoori et al. 2015, Saxami et al. 2016). Relatively to GC, it seems that its incidence is constantly decreasing, possibly due to the fall in the H. pylori (HP) prevalence. Oesophageal adenocarcinoma (OAC) was found inversely related with the presence of HP in the stomach. Small intestine cancers have been related to chronic inflammation and immune dysfunction (especially Crohn's disease and celiac disease) and also to polyposis syndromes (Serban 2014).

Experimental evidence suggests that probiotics might have beneficial effect on the toxicity of anticancer therapy, such as chemotherapy and radiation therapy (Mego et al. 2013).

4.5. Cholesterol

Cholesterol has a unique structure of four fused hydrocarbon rings with a polar hydroxyl group at one end and an eight-carbon branched aliphatic tail at the other end. It is a component of cell membranes and nerve cells and acts as a precursor to certain hormones and vitamins and it is essential for many functions in the human body, being an essential component of mammalian cells. It is synthesized in a complex series of enzymatic steps in the endoplasmic reticulum and is eventually transported through the Golgi to the plasma membrane where its concentration is much higher than in other cellular compartments. However, elevated levels of total blood cholesterol, or other blood lipids, such as triglycerides or low LDL, constitute a risk for developing coronary heart disease. In normal conditions, humans synthesize cholesterol to maintain its levels at the minimum required for biological functioning. However, sometimes the cholesterol levels are increased beyond the desirable values, much due to the ingested diet, which plays an important role on this subject (Epand et al. 2016, Sung-Tae Yang et al. 2016).

Probiotic cultures have been evaluated for their effect on serum cholesterol levels, and clinical studies highlight that probiotic bacteria help lowering cholesterol or LDL levels. Besides, the removal ability mechanisms have also been evaluated and fully established in many cases (Anandharaj and Sivasankari 2014, Aydaş and Aslim 2016, Choi and Chang 2015, Miremadi et al. 2014, 2016, Shehata et al. 2016).

4.6. Diabetes

Diabetes mellitus is a looming epidemic worldwide, which affects almost all major groups of society, and generates high burdens on global health and economy. A significant number of studies have recognized a series of multiple risk factors associated with increased incidence of type 2 diabetes mellitus (T2DM), like for example: genetic predisposition, epigenetic changes, unhealthy lifestyle, and altered gut microbiota. These factors cause increased adiposity, β-cell dysfunction, hyperglycemia, hypercholesterolemia, adiposity, dyslipidaemia, metabolic endotoxemia, systemic inflammation, intestinal permeability (leaky gut), defective secretion of incretins and oxidative stress associated with T2DM (Panwar et al. 2013). Probiotics, particularly lactobacilli and bifidobacteria, have been recognized, based

both on *in vitro* and *in vivo* animal models, as the prospective biotherapeutics for the prevention and treatment of T2DM (Panwar et al. 2013).

Samah et al. (2016) presented a recent review on the effect of probiotics for the management of T2DM and found evidence of a moderate hypoglycaemic effect of probiotics, with a significantly lower fasting blood glucose (FBC). However, regarding glycated haemoglobin (HbA_{1c}), anti-inflammatory and anti-oxidative effects of probiotics the findings are not so consistent. Therefore, the authors suggest the need for well-designed clinical studies to further assess the potential beneficial effects of probiotics in management of T2DM.

4.7. Diarrhoea

Infectious diarrhoea remains a major cause of morbidity and mortality worldwide. Viruses, bacteria and protozoa are responsible for the majority of infections, which are transmitted most commonly by the faecal–oral route through water, food and person-to-person transmission. Many types of diarrheal illnesses disrupt intestinal function, owing to many different causes, including antibiotics administration, which cause the death of beneficial bacteria together with the targeted harmful bacteria. The ability of probiotics to decrease the incidence or duration of certain diarrheal episodes is among the most substantiated of the health effects of probiotics (Paul Kelly 2015, Kilpinen et al. 2015, Selinger et al. 2013, Xie et al. 2015).

However, studies evaluating the effect of probiotics on travelers' diarrhea are recent and are not concluding as to the benefits of probiotics is this particular field (Briand et al. 2006, McFarland 2007). McFarland (2015) analysed the effects of probiotics in protecting elderly from travelers' diarrhea but unfortunately, they were not able to clearly establish any recommendations for this emerging population of travelers, who might be at higher risk for travel-related illnesses.

4.8. Helicobacter Pylori

Helicobacter pylori is a ubiquitous Gram negative, flagellated, spiral organism which survives in the mucus layer overlying the gastric mucosa, thus colonizing the stomach (Boltin 2016). *H. pylori* is a highly prevalent pathogen that colonizes approximately half of the world's population and although most subjects colonized by *H. pylori* are asymptomatic, this pathogen is strongly associated with gastritis, peptic ulcers, and gastric cancer in humans (Pan et al. 2016). Its presence is associated with gastric ulcers and gastric cancer, although its role in the etiology of these diseases is still under investigation. The effect of probiotics on *H. pylori* has been object of some studies, which show that antibacterial substances including (but not limited to) organic acids produced by some lactobacilli inhibit the growth and survival of this pathogen. Results demonstrate that some lactobacilli inhibit *H. pylori* attachment and prevent colonization. Probiotics have an *in vitro* inhibitory effect on *H. pylori*, as demonstrated in preclinical and animal clinical studies using animals and humans, thus demonstrating that probiotic treatment is effective in reducing *H. pylori*-associated gastric inflammation (Chitapanarux et al. 2015, Lunder 2015b, Pan et al. 2016, Patel et al. 2014).

However, although prebiotics can help to control gastric infections originated by *H. pylori*, they are not able to eradicate it from the stomach (Sgouras et al. 2004).

4.9. Hepatic Encephalopathy and Other Liver Diseases

Hepatic encephalopathy (HE) is a disorder of mental status and cognitive function as a result of liver failure and/or portosystemic shunt. It is a liver disease and its effects can be life threatening, impairing the patient's quality of life and daily functioning (Stepanova et al. 2012). The exact pathogenesis of HE still remains unknown, but some probiotics containing therapeutic effect have multiple mechanisms of action that could disrupt the pathogenesis of HE. This makes them superior to conventional treatment and with a reduction in the risk of bleeding (De Santis et al. 2000, Solga 2003).

Liver cirrhotic patients with hepatic encephalopathy have poor prognosis. Probiotics alter the intestinal microbiota and reduce the production of ammonia. Xu et al. (2014) conducted a meta-analysis about the role of probiotics on liver cirrhotic patients with HE and concluded that probiotics were able to decrease overt HE in patients with liver cirrhosis. Other studies corroborate this role of probiotics in HE (Lunia et al. 2014, Zhao et al. 2015).

The liver is permanently exposed to several noxious and beneficial products and microorganisms derived from blood current (1000–1200 mL/min) via the portal vein, which carries blood out of the spleen and intestines. Probiotics have demonstrated useful to treat hepatic diseases owing to their potential ability to modulate changes in the gut microbiota, intestinal permeability, as well as immune and inflammatory responses (Chávez-Tapia et al. 2015).

The probiotics VSL#3, *Lactobacillus rhamnosus* and *acidophilus* have proven to decrease endotoxin levels and to mildly decrease intrahepatic lymphocytes in a high-fat diet model in patients with alcoholic liver disease (Chang et al. 2013, Hong et al. 2015). Also *Saccharomyces boulardii* was demonstrated as able to reduce hepatic steatosis through lowering the hepatic lipid content and low-grade systemic inflammation (Everard et al. 2014).

4.10. Hypertension

The evidence from scientific research suggests that the consumption of certain lactobacilli, or products derived from them, may reduce blood pressure in mildly hypertensive people.

Lollo et al. (2015) investigated the effects of ingestion of a probiotic cheese on hypertension parameters in an *in vivo* model and their results suggest that consumption of probiotic cheese can be potentially useful to improve the cardiovascular health parameters.

Antihypertensive effects have been documented in mildly hypertensive adults for three compounds derived from the growth of certain lactobacilli: 1) fermented milk containing two tripeptides derived from the proteolytic action of *L. helveticus* on casein in milk; 2) bacterial cell wall components from cell extracts of lactobacilli; and 3) fermented milk containing fermentation-derived gamma amino butyric acid (Parvez et al. 2006, Sydorchuk et al. 2005).

4.11. Immune System Stimulation

The immune system provides the primary defence against microbial pathogens that enter the human body. It is extremely complex, involving both cell-based and antibody-based responses to potential infectious agents.

Immunodeficiency can result from certain diseases (e.g., cancer, AIDS, leukemia) or, to a lesser extent, from more normal conditions such as old age, pregnancy, or stress. Autoimmune diseases (like for example allergies, rheumatoid arthritis or inflammatory bowel diseases) also can occur due to misdirected immune system activity. Probiotic cultures have proved to stimulate certain cellular and antibody functions of the immune system, providing an additional tool to help the human body to protect itself. Moreover, certain probiotic bacteria show the ability to modulate immune deregulation and stimulate the immune response mechanisms. The protective effects associated with these microbes are mediated by multiple mechanisms involving T cells, dendritic cells (DCs) and epithelial cells (Dwivedi et al. 2016, Galdeano and Perdigón 2006, Geuking et al. 2014, Konieczna et al. 2012).

4.12. Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) are heterogeneous diseases that likely arise from the convergence of genetic, microbial, and environmental factors. They fall into 2 main clinical phenotypes: ulcerative colitis (UC) and Crohn's disease (CD), being serious intestinal diseases that can ultimately lead to the surgical removal of the colon. UC is characterized by mucosal inflammation in colon, while CD can affect any part of the gastrointestinal (GI) tract and its lesions are focal and transmural, progressing to deep ulcerations that can result in development of fistulas, abscesses, and strictures (Miyoshi and Chang (n.d.)).

What causes these diseases is not fully understood, but it seems that an intolerance to the normal flora in the gut may lead to inflammation, thus originating the pathology. Many studies of the genetic basis of IBD, including genome-wide association studies, have revealed key insights and improved our understanding of IBD pathogenesis. Besides the host genetic factors affecting IBD, also some environmental risk factors are highlighted, such as hygienic conditions, diet, environment, and lifestyle (Molodecky et al. 2012). The role of gut flora in the progression of these diseases has led to research concerning the impact of certain probiotic bacteria on the maintenance of reduced inflammation that occurs during remission stages. Several preclinical and clinical studies have shown that certain probiotics can exert a positive effect on IBD (Eppinga et al. 2016, Fedorak and Demeria 2012, Jonkers et al. 2012, Lichtenstein et al. 2016, Lunder 2015b, Steppe et al. 2014).

4.13. Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a functional bowel disorder that can be characterized by symptoms of abdominal pain, cramps, gas, bloating, diarrhoea and constipation. IBS is the most frequently diagnosed gastrointestinal disorder in clinical practice. It is estimated that the prevalence rate ranges from 10% to 20% of the adult population, and that its incidence in women is three times greater than in men. The symptoms of this clinical condition cannot be

explained by organic, metabolic, or underlying infectious causes (Carmona-Sánchez et al. 2016, Thomas and Quigley 2016).

The studies involving probiotics and IBS indicate important symptom relief using probiotics like lactobacilli (for example *Lactobacillus acidophilus*), bifidobacteria (ex. *Bifidobacterium animalis*), and VSL#3 (Bogovič Matijašić et al. 2016, Camilleri 2006, Glickman-Simon and Wallace 2015, Hod and Ringel 2016, Quigley 2016, Šmid et al. 2016).

4.14. Kidney Diseases

Humans do not possess the enzymes necessary to metabolize endogenous and dietary oxalate, a toxic compound causing hyperoxaluria and calcium oxalate urolithiasis. Oxalate in humans can be eliminated through (1) excretion in urine, (2) forming insoluble calcium oxalate and elimination in feces, or (3) oxalate degradation by gastrointestinal (GIT) microorganisms. High levels of oxalate in the urine constitute a risk factor for the development of kidney stones. The presence of some microbes in the intestine may limit its absorption, and therefore, the manipulation of the gut flora with the right probiotic bacteria (like *Lactobacillus* and *Bifidobacterium* species) may have a positive impact on gastrointestinal tract oxalate levels, thus decreasing oxalate absorption. On the other hand, the gut inhabiting bacterium *Oxalobacter formigenes* may be used as probiotic treatment to reduce urinary oxalate (Abratt and Reid 2010, Azcarate-Peril et al. 2006, Bose et al. 2016). Other studies highlighted the role of probiotics in chronic kidney diseases (Kirk and Dunker 2014).

4.15. Lactose Intolerance

Lactose is a major disaccharide present in milk, composed by two simple sugars: glucose and galactose. The incapacity of some adults to digest lactose, or milk sugar, results from a poor ability to produce the lactose-digesting enzyme β-galactosidase enzyme, lactase-phlorizin hydrolase, generally called lactase. Consumption of lactose by those lacking adequate levels of lactase produced in the small intestine can result in symptoms of diarrhoea, bloating, abdominal pain and flatulence, both in men and women. These symptoms are due to the undigested lactose, which reaches the large intestine and is there fermented by the colonic microbes (Lule et al. 2016, Lunder 2015b).

The consumption of dairy products is important for supplying calcium and preventing osteoporosis, and, in the case of lactose intolerance, can be facilitated by the presence of probiotic bacteria. Scientific studies have proved that many lactose intolerant individuals show a better tolerance to the consumption of fermented dairy products, such as yogurt, when compared to unfermented milk. In fact, they reveal less symptoms when exposed to the same amount of lactose from a source containing probiotic bacteria. The lactic acid bacteria present in yogurt produce lactase, thus helping in the digestion of lactose before it reaches the colon. The yogurt starter bacteria, *L. acidophilus* and bifidobacteria have been shown to improve digestion of lactose, although in a lesser extent than the yogurt starter cultures, *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (Lule et al. 2016, Ockeloen and Deckers-Kocken 2012, Perets et al. 2014).

4.16. Osteoporosis

Osteoporosis is a multifactorial bone disease, with increasing prevalence and importance also due to increased life expectancy (Wright et al. 2014). The prevention and treatment of osteoporosis strongly relies on nutrition and focus is given to substances/organisms that can enhance the bioavailability in the diet thus increasing calcium absorption and bone mineral content (BMC) (Scholz-Ahrens et al. 2016). Prebiotics have shown to increase the absorption of minerals and trace elements, that have an impact on the mineralization of bone, increased BMC and bone trabecular structure, or reduced estrogen-deficiency-induced bone loss (Roberfroid et al. 2010, Scholz-Ahrens et al. 2007).

4.17. Small Bowel Bacterial Overgrowth

When the microbial populations in the small intestine overgrow beyond normal levels, that condition is referred to as small bowel bacterial overgrowth. This pathology happens under certain conditions such as production of low stomach acid or kidney dialysis. This excessive microbial population can produce toxic by-products, as a result from its growth, but investigations have discovered that feeding high levels of certain probiotic strains can control the toxic effects of these microbes (Besselink et al. 2004, Guiné et al. 2010).

4.18. Respiratory Infection

Probiotics have been used for the prevention and control of many infectious diseases, and studies have demonstrated their effectiveness against not only infections in the gastrointestinal tract, but also those in the respiratory tract (Hao et al. 2015, King et al. 2014). Many clinical trials have proven the efficacy of several probiotic strains against upper respiratory tract infections (URTIs), such as the common cold and influenza. These clinical trials have focused mainly on infants, children, students, and elderly people, whose immune defences are more vulnerable (Makino et al. 2010, Merenstein et al. 2010, Taipale et al. 2011, Waki et al. 2014). However, some other studies have also investigated the efficacy of probiotics in healthy adults (Gleeson et al. 2011, Guillemard et al. 2010, Shida et al. 2015). It is proposed that the daily ingestion of probiotic beverages might maintain normal immune function and control URTIs (Shida et al. 2015).

4.19. Vaginosis and Urogenital Tract Infections

Vaginal microbiota forms a mutually beneficial relationship with their host and has a major impact on health and disease. In the vaginal microbiome, lactobacilli constitute the dominant proportion (80%) of bacteria inhabiting the healthy women's vagina. The vagina and its microbiota form a finely balanced ecosystem, which, if disrupted, can lead to symptoms of vaginosis. This used to be considered a mere annoyance, but now is being looked at more seriously because it is associated to a wide range of complications, such as

pelvic inflammatory disease, problems related to pregnancy (low birth weight babies, etc.), and increased susceptibility to AIDS infection (Nader-Macías and Juárez Tomás 2015).

Vaginosis can be caused by several different organisms, which in many cases, are not identified. A healthy vagina contains predominantly lactobacilli, and a lack of these (especially the ones producing hydrogen peroxide) constitutes an increasing risk of developing vaginosis.

It is hypothesized that the lactobacilli help maintaining a favourable vaginal pH in the acidic range and contribute to inhibit pathogens, possibly through the production of hydrogen peroxide and other antimicrobial factors. So far, research suggests that lactobacilli may be helpful in controlling the incidence and duration of vaginal infections (Brown and Valiere 2004, Burton et al. 2003, Reid et al. 2003, 2004).

The urogenital microbiome composition is influenced by genetic factors, hormone levels, hygiene, disease and use of contraceptives and antibiotics, among others factors The imbalance of the female urogenital ecosystem, which can be caused by different external or internal factors, negatively affects the protective, indigenous microbiota thus favouring the appearance or the overgrowth of pathogenic or potential pathogens causing urogenital tract infections (UGTI) (Brotman et al. 2014, Hickey et al. 2012, Bing Ma et al. 2012).

5. NANOTECHNOLOGY APPLICATIONS

Nanoscience and nanotechnology are new frontiers of this century and food nanotechnology is an emerging technology. Their applications to the agriculture and food sector are relatively recent compared with their use in drug delivery and pharmaceuticals. Many scientists and engineers have recognized well the potential of nanotechnology to lead all the food industries in the 21st century (Ravichandran 2010).

Nanotechnology is concerned with the manipulation of materials at the atomic and molecular scales to create structures that are less than 100 nm in size in one dimension (Singh 2016). By reducing the size of the particles, nanotechnology can improve the properties of bioactive compounds, such as controlled release, solubility, prolonged residence time in the gastrointestinal tract and efficient absorption by the cells (Bernardes et al. 2014).

In the recent past, there has been an explosion of functional food, such as probiotic and prebiotic, health-based products. However, the survival in the human gastro-intestinal system is questionable. Providing probiotic and prebiotic with a physical barrier against adverse environmental conditions is therefore an approach currently receiving considerable interest.

The development of a suitable technology for the production of probiotics is a key research for industrial production, which should take into account the viability and the stability of the organisms involved. Microbial criteria, stress tolerance during processing and storage of the product constitute the basis for the production of probiotics (Sarao and Arora 2015).

5.1. Probiotics and Prebiotics Viability

The viability and survival of probiotics are strain specific. Probiotic survival in products is affected by a range of factors including pH, post-acidification during products fermentation, hydrogen peroxide production and storage temperatures (Kailasapathy 2009, Song et al. 2012). In order to be effective and confer health benefits to the host, probiotics must be able to survive passage through the stomach and upper intestine and be present in sufficient amount to impact the colon microenvironment. This means that they must tolerate the acidic and protease rich conditions of the stomach and survive and grow in the presence of bile acids (Del Piano et al. 2006).

To be an effective prebiotic, an ingredient must neither be hydrolysed nor absorbed in the upper part of the gastrointestinal tract, have a selective fermentation such that the composition of the large intestinal microbiota is altered towards a healthier composition (Gbassi and Vandamme 2012).

5.2. Encapsulation

Encapsulation may be defined as a process of entrapping one substance (active agent) within another substance (wall material). The substance that is encapsulating is often called the coating, membrane, shell, capsule, carrier material, external phase, or matrix (Fang and Bhandari 2010, Wandrey et al. 2010). The encapsulation technology has been used by the food industry for several years and is a useful tool to improve delivery of bioactive molecules and living cells into foods (Saravacos et al. 2011).

Microencapsulation is the process by which small particles or droplets are surrounded by a coating to produce capsules in the micrometre to millimetre range known as microcapsules (Del Piano et al. 2006, Pimentel-González et al. 2009). The concept of microencapsulation allows the functional core ingredient (in this case the probiotic bacteria) to be separated from its environment by a protective coating. Separation of the functional core ingredient from its environment continues until the release of the functional ingredient is desired (post-stomach for the probiotic). This technique has emerged as an alternative for protection of probiotics, providing a particular and convenient micro-environment for the encapsulated microorganism, enhancing their viability, and enabling controlled release of cells in the intestinal tract. Encapsulation technology has been proved to be one of the most effective ways to protect probiotics during processing and subsequent storage (Gbassi and Vandamme 2012, Ozyurt and Ötles 2014). It also helps food materials to resist processing and packaging conditions, improving taste, aroma, stability, nutritional value and product appearance (Huertas 2011).

5.2.1. Materials Employed for the Encapsulation of Probiotics

Carrier materials should serve as protection for probiotics and also be safe for consumption, that is, Generally Recognized As Safe (GRAS) and cost effective, since a high cost will directly influence the value of the final product. Low cost carrier materials include starches, inulin, pectin and most carbohydrates (de Vos et al. 2010).

Each one of the encapsulating materials has its own unique characteristics of capsule formation and provision of shape, appearance, and strength to microbeads. The type of encapsulating material also influences the viability of probiotics during storage, processing, and in the gastrointestinal tract. The effectiveness of any material depends not upon its capsule forming capability, strength, and enhancing viability but also on its cheapness, availability, and biocompatibility (Riaz and Masud 2013).

5.2.1.1. Alginate

Alginates are a group of viscous polysaccharides derived from brown seaweeds and produced as an extracellular matrix by some bacterial species. They are composed of β-D-mannuronic acid (M) and α-Lguluronic acid (G) which are linked by 1→4 glycosidic bonds (Brownlee et al. 2009). The composition of the polymer chain varies in amount and in sequential distribution according to the source of the alginate and this influences functional properties of alginate as supporting material (Burgain et al. 2011, Rowley et al. 1999). According to Krasaekoopt et al. (2003) calcium alginate is preferred for encapsulating probiotics because of its simplicity, non-toxicity, biocompatibility and low cost.

However, its two major limitations are encapsulant leaching during preparation (low encapsulation efficiency) and rapid dissolution (burst release) in the intestinal pH or in presence of sodium ions (Fathi et al. 2014). The defects can be by mixing alginates with other polymer compounds, coating the capsules by another compound or applying structural modification of the alginate by using different additives (Krasaekoopt et al. 2003). For example, George and Abraham (2007) included guar gum in an alginate matrix along with a cross linking agent (glutaraldehyde) to overcome these limitations. In contrast to alginate, chitosan is insoluble in intestinal pH and therefore alginate/chitosan nanoparticles could be used together to inhibit burst release in intestinal condition (Meera George and Abraham 2006, Li and McClements 2011). Is also commonly used mix alginate with starch and it has been shown that this method results in an improvement of probiotic encapsulation effectiveness (Hansen et al. 2002, Krasaekoopt et al. 2003, Sultana et al. 2000, Sun and Griffiths 2000).

5.2.1.2. k-Carrageenan

k-Carrageenan is a natural polysaccharide extracted from marine macroalgae, commonly used as a food additive. In order to use this compound is necessary a temperature range of 40 and 50 ^{0}C at which the cells are added to the polymer solution. The gelation occurs when the mixture is brought to room temperature and then the microparticules are stabilised by adding potassium ions (Krasaekoopt et al. 2003). The probiotic bacteria cells are kept in a viable state when encapsulated in k-carageenan (Dinakar and Mistry 1994) but the gels obtained by this method are unable to withstand stress and are brittle (Chen and Chen 2007).

5.2.1.3. Gellan Gum and Xanthan Gum

Gellan gum is a polysaccharide derived from *Pseudomonas elodea* and consists of repeating unit of four monomers namely glucose, glucuronic acid, glucose and rhamnose (Chen and Chen 2007). Encapsulation of probiotic cells was carried out by using a mixture of xanthan–gelan gum (Sultana et al. 2000, Sun and Griffiths 2000) and as compared to the use of alginate exhibited high resistance towards acid conditions.

5.2.1.4. Chitosan

Chitosan is a linear polysaccharide composed of glucosamine units that in the presence of polyanions can polymerise by formation of a cross-link formation (Burgain et al. 2011, Sarao and M 2015). According to Chávarri el al (2010) a good method of delivering viable bacterial cells to the colon is encapsulation of the probiotic cells using a coating of alginate and chitosan as this provides protection in the simulated gastrointestinal conditions.

The use of chitosan also exhibits some disadvantages, such as inhibitory effects on lactic acid bacteria (Groboillot et al. 1993). As was reported by Mortazavian et al. (2008) encapsulation using chitosan does not exhibit appreciable efficiency for enhancing the viability of the cells. Therefore, chitosan is used more as a coat rather than as a capsule.

5.2.1.5. Starch

Chemically, starch is a polysaccharide composed of a large number of glucose units joined together by glucosidic bonds. It consists mainly of amylose, a linear polymer of D-glucopyranose joined by α-1-4 glucosidic bond and amylopectin, a branched polymer of glucose joined by α-1-4 glucosidic bond and α-1-6 glycosidic bond for ramification (Sajilata et al. 2006).

Resistant starch is defined as the total amount of starch and the products of starch degradation that resists digestion in the small intestine. Starches that were able to resist the digestion will arrive at the colon where they will be fermented by the gut microbiota (Zaman and Sarbini 2016). Resistant starch can be used to ensure the viability of probiotic populations from the food to the large intestine. It also offers an ideal surface for adherence of the probiotics to the starch granule during processing, storage and transit through the upper gastrointestinal tract, providing robustness and resilience to environmental stresses. Bacterial adhesion to starch may also provide advantages in new probiotic technologies to enhance delivery of viable and metabolically active probiotics to the intestinal tract (Anal and Singh 2007, Crittenden et al. 2001).

According to the study developed by Fahimdanesh et al. (2012) encapsulation with resistant starch can significantly enhance the survival of probiotic bacteria in mayonnaise sauce during storage. Furthermore, microencapsulation with resistant starch may improve viable cells of bacteria in acidic foods (Homayouni et al. 2008, Kebary and Hussein 1999, Krasaekoopt et al. 2003, Mirzaei et al. 2012, Sultana et al. 2000).

5.2.1.6. Gelatin

Gelatin is useful as a thermally reversible gelling agent for encapsulation. Because of its amphoteric nature, it is also an excellent candidate for incorporating with anionic-gel-forming polysaccharides, such as gellan gum These hydrocolloids are miscible at pH higher than 6, because they both carry net negative charges and repel one another (Anal and Singh 2007). However, when the pH is adjusted below gelatin's isoelectric point, the net charge on the gelatin becomes positive, causing an interaction with the negatively charged gellan gum (Merck and King 1995).

5.2.1.7. Milk Proteins

Milk proteins are natural vehicles for probiotic cells and because of their structural and physicochemical properties they can be used as a delivery system (Livney 2010). Based on

their specificity of possessing excellent gelation properties, Heidebach et al. (2009a, 2009b) encapsulated probiotic cells in these proteins. The results of these studies are quite promising and using milk proteins for encapsulation is an attractive method because of their biocompatibility (Livney 2010).

5.2.1.8. Cellulose Acetate Phthalate

Cellulose acetate phthalate has a safe nature and because of that is used for controlling drug release in the intestine (Mortazavian et al. 2008). Due to the presence of ionisable phthalate groups, this polymer is insoluble in acid media at a pH of 5 or lower but is soluble when the pH is increased to 6 or higher. This is one of the main advantages of using this encapsulating material (Anal and Singh 2007, Krasaekoopt et al. 2003, Sarao and Arora 2015). According to Fávaro-Trindade et al. (2011) the encapsulation of probiotic bacteria using cellulose acetate phthalate provides good protection for microorganisms in simulated gastrointestinal tract conditions.

5.2.1.9. Supercritical Carbon Dioxide Coating

Traditional encapsulation methods in fortified foods and drug delivery applications have many difficulties for the probiotic cells because of their sensitivity to exposure to water, solvents, heat, or oxygen. A novel encapsulation technology, based on interpolymer complex formation in supercritical carbon dioxide, avoids such exposure during the encapsulation process (Riaz and Masud 2013). In a study developed by Moolmmman et al. (2006), this method was used to encapsulate *Bifidobacterium longum* in a poly (vinyl pyrrolidone)–poly (vinyl acetate–co-crotonic acid) interpolymer complex. The results showed that the encapsulation matrix was stable at low pH, but disintegrated at higher pH, causing the release of the encapsulated material. Therefore, this technology could find applications in the food and pharmaceutical industries.

5.2.1.10. Prebiotics

Prebiotics are the materials that are actually used to enhance the growth of probiotics, which means that prebiotics serve as probiotics food. Adding the prebiotic inulin to yoghurt boosted the growth of probiotic bacteria and, when used in a novel double-microencapsulation, extended the survival rates of the friendly bacteria. The various prebiotic fibres protect the stability and viability of probiotic *Lactobacillus rhamnosus* strains during freeze-drying, storage in freeze-dried form, and after formulation into apple juice and chocolate-coated breakfast cereals (Riaz and Masud 2013).

5.2.2. Main Techniques for Microencapsulation of Probiotics

Selecting the encapsulation technology is very important. Whereas probiotics are living cells, the conditions for implementation of this technology are designed to maintain cell viability, and solvents involved in the encapsulation technology must be non-toxic. Furthermore, assess the release conditions of encapsulated probiotics in a gastrointestinal tract model is an essential approach, which would give an idea of the cells' behaviour (Gbassi and Vandamme 2012).

5.2.2.1. Extrusion Technique

Extrusion is the most popular microencapsulation technique for microorganisms, since it can be very simple, low cost and gentle formulation conditions that ensure high cell viability (Krasaekoopt et al. 2003). This technique involves preparing an aqueous hydrocolloid solution, adding concentrated microorganisms to it, and extruding the cell suspension through a nozzle that forms droplets that fall into a hardening solution (Heidebach et al. 2012).

When the formation of droplets happens in a controlled environment way (as opposed to spray-drying), the technique is known as prilling. This is done by the pulsation or vibration of the jet nozzle. The other common technique to form small droplets is the use of coaxial flow or an electrostatic field. When an electrostatic field is applied, the electrostatic forces disrupt the liquid surface at the needle tip, forming a charged stream of small droplets (Kailasapathy 2002, Martín et al. 2015).

This technology does not involve deleterious solvents and can be performed under aerobic and anaerobic conditions. The most important disadvantage of this method is that it is difficult to use in large scale production owing to the slow formation of the microbeads. Nevertheless, mass production of beads can either be achieved by multi-nozzle system or using a rotating disc. Another process is the centrifugal extrusion which consists of a coextrusion process. It utilizes a nozzle with concentric orifices located on the outer circumference of a rotating cylinder. The core material is pumped through the inner orifice and a liquid shell material through the outer orifice. When the system rotates, the extruded rod breaks up into droplets that form capsules (Kailasapathy 2002).

Various polymers can be used to obtain capsules by this method. However, the most used agents are alginate, k-carrageenan and whey proteins. Among these materials, alginate has been the most applied for extrusion (Rokka and Rantamäki 2010).

There are several studies with the extrusion techniques for probiotic protection and stabilization (Cui et al. 2000, Khalil and Mansour 1998, Liserre et al. 2007). All of them had similar results to the ones obtained in the study of Chávarri et al. (2010), where chitosan was used as coating material to improve the stability of alginate beads with probiotics. In this study, with extrusion technique, the results showed an effective means of maintaining survival under simulated human gastrointestinal conditions.

5.2.2.2. Emulsion Technique

Emulsion is a chemical technique based on the relationship between the discontinuous and the continuous phases. It is used to encapsulate probiotic living cells and uses hydrocolloids (alginate, carrageenan, pectin) as encapsulation materials. For carrying out encapsulation in an emulsion an emulsifier and a surfactant are required. So, a solidifying agent such as calcium chloride is added to the emulsion (Chen and Chen 2007, Kailasapathy 2009, de Vos et al. 2010). The production of the microcapsules of the desired size is achieved by this method by variation of agitation speed and the water/oil ratio.

According to Chen and Chen (2007) emulsion technique is easy to scale-up and gives a high survival rate of the bacteria and the obtained capsules have a small diameter. The major disadvantage of this technique is that it provides capsules with an extremely large size distribution. For that reason, there are several industrial efforts to achieve a narrow particle size distribution controlling the stirring and homogenization of the mixture.

There are also double emulsions, such water-in-oil-in-water (w/o/w). This technique is a modification of the basic technique in which an emulsion is made of an aqueous solution in a

hydrophobic wall polymer. This emulsion is then poured with vigorous agitation into an aqueous solution containing stabilizer. The loading capacity of the hydrophobic core is limited by the solubility and diffusion to the stabilizer solution (Chavarri et al. 2012).

According to Hou et al. (2003), entrapment of cells of lactic bacteria (*Lactobacillus delbrueckii* ssp. *bulgaricus*) in the droplets of reconstituted sesame oil body emulsions increased approximately 10^4 times their survival rate compared to free cells when subjected to simulated gastrointestinal tract conditions. However those results were not similar to the ones found in the study of Mantzouridou et al. (2012). In that study was investigated the effect of cell entrapment inside the oil droplets on viable cell count over storage and under gastrointestinal simulating conditions, according to the type of emulsifier used: egg yolk, gum arabic/xanthan. The investigation was performed with *Lactobacillus paracasei* and their entrapment in the oil phase of protein-stabilized emulsions protected the cells when exposed to gastrointestinal tract enzymes, provided that the emulsions were freshly prepared. Following, however, treatment of aged for up to 4 weeks emulsions under conditions simulating those of the human gastrointestinal environment, the microorganism did not survive in satisfactory numbers. The probiotic cells survived in larger numbers in aged emulsions when the cells were initially dispersed in the aqueous phase of a yolk-stabilized dressing-type emulsion and their ability to survive enzymatic attack was further enhanced by inulin incorporation.

In some countries the use of coatings such as alginate, k-carrageenan gellan-gum or xanthan for encapsulation is not permitted for dairy products. The alternative to this method is the use of milk proteins in which the encapsulation of the probiotic cells can be done by employing the method of enzymatic induced gelation (Heidebach et al. 2009a, 2009b).

Interfacial polymerization is an alternative technique which is performed in a single step. It requires the formation of an emulsion: the discontinuous phase contains an aqueous suspension with the probiotic cells and the continuous phase is an organic solvent. To initiate the polymerisation reaction is added a biocompatible agent which is soluble in the continuous phase. The droplets obtained containing probiotic cells are enveloped in a thin and strong membrane (Kailasapathy 2002). In order to improve their productivity in fermentation, interfacial polymerization is used to encapsulate microorganisms (Yáñez-Fernández et al. 2007).

5.2.2.3. Spray Drying

Spay drying is one of the most commonly applied technologies for encapsulation. The energy consumption of spray drying is 6 to10 times lower compared to freeze drying and it produces a good quality product. The process involves the dispersion of the core material, forming an emulsion or dispersion, followed by homogenization of the liquid, and then the atomization of the mixture into the drying chamber. This leads to evaporation of the solvent. It is important to underline that in this technique, the product feed, gas flow and temperature should be controlled (Martín et al. 2015).

The main advantages of this technique are the rapidity and the relatively low cost of the procedure, it is highly reproducible and the most important is that it is suitable for industrial applications. The major disadvantage of this method is that the use of high temperature is not compatible with the survival of bacteria. In fact outlet temperatures greater than 85–90°C are lethal for probiotics. Therefore, in order to increase the probiotic survival protectants can be added to the media prior to drying. For example, granular starch improves culture viability

during drying and storage, soluble fiber increases probiotic viability during storage and trehalose is a thermoprotectant. Moreover, spray-dried capsules can be coated by an additional layer in order to give a protection against acidic environment of the stomach or to reduce the deleterious effect of bile salts (Semyonov et al. 2010).

Many studies have reported the successful spray drying of *Lactobacillus* and *Bifidobacterium* for a number of different strains, such as *Lactobacillus paracasei* (Desmond et al. 2002, Gardiner et al. 1998), *Lactobacillus curvatus* (Mauriello et al. 1999), *Lactobacillus acidophilus* (Prajapati et al. 1987), *Lactobacillus rhamnosus* (Corcoran et al. 2004) and *Bifdobacterium ruminantium* (O'Riordan et al. 2001).

The most typical materials used to encapsulate probiotic bacteria are proteins and/or carbohydrates, which may be in the glassy state at storage temperatures to minimize molecular mobility and thus degradation. The presence of some prebiotics in the encapsulating material shows higher count after spray drying for *Bifidobacterium*, depending of the physical properties of the prebiotic compound selected (thermoprotector effect, crystalinity, etc.) (Fritzen-Freire et al. 2012, Rodríguez-Huezo et al. 2007) and a similar effect occurs for *Lactobacillus* bacteria (Corcoran et al. 2004, Ying et al. 2012).

5.2.2.4. Freeze Drying

Freeze drying is a technique whose principle is based on the freezing of the product and then reducing the pressure in the external environment to allow the frozen water to sublime directly from the solid phase to the gas phase. The process is carried out by freeze probiotics in the presence of the support material at low temperatures, followed by sublimation of water under vacuum (Chavarri et al. 2012). Although this technique has been used to manufacture probiotic powders for decades, the combination of freeze drying and encapsulation is a relatively new concept.

Several advantages arise from the use of this technique, like providing controlled size and larger specific surface area than spray dried capsules. Some disadvantages are the use of high energy, the long processing time and the cost which is 30–50 times more expensive than spray drying (Zuidam and Shimoni 2010). In order to give protection against adverse environmental conditions, capsules can be coated by an additional shell (Semyonov et al. 2010).

5.2.2.5. Fluid Bed Agglomeration and Coating

Fluid bed is a process in which cell suspension is sprayed and dried on inert carriers using a Wurster based fluidized bed system. Before drying it is important that the probiotic culture is encapsulated in a supporting material such as skimmed milk calcium alginate or fats (Martín et al. 2015) Shellac has also been used as a supporting material, and a purified product of the resinous secretion of the insect *Kerria lacca.* The physicochemical properties of shellac are dependent on the strain of insect, host trees and refining methods (Buch et al. 2009). Due to its natural origin, shellac is an acceptable coating material for food supplement products. Generally, shellac possesses good resistance to gastric fluid, suggesting its use for enteric coating purposes. Despite that, shellac has a low solubility in the intestinal fluid, especially in the case of enteric coating of hydrophobic substances. For that reason, its use as an enteric coating polymer is limited. In order to improve the properties of the enteric coating of shellac, in a study performed by Stummer et al. (2010) it was used sodium alginate,

hydroxypropyl methylcellulose and polyvinylpyrrolidone as additional water-soluble polymers, and glyceryl triacetate and glycerol as plasticizers.

Fluid bead is easy to scale up and for that reason it is one of the encapsulation technologies most applied commercially to probiotics. For example, some companies have developed products using Probiocap® and Duaolac® (Burgain et al. 2011).

This technique has both advantages and disadvantages. The advantages are total control over the temperature and lower comparable cost. The disadvantages are that this technology is difficult to master and is of relatively longer duration (Martín et al. 2015).

Stummer et al. (2012) evaluated the use of this method for the dehydration of *Enterococus faecium*. Their results showed that using fluidized-bed technology is a feasible alternative for the dehydration of probiotic bacteria by layering the cells on spherical pellets, having tested different protective agents as glucose, maltodextrin, skim milk, trehalose or sucrose, preferably skim milk or sucrose.

5.2.2.6. Coacervation

Coacervation is a technique that involves the precipitation of a polymer or several polymers by phase separation, depending if it is simple or complex coacervation, respectively. On the case of simple coacervation, the concept behind it is based on "salting out" of one polymer by addition of agents as salts that have higher affinity to water than the polymer. This means it is essentially a dehydration process whereby separation of the liquid phase results in the solid particles or oil droplets (starting in an emulsion process) becoming coated and eventually hardened into microcapsules. In the case of complex coacervation, it is formed a polyelectrolyte complex. This process requires the mixing of two colloids at a pH at which both polymers are oppositely charged (i.e., gelatine (+) and arabic gum (-)), leading to phase separation and formation of enclosed solid particles or liquid droplets (Chavarri et al. 2012).

Complex coacervation is one of the most frequently used techniques to flavour microencapsulation, but can also be used to encapsulate probiotics. In the case of probiotics encapsulation the most frequent medium used might be a water-in-oil emulsion (Nag et al. 2011).

Compared with other methods the advantages of using the complex coacervation to the encapsulation of probiotics are a relatively simple low-cost process and allows the incorporation of a large amount of microorganisms in relation to the encapsulant. However, the scale-up of coacervation is difficult, since it is a batch process that yields coacervate in an aqueous solution. Therefore, to extend its shelf-life, an additional drying process should be applied, which can be harmful to cells (Chavarri et al. 2012).

In the study of Oliveira et (2007) microcapsules of *Bifidobacterium lactis* and *Lactobacillus acidophilus* were produced by complex coacervation using a casein/pectin complex as the wall material, followed by spray drying. The results showed that the process used and the wall material were efficient in protecting the microorganisms under study against the spray drying process and simulated gastric juice. However, microencapsulated *Bifidobacterium lactis* lost its viability before the end of the storage time.

5.2.2.7. Electrospinning

Electrospinning technology utilizes electrostatic forces to generate polymer fibers, and consists of a capillary through which the liquid to be electrospun is pumped; a high voltage

source (1–30 kV) with positive or negative polarity, generates a charge in the polymer solution and a grounded collector, which is brought into contact with the counter electrode (Bhardwaj and Kundu 2010, Greiner and Wendorff 2007, Sill and von Recum 2008). The high voltage is applied to the droplet or to the needle (if it is conductive) leading to formation of repulsive interactions between the same charges in the liquid and the attractive forces between the oppositely charged liquid and collector and consequently elongating the pendant drop (Fathi et al. 2014).

According to Agarwal et al. (2008) the major advantages of this technique are the production of very thin fibers or capsules to the order of few nanometers with large surface areas. In addition, the possibility of large scale productions combined with the simplicity of the process makes this technique very attractive for many different applications.

Probiotic encapsulation has been carried through electrospinning using a protein based matrix (whey protein concentrate) and a carbohydrate based matrix (pullulan). Whey protein concentrate have demonstrated greater protection ability as encapsulation material than pullulan as it effectively prolonged the survival of the cells even at high relative humidity (López-Rubio et al. 2012).

5.2.2.8. Hybridization System

Hybridization system is a dry technique and consists of a high-speed rotating rotor with six blades, a stator and a powder recirculation circuit. The powder mixture (host and guest particles) placed in the vessel is subjected to high impaction in air stream generated by the blade rotating at high speed. During the process, the particles form ordered mixture by embedding or filming of the guest particles onto the surface of the host particles. When compared with other microencapsulation techniques, including spray-drying, the hybridization system results in high yields of microcapsules and minimizes heat induced bacterial damage using a cooling system that maintains temperatures below 30°C (Takafumi et al. 1993).

There are some prebiotic substances that have been tested with this technique, such as sorbitol, mannitol, lactulose, xylitol, inulin, fructo-oligosaccharide and raffinose. The results indicate that double microencapsulation by hybridization is useful to effectively provide beneficial effects of probiotics for the host (Ann et al. 2007).

5.2.2.9. Impinging Aerosol Technology

Impinging aerosol technology is a new continuous encapsulation process that uses separate impinging aerosols of sodium alginate solution and calcium chloride cross-linking solution to produce water insoluble cross-linked alginate micro beads with an average diameter of less than 40 μm (Sohail et al. 2011). Since there is no heat and no solvent is used, this method is suitable for encapsulating heat labile and solvent sensitive materials. The method has a great deal of production capacity and microspheres can be spray or freeze dried (Sohail et al. 2012).

In the study of Sohail et al. (2011) it was demonstrated that the microbeads obtained by impinging aerosol technology and extruded macrobeads (approximately 2 mm diameter) offered similar protection to *Lactobacillus rhamnosus* GG in the acid and bile tolerance study. Sohail et al. (2012) investigated the effect of the microencapsulation on the survival of *Lactobacillus rhamnosus* GG and *Lactobacillus acidophilus* NCFM and their acidification in orange juice at 25°C for nine days and at 4°C over thirty five days of storage. The results

showed that unencapsulated *Lactobacillus rhamnosus* GG was found to have excellent survivability in orange juice at both temperatures. However, unencapsulated *L. acidophilus* NCFM showed a significant reduction in viability. The encapsulation of these two bacteria did not significantly enhance survivability but did reduce acidification at 25°C and 4°C. In conclusion, *Lactobacillus rhamnosus* GG showed excellent survival in orange juice and the microencapsulation has potential in reducing acidification and possible negative sensory effects of probiotics in orange juice and other fruit-based products.

5.2.3. Sensory Quality of Foods with Microencapsulated Probiotics

Despite the benefits of probiotic encapsulation, there are some consequences as well, such as the sensory quality of foods. The shape and size of capsules affecting the sensory quality of final products are important issues for industrial production (Iravani et al. 2015, Rokka and Rantamäki 2010). For example, in spray drying, the outlet temperature may affect the colour of the capsules due to a Maillard reaction (McMaster and Kokott 2005, O'Riordan et al. 2001, Su et al. 2007). In the case of the yoghurts, the addition of encapsulated probiotic bacteria did not significantly change the appearance and color, acidity, flavor or aftertaste, but significantly affected their textural properties (smoothness) (Krasaekoopt et al. 2006).

Although microencapsulation of probiotic cells can be applied as an efficient method to improve the sensory attributes of the probiotic fermented milks, its unsuitable usage might lead to the off flavor and/or off texture of the final product (Iravani et al. 2015). According to Hansen et al. (2002) the encapsulation of *Bifidobacterium longum* and *Bifidobacterium lactis* in milk led to an especial off-flavor which was not observed in the product containing free cells of the same bacteria. This fact was attributed to changes in the metabolic pathways of the encapsulated cells which caused production of small-bitter peptides.

Adhikari et al. (2002) observed that encapsulation lowered the acetic acid content in yoghurt significantly if bifidobacteria were added to the product before fermentation. The study of Kailasapathy et al. (2006) has shown that addition of probiotic capsules did not significantly alter the appearance and colour, acidity, flavour and after taste attributes of the yoghurts, however, significantly altered the textural properties (smoothness) of yoghurts. The panelists reported grittiness in yogurts with encapsulated probiotic cultures. This study also showed that the starch and sodium alginate used in the capsular matrix may have an influence on the mouthfeel of the product and that microencapsulation enhanced the survival of probiotic cultures compared to free cells in yogurts stored over 7 weeks. On the other hand, McMaster et al. (2005) detected no grittiness or texture change in the African fermented foods *mahewu* and *amasi* containing *Bifidobacterium lactis.* These results are similar to those obtained in the study of Muthukumarasamy and Holley (2006), in which no significant difference in sensory quality was found between control and sausages containing either unencapsulated or microencapsulated *Lactobacillus reuteri.*

In the study developed by Gardiner et al. (2002) they found that the composition of Cheddar cheese was not affected by the addition of *Lactobacillus paracasei* spray dried with skimed milk. Its sensory quality also reached the scores required for commercial-grade Cheddar cheese in Ireland.

According to Hansen et al. (2002) it is desirable to obtain a size below 100 μm in order to prevent negative sensorial impacts of microcapsules in the food products.

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Honors:

- 2015: Paper awarded a certificate of Best Scientific paper CI&DETS published in 2014: Silva V, Figueiredo AR, Costa JJ, Guiné RPF. (2014) Experimental and mathematical study of the discontinuous drying kinetics of pears. Journal of Food Engineering, 134, 30-36.
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Chapter 6

PROBIOTIC YOGURT AS A FUNCTIONAL FOOD

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ABSTRACT

Yogurt has long been known as a product with many desirable effects for health. The excellent sensory properties and the health benefits of yogurt can be credited to the action of yogurt bacteria and their metabolites. Probiotic is a dietary supplement of live microorganism that contributes to the health of the host. The most common types of microbes that used as probiotic are lactic acid bacteria (LAB) particularly Lactobacilli, Streptococci and Bifidobacteria. They are the most important microorganisms associated with the health status of human gastrointestinal tract which justifies the reason for calling them friendly bacteria. Therefore, this study is to investigate the inclusion of probiotics in yogurt as functional food.

Keywords: yogurt, probiotics, milk fermentation

1. INTRODUCTION

Functional food is not only a dietary product providing basic nutritional function of supplying nutrients but it is also known as a health-promoting and/or disease-preventing (Korhonen and Pihlanto-Leppälä, 2004). Yogurt is one of the most-widely studied functional food. The major beneficial function of yogurt is played by the lactic acid bacteria (LAB). Yogurt is defined as a coagulated milk by lactic acid bacteria *Lactobacillus bulgaricus* and *Streptococcus thermophilus* which produce lactic acid in milk during fermentation. The word yogurt derives from the Turkish yoğurt, originates from the adjective 'yoğun', which means “dense” and “thick”. It is a smooth pleasant, firm, tenacious, custard-like milk with high acidity and the characteristic sour taste (Hoier, 1992).

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The nutritional value of yogurt is made up of milk's nutrients and the byproducts of probiotic fermentation of milk. It is believed that these nutrients are capable to confer a variety of important nutritional and therapeutic benefits to consumers (Naidu and Clemens, 2000). The growth of probiotic in milk resulted in several important changes in the milk components which include reduction in lactose concentration and milk components degradation associated with lipolysis and proteolysis. Reduction in lactose concentration made milk more acceptable to lactose intolerant consumers, whereas lipolysis made milk more digestible and also yield few fatty acids, some of which (e.g., palmilk acid) may confer antimutagenic activities. Therefore, this study is to investigate the inclusion of probiotics in yogurt as functional food and their health properties.

2. WHAT ARE FUNCTIONAL FOODS?

Foods are functional when they provide additional properties other than nutritive values. However, added physiologic benefits to foods are now being examined intensively which may either a state of well-being and health and/or to the reduction of the risk of a disease. Functional foods have no universally accepted definition. The concept was first developed in Japan in the 1980s when the Ministry of Health and Welfare introduced a controlling system to approve certain foods with recognized health benefits in hopes of developing the health of the nation's aging population (Yamada *et al*., 2008). These foods are also called "Foods for Specified Health Use" (Yamada *et al*., 2008). Functional foods may also be defined as "any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (Hasler, 2002). The International Life Sciences Institute defines them as further refinement i.e., "foods that, by virtue of the presence of physiologically-active components, provide a health benefit beyond basic nutrition" emphasise the importance of therapeutical values inherent in functional foods. Functional foods must not be consumed as medicine but rather as foods that are "whole, fortified, enriched or enhanced" but more importantly, states that such foods must be consumed as "part of a varied diet on a regular basis, at effective levels " for consumers to reap their potential health benefits" (American Dietetic Association, 1999). Nutraceuticals is another term commonly used with functional foods. This term created in 1991 by the Foundation for Innovation in Medicine refers to nearly any bioactive component that provides a health benefit.

A food can be assumed to be functional if it follows one of the next criteria (Ramchandran and Shah, 2009):

a It comprises a food component (being nutrient or not) which have positive effects targeted one or a limited number of function(s) in the body.
b It has physiological or psychological properties further than the traditional nutritional effect.

The component that makes the food "functional" can be 'either an essential macronutrient with particular physiological properties or an essential micronutrient for body needs on a daily basis. In addition, some of food components may not recorded as essential, such as

some oligosaccharides, or they have no nutritive value, such as live microorganisms or plant chemicals' (Nakakuki, 2002). The main types of functional foods are indicated in Table 1.

Table 1. Different types of functional foods

Type	Description	Some examples
Fortified products	Increasing the content of existing nutrients	Grain products fortified with folic acid, fruit juices fortified with additional vitamin C
Enriched products	Adding new nutrients or components not usually present in a certain food	Fruit juices enriched with calcium, foods with probiotics and prebiotics
Altered products	Replace existing components with beneficial components	Low-fat foods with fat replacers
Enhanced commodities	Changes in the raw commodities that have altered nutrient composition	High lysine corn, carotenoid containing potatoes, lycopene enhanced tomatoes

(Source: Spence, 2006)

3. FUNCTIONAL DAIRY PRODUCTS

Dairy products are established as healthy natural products and they form one of the four major food groups (the other three being protein, fruits and vegetables and grains) that make up a balanced diet (Ramchandran and Shah, 2009). Regular consumption of certain dairy products has beneficial effects in the prevention of disease (Bozanic *et al*., 2001) because they contain a number of active compounds with putative roles in both nutrition and health protection (Table 2).

4. YOGURT

Yogurt is defined by the Codex as a coagulated milk product that results from the fermentation of lactic acid in milk by *Lactobacillus bulgaricus* and *Streptococcus thermophilus.* The word yogurt derives from the Turkish yogurt, originates from the adjective yogun which means dense and thick. It is a smooth pleasant, firm, tenacious, custard-like milk with high acidity and the characteristic sour taste (Davis *et al*., 1971). Any sort of milk may be used to make yogurt but modern production is dominated by cow's milk. Under normal storage conditions at low temperatures, yogurt has an expected shelf life of 30 days.

Yogurt is a product which is obtained from a biological type of food preservation (fermentation) or more precisely, from the spontaneous or controlled acidification of milk. The product's acidification happens when lactose (milk's sugar) is converted into two simpler components, glucose and galactose with the production of lactic acid. This quality makes the product more suitable for individual suffers from milk intolerance that caused by the lack of an enzyme lactase.

Table 2. Dairy components and ingredients in functional foods and their health claims

Component	Sources	Claim areas
Minerals	Calcium Casein peptides	Optimum growth and development, dental health, osteoporosis
Fatty acids	Conjugated linoleic acid (CLA)	Heart disease, cancer prevention, weight control
Prebiotics/carbohydrates	Galactooligosaccharides Lactulose Lactose	Digestion, pathogen prevention, lactose intolerance, immunity and gut flora balance
Probiotics	Lactic acid bacteria Bifidobacteria	Immunity, heart disease, digestion, vitamin production, remission of inflammatory bowel disease, antitumor activity, alleviation of diarrhea and prevention of allergy
Proteins/peptides	Whey proteins, caseins, lactoferrin, immunoglobulins, glycoproteins, specific peptides	Growth, antibacterial activity, dental health, immunomodulation and hypertension regulation (angiotensin inhibitors)

(Source: Shortt *et al*., 2004)

The fermenting process is triggered by lactic acid bacteria (e.g., *L. delbrueckii subsp. bulgaricus* and *S. thermophilus*). The lactic cultures are mono-cellular organisms and there should be at least 2 million viable bacteria per gram consumed to present a live in a quality yogurt (Viljoen *et al.,* 2001). The lipidic part of the fermented product remains almost identical to that of original milk while the proteins (milk casein) are partially hydrolysed and become more digestible (Viljoen *et al.,* 2001). In comparison with cheeses, the serum proteins in yogurt (lacto-albumin and lacto-globulin) remain within the product and the simultaneous presence of lactose and lactic acid allows micro-components such as calcium and phosphorus which can be found in abundance in both milk and yogurt to become more widely and immediately available (Haertlle *et al.*, 2002) The fermented product's high level of acidity encourages the development of the intestine's bacterial flora which is capable of successfully blocking the putrefactive phenomena within the human intestine.

In recent years the consumption of yogurt has increased rapidly owing to the fact that this dairy product fulfils many of the current dietary needs. It is a ready-to-eat food, relatively low in fat and rich in nutritional components. Furthermore, the demand for functional foods has been boosted in recent years as a result of growing awareness among consumers of the link between diet and health (FitzGerald *et al*., 2004). These are briefly outlined in the following sections.

5. FERMENTATION PRODUCTS OF YOGURT

During the production of yogurt, changes to the milk constituents refer to fermentation and the ingredients added during manufacturing. Changes induced during fermentation

include the fermentative action of the inoculated starter cultures, the secretion of nutritional and chemical substances by the microorganisms as well as the presence of the microorganisms and their associated enzymes (Gurr, 1987).

The primary role of lactic acid bacteria is to utilize lactose as a substrate and convert it into lactic acid during fermentation of milk. Lactose is taken up as the free sugar and split with b-galactosidase to glucose and galactose. Both glucose and galactose are metabolized simultaneously via the glycolytic and d-tagatose 6- phosphate pathways respectively (Thomas and Crow, 1984). In addition galactose can also be further metabolised by enzymes of the Leloir pathway (Hutkins *et al*., 1985). The lactic acid present in yogurt is produced from the glucose moiety of lactose rather than the galactose moiety. Hence, galactose accumulates in fermented milk products. Free galactose can later be utilised by *S. thermophilus* or *L. bulgaricus*. This suggests that the enzymes for galactose metabolism are present but at low activity (Thomas and Crow, 1984). The lactose concentration in yogurt is lowered provided that no milk powder was added while the concentration of galactose present is higher compared to milk. The protein content of protein-enriched yogurt (addition of milk powder) is increased to 4–5% whereas normal yogurt exhibits an average protein content of 2% (Renner, 1983). The total amino acid content of yogurt does not differ from milk but the free amino acid content is higher due to proteolytic activity of microorganisms (Rasic and Kurmann, 1983).

The microbial inoculum has a substantial influence on the vitamin content of yogurt. Some bacteria require B vitamins for growth. Fermentation has little effect on the mineral content of milk and the total mineral content remains unaltered in the yogurt (Gurr, 1987).

In summary, the concentrations of lactic acid, galactose, free amino acids and fatty acids increase as a result of fermentation while lactose concentration decreases, whereas the addition of ingredients mainly increases the protein and sugar content.

6. PROBIOTIC

Interest in the role of probiotics for human health goes back to 1908 when Metchnikoff suggested that man should consume milk fermented with *lactobacilli* to prolong life (O'Sullivan *et al*., 1992). It is only recently understood that the interrelationship between intestinal microorganisms and the health benefits is derived from milk fermentation.

An optimum balance in microbial population in our digestive tract is associated with good nutrition and health (Rybka and Kailasapathy, 1995). The microorganisms primarily associated with this balance are *lactobacilli* and *bifidobacteria*. Negative factors that influence the interaction between intestinal microorganisms such as stress and diet, lead to detrimental effects in health. Increasing evidence indicates that consumption of probiotic microorganisms can help maintain such a favourable microbial profile and results in several therapeutic benefits. In recent years probiotic bacteria are incorporated into foods as dietary adjuncts.

One of the most popular dairy products for the delivery of viable *L. acidophilus* and *B. bifidum* cells is bio-yogurt. Minimum numbers of viable cells required to achieve the therapeutic properties need to be consumed regularly for transfer of the probiotic effect to consumers. Consumption should be more than 100 g per day of bio-yogurt containing more

than 10^6cfu/ml (Rybka and Kailasapathy, 1995). Survival of these bacteria during shelf life and until consumption is an important consideration.

6.1. Definition of Probiotics

The word probiotic" was derived from the Greek language which means for life" (Fuller, 1989). Probiotic would also substances produced by protozoa that stimulate the growth of other organisms and substances that have a beneficial effect on the host animal by contributing to its intestinal microbial balance. These general definitions were unsatisfactory because substances include chemicals such as antibiotics. The definition of probiotics was expanded to stress the importance of live cells as an essential component of an effective probiotic. Huis and Havenaar (1991) broadened the definition of probiotics to being a mono- or mixed-culture of live microorganisms which applied to man or animal (e.g., as dried cells or as a fermented product) beneficially effects the host by improving the properties of the indigenous microflora. This definition implies that probiotic products for example bio-yogurt contain live microorganisms and improve the health status of the host by exerting beneficial effects in the gastrointestinal tract.

6.2. Therapeutic Potentials of Probiotics

6.2.1. Reducing Lactose Intolerance

Some people are unable to digest lactose adequately due to the absence of beta-d-galactosidase in the human intestine and this leads to various degrees of abdominal discomfort (Kim and Gilliland, 1983). Lactic acid bacteria commonly used as starter cultures in milk and fermentation such as *L. acidophilus* and *B. bifidum* produce β -d-galactosidase. This utilisation is referring to intra-intestinal digestion by β-d-galactosidase. On the other hand, some lactic acid bacteria hydrolyse lactose by phosphor β-galactosidase which may not be as effective in the intestine (Kim and Gilliland, 1983). Investigated showed the effect of *L. acidophilus* as a dietary adjunct in milk to aid lactose digestion in humans. Improved digestion of lactose was shown not caused by hydrolysis of the lactose prior to consumption but rather after the consumption of milk that contains *L. acidophilus.* The continued utilisation of lactose within the gastrointestinal tract was suggested to depend on the survival of the *lactobacilli* in that environment.

6.2.2. Control of Intestinal Infections

Probiotic bacteria such as *bifidobacteria* and *lactobacilli* possess antimicrobial properties (Hughes and Hoover, 1991). Both L. *acidophilus* and *B. bifidum* have been shown to inhibit many commonly known food borne pathogens (Rasic and Kurmann, 1983; Lim *et al*., 1993). Several studies indicated the preventive control of intestinal infections through administering milk cultured with *L.acidophilus* or *B. bifidum* or both (Rasic and Kurmann, 1983, Gorbach *et al*., 1987).

Mechanisms for the inhibition of pathogens ascribed to lactobacilli and bifidobacteria include:

- The production of inhibitory/antimicrobial substances such as organic acids, hydrogen peroxide, bacteriocins, antibiotics and deconjugated bile acids.
- Their acting as competitive antagonists i.e., competition for adhesion sites and nutrients.
- Stimulation of the immune system.

Production of organic acids by the probiotics lowers the pH and alters the oxidation–reduction potential in the intestine. Antimicrobial action combined with the limited oxygen content in the intestine and organic acids will inhibit especially pathogenic gram-negative bacteria type's e.g., coliform bacteria (Sandine, 1979).

Bifidobacteria produce both lactic and acetic acids, but higher amounts of acetic acid produced exhibits a stronger antagonistic effect against gram negative bacteria than lactic acid (Rasic and Kurmann, 1983). Probiotic microorganisms may prevent harmful bacterial colonisation either by competing more effectively than an invading strain for essential nutrients or adhesion sites or by making the local environment unfavourable for the growth of the invader by producing antibacterial substances (Sandine, 1979; Gurr, 1987). Regular consumption of probiotic bacteria may induce an improved immunological response in humans (Rasic and Kurmann, 1983).

6.2.3. Reduction in Serum Cholesterol Levels

Consumption of fermented milk significantly reduces serum cholesterol (Gilliland *et al*., 1985; Gilliland, 1989). For hypercholesterolemic individuals, significant reductions in plasma cholesterol levels are helps to reduce the risk of heart attacks. The principal site of cholesterol metabolism is the liver, although appreciable amounts are formed in the intestines. Certain *L. acidophilus* strains and some *bifidobacteria* species are able to lower cholesterol levels within the intestine. Lactic acid bacteria produce the lactic acid that causes the declines of pH. This results in cholesterol to co-precipitatate with deconjugated bile salts (Marshall, 1996). The role of bifidobacteria cultures in lowering serum cholesterol is not yet understood. In rat models, serum cholesterol was lowered by feeding bifidobacteria in a mechanism involving HMG-CoA reductase (Homma, 1988). Gilliland, (1989) concluded that unknown factor is produced in the fermented milk that inhibits cholesterol synthesis in the body.

Another theory is that the deconjugated of bile acids into free acids (by *L. acidophilus*) are excreted more rapidly from the intestinal tract than conjugated bile acids. Free bile salts are excreted from the body necessitates the synthesis of new bile acids from cholesterol to reduce the total cholesterol concentration in the body (Gilliland and Speck, 1977). A third hypothesis is reduction of cholesterol may be due to a co-precipitation of cholesterol with deconjugated bile salts at lower pH values as a result of lactic acid production by the bacteria (Kailasapathy and Rybka, 1997).

6.2.4. Anticarcinogenic Activity

The antitumour action of probiotics is thought to occur via the inhibition of carcinogens and/or procarcinogens formation, inhibition of bacteria that convert procarcinogens to carcinogens (Gorbach *et al*., 1987; Gilliland, 1989), activation of the host's immune system (Rasic and Kurmann, 1983) and/or reduction of the intestinal pH to reduce microbial activity. A report from several animal studies has established the intake of yogurt and fermented milks

containing probiotic bacteria inhibited tumour formation and proliferation (Kailasapathy and Rybka, 1997).

Yogurt has similar nutritional values as milk. Yogurt excelled milk in terms of better digestibility and improved nutrition absorption. Lactase-deficient individuals have impaired calcium absorption. Thus, the consumption of yogurt provides them an alternative to prevent osteoporosis.

6.2.5. Discouraging Vaginal Infection

Candida or yeast vaginal infections are a common problem for women with diabetes. *Lactobacillus acidophilus*, a strain of friendly bacteria is an integral part of normal vaginal flora. Lactobacilli prevent overgrowth of unfriendly bacteria and *Candida*. Besides, these friendly bacteria compete with other organisms for the utilization of glucose. Furthermore, the acidic pH which acts like a natural antibiotic (Sandine, 1979) needed for healthy vaginal flora is maintained by the presence of *lactobacilli* that produce lactic acid and hydrogen peroxide.

7. APPLICATION OF PROBIOTIC MICROORGANISMS IN FUNCTIONAL FOODS

Consumption of probiotic bacteria via food products is an ideal way to re-establish the intestinal microflora balance. For a culture to be considered a valuable for use as a dietary adjunct and to exert a positive influence, it must conform to certain requirements (Martin and Chou, 1992) such as the culture must be a normal inhabitant of the human intestinal tract, survives passage through the upper digestive tract in large numbers, be capable of filling an ecological niche and have beneficial effects in the intestine (Gilliland, 1989). In order to survive, the strain must be resistant to bile salts present in the lower intestine, gastric conditions (pH 1–4), enzymes present in the intestine (lysozyme) and toxic metabolites produced during digestion (Hoier, 1992). The bacteria used in traditional yogurt fermentation *L. bulgaricus* and *S. thermophiles* do not belong to the indigenous intestinal flora, not bile acid resistant and do not survive passage through the gut (Gilliland, 1979). These traditional yogurt bacteria may have positive effects as a result of fermentation metabolites either by an inhibitory action towards pathogens or improvement of lactose digestion (Hoier, 1992). Not less than a million viable cells/mL probiotic products have to be present for transfer of the probiotic effect to consumers (Rybka and Kailasapathy, 1995). It is desirable that probiotic culture multiply to reach high cell counts in the fermented product and possess a high acid tolerance to ensure high viable cell numbers during storage. The selected strains must be able to ferment milk quickly either alone or in combination with other strains.

The possibility of influencing the composition of the intestinal flora by consuming probiotic bacteria partly depends on the dose level. It is generally recognised that 10^8–10^9 bacteria are necessary at the time of consumption (Speck, 1978). Therefore, the probiotic culture must remain viable in the food carrier up to consumption.

A number of food bioproducts have been employed or in the process of being developed to enhance their usage as delivery vehicles of probiotic cells fed to humans. Approximately 80 bifid-containing products are estimated to be on the world market (Hughes and Hoover, 1991). Most of these products are of dairy origin and include fresh milk (Klaver *et al*., 1993),

fermented milk (Tamime *et al*., 1995), beverages, cheese (Gomes *et al*., 1995; Roy *et al*., 1995), cottage cheese (Blanchette *et al*., 1995), powdered milk, cookies, health foods, ice cream (Hekmat and McMahon, 1992) and dairy desserts (Laroia and Martin, 1991).

Some of these products in addition to the probiotic contain inulin or oligofructose as bifidogenic factors also called prebiotics. While bifidobacteria are difficult to propagate in food due to oxygen sensitivity and low acid tolerance the addition of prebiotics to dairy foods may lead to promising results to ensure the presence of high numbers of bifidobacteria during normal shelf life of the dairy products (Modler *et al*., 1990). Prebiotics are used to supplement human diets and support the growth of bifidobacteria in the intestine hence the name bifidogenic factors (Modler *et al*., 1990). Prebiotics are complex sugars that cannot be metabolised directly by humans but serve as a carbohydrate source for intestinal flora. Oligosaccharides such as fructo-oligosaccharides, lactulose, raffinose, stachyose and inulin oligomers are used as prebiotics or bifidogenic factors.

8. YOGURT AS A PROBIOTIC CARRIER FOOD

Since the renewed interest in probiotics, different types of products were proposed as carrier foods for probiotic microorganisms by which consumers can take in large amounts of probiotic cells for the therapeutic effect. Yogurt is recognised as a product with many desirable effects for consumers and most consumers consider yogurt to be healthy. Significant increase in the popularity of yogurt (Hamanna and Marth, 1983) as a food product was attributed to the incorporation of *L. acidophilus* and *B. bifidum* into yogurt to add extra nutritional-physiological value. This is because conventional yogurt starter bacteria, *L. bulgaricus* and *S. thermophilus* lack the ability to survive passage through the intestinal tract and because of that do not play a role in the human gut (Gilliland, 1979). In recent years some yogurt products have been reformulated to include live strains of *L. acidophilus* and species of *Bifidobacterium* (known as AB-cultures) in addition to the conventional yogurt organisms, *S. thermophilus* and *L. bulgaricus*. Therefore, bio-yogurt is yogurt that contains live probiotic microorganisms that give beneficial health effects.

In conclusion, it has been believed that the consumption of bio-yogurt provides many health benefits. Recent studies of the possible health benefits of yogurt can be increased by manipulation of bacterial fermentation of milk in the presence of herbal extracts. This may imply unique properties related to the interaction between herbal extracts and milk in the presence of probiotics resulted in formation of bioactive health compounds. Therefore, further studies are need to investigate the ability of herbal medicines and phytonutrients or nutraceuticals to improve the health benefits of bio-yogurt.

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Chapter 7

ENHANCING YOGURT HEALTH BENEFITS: DEVELOPMENT OF STARTERS FOR DAIRY AND NON-DAIRY YOGURT

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ABSTRACT

Yogurt supplies the organism with proteins, lipids, carbohydrates, vitamins and microelements and imports active yogurt microflora. Therefore traditional yogurt is a suitable basis for the development of new functional foods.

Starter culture development, including strain property studies and development of symbiotic relationships between the strains in the starter composition, is of paramount importance in yogurt manufacture. Any failure in the starter development will, in most cases, lead to detrimental effects on the quality of the final product. One of the aims of the present chapter is to present our experience in the development of yogurt starters, including the selection of strains of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* and the development of symbiotic starters for traditional yogurt.

Nowadays, most consumers choose foods not only based on their flavor and direct nutritional value, but also on their potential health benefits. Functional foods fulfill the organism's nutritional needs and improve its general condition (e.g., pre- and probiotics), reduce the risk of certain diseases (e.g., cholesterol-lowering products), and can be used to treat other diseases. Development of new functional foods is a good opportunity to improve food quality and enhance its health benefits; thus, they are the subject of growing industrial and social interest in contemporary society.

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Only species and strains that meet the specified requirements for probiotic strains can be included in the composition of probiotics and milk-based and non-milk-based probiotic foods. For each food type are selected microorganism strains that by performing their inherent metabolic processes are involved in flavor and aroma formation, influence the shelf life of the finished foods and contribute to the health benefits of their consumption.

Another aim of the present chapter is to present our experience in the development of multistrain starters for yogurt series with the inclusion of different probiotic strains - yogurt enriched with *Lactobacillus delbrueckii* ssp. *bulgaricus*; *Lactobacillus gasseri*; *Lactobacillus acidophilus*; *Bifidobacterium* strains - *Bifidobacterium longum*, *Bifidobacterium breve*, and *Bifidobacterium infantis*. The resulting yogurts retain high concentration of viable cells of the probiotic strain included, preserve their organoleptic characteristics and moderate titratable acidity for 30 days of refrigeration storage.

Proteins of plant origin present a good alternative for partial substitution of animal milk in the production of different dairy foods and beverages. Their application as substrates for lactic acid bacteria and bifidobacteria growth allows the development of non-milk-based probiotic fermented foods that bring the valuable characteristics of plants and the biological activity of probiotic microorganisms together. We have developed soy yogurts (soy acidophilic yogurt and soy bifid yogurt and yogurt beverages). The active cell concentration in the finished products exceeds 10^9 cfu/cm^3, and their shelf life is more than 20 days at refrigeration conditions. The organoleptic evaluation showed blinding of the soy off-flavor.

The wide range of milk-based and non-milk-based yogurts can be used as functional foods and beverages, providing the organism with both the inherent traditional yogurt health benefits and the required amount of beneficial microflora to exert its preventive role.

Keywords: yogurt, symbiotic starter, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*, probiotic, soy yogurt

INTRODUCTION

1. Yogurt Production

The development and production of fermented dairy products like yogurt dates back to 1300 BC in the Middle East (Harding 1995). Nomadic people from the Near East are supposed to have brought them into European and Russian areas from where they were spread all over the world (Chandan 2006).

The first scientific notion of probiotic microorganisms can be traced to the Nobel Laureate Ilya Metchnikoff, who noticed that the long healthy life of Bulgarian people was due to the consumption of yogurt containing high concentrations of live lactic acid bacterial cells (Vasiljevic and Shah, 2008; Figueroa-Gonzalez et al., 2011).

Henry Tissier also gave a push to the exploration of lactic acid bacteria. He investigated the relationship between bifidobacteria contained in breast milk and the prevention of nursed infants' infections and he found out that babies consuming breast milk suffer less from infections (Farnworth 2003). Metchnikoff's and Tissier's work put the foundations for the production of probiotic foods and beverages including live probiotic bacterial cells.

Codex Almentarius defines fermented milk as a "milk product obtained by fermentation of milk, which milk may have been manufactured from products obtained from milk with or without compositional modification, by the action of suitable microorganisms and resulting in reduction of pH with or without coagulation (iso-electric precipitation)" (Mohammadi et al., 2012)

Dairy-based functional foods account for almost 43% of the market of functional foods, which is almost entirely made up of fermented dairy products. Saxelin et al., 2003 divide dairy products into three groups:

1. Basic milk products, which include classical dairy products such as yogurt, cheese, butter, ice cream, etc.
2. Added-value products, which include low-lactose or lactose-free products, hypoallergenic formulae with hydrolysed protein for milk-hypersensitive infants, enriched milk with Ca, vitamins, etc.
3. Functional dairy foods, which include products that are enriched with functional food components originating from dairy and nondairy sources. The products in this group demonstrate health benefits beyond their basic nutritional value. Probiotics are the major bioactive components included in the composition of fermented functional dairy foods and numerous economic indicators demonstrate that probiotic-enriched products are still in the forefront of innovation in the functional food sector (Champagne, 2009).

Modern yogurt production is a well-controlled fermentation process that utilizes milk, milk powder, sugar, fruit, flavours, colouring agents, emulsifiers, stabilizers, and specific pure cultures of lactic acid bacteria (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*) to conduct the fermentation process.

A number of processes occur during yogurt fermentation: milk proteins hydrolysis, dropping of the pH, increasing of the viscosity, and synthesis of bacterial metabolites that contribute to the taste and possibly to the health promoting properties of yogurt (Farnworth et al., 2007).

Fermented foods are widely known to possess various nutritional and therapeutic properties. Lactic acid bacteria (LAB) play a major role in determining the beneficial health effects of fermented milk and related product consumption (Santiago-Lopez et al., 2015).

As a result of the fermentation process is obtained a product with high concentration of viable cells of the strains from the composition of the starter, which is retained during refrigeration storage (Codex of Fermented milk, 2003). The classical scheme for the production of traditional Bulgarian yogurt is given in Figure 1.

Lactic acid bacteria are susceptible microorganisms and their growth is often limited by the lack of some nutrients in milk.

Streptococcus thermophilus and *Lactobacillus delbrueckii* ssp. *bulgaricus* exhibit a symbiotic relationship during the processing of yogurt, with the ratio between the species changing constantly (Lynradke-Mitchell and Sandine, 1984). Both bacteria can grow separately in milk, but in combination a mutually beneficial interaction called protocooperation is observed. This positive relationship between the two microorganisms has a positive effect on the growth and development of the two starter species, on the production

of lactic acid and flavoring components. Protocooperation is a result of the metabolic activity of the two microorganisms.

During fermentation, *Streptococcus thermophilus* grows quickly at first, utilizing essential amino acids produced by *Lactobacillus delbrueckii* ssp. *bulgaricus* as a result of milk protein hydrolysis. *Streptococcus thermophilus*, in return, produces lactic acid, which results in pH reduction to an optimal level for *Lactobacillus delbrueckii* ssp. *bulgaricus* growth. The lactic acid and lesser amounts of formic acid produced stimulate *Lactobacillus delbrueckii* ssp. *bulgaricus* growth. Both species produce β-galactosidase and are able to utilize lactose to produce D (-) and L (+) lactic acid which plays a significant role in pH dropping of the medium to pH=4,8 – 4,2 (Lourens-Hattingh and Viljoen, 2001). At these pH values are observed structural changes in the casein-calcium complex that lead to milk coagulation. The concentration of lactic acid, the released galactose, free amino acids and fatty acids, the released acetaldehyde form the yogurt sensory profile. Of particular importance for yogurt quality is the ratio of *Lactobacillus delbrueckii* ssp. *bulgaricus* to *Streptococcus thermophilus* in the composition of the symbiotic starters. The most appropriate ratio of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* is 1:2 to 1:4. The balance between lactobacilli and streptococci determines yogurt taste. When streptococci prevail yogurt with gently sour taste with stronger diacetyl and acetaldehyde flavor is obtained, while the dominance of lactobacilli results in a sharp, sour flavor and good inherent yogurt aroma (Marshall, 1987). This ratio is influenced by the culturing temperature and the environmental conditions.

The mutual growth of both cultures in the composition of the starter leads to a more efficient acid formation, a higher final concentration of viable cells in resulting yogurt (Amoroso and Nanca de Nandra, 1990) and an increase in the specific growth rates (Driessen et. al.1981) compared to separate cultivation.

The positive effect of the protocooperation between the two starter species is confirmed by the following features of their metabolism during their mutual growth in milk:

1. The two bacterial starter species coagulate separately the sterilized milk at 45°C for 6-10 h, but in co-culturing the milk coagulates for 2.0 - 2,5 h (Kondratenko et al., 1985; Chomakov, 1986)
2. The milk coagulated with monocultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, is characterized by texture, taste and flavor different than the inherent ones of milk coagulated with mixed culture. In the joint development of the two thermophilic species the coagulated milk is thick in texture with pronounced inherent yogurt flavor (Kondratenko et al., 1985; Matalon and Sandine, 1986);
3. More volatile flavoring agents, acetaldehyde, diacetyl and acetone are produced in the co-culture of the two bacterial starter species in milk (Chomakov, 1986; Obretenova, 1987; Simova, 2007);
4. In co-culturing both starter species possess higher acid resistance (Obretenova, 1987);
5. In separate cultivation *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* quickly lose their inherent morphological properties and degenerate, while in their association they retain these qualities for a long time (Tamime and Robinson, 2003; Chomakov, 1986; Kondratenko et al., 1985).

The contribution of each culture in the symbiotic development is considered in a number of publications (Driessen et al., 1981; Thunell and Sandine, 1985) and some key elements in stimulating the mutual growth of lactobacilli and streptococci are established. The cells of *Streptococcus thermophilus* produce a large amount of carbon dioxide (CO_2) which is not formed from the lactose metabolism of *Lactobacillus delbrueckii* ssp. *bulgaricus* as *Lactobacillus delbrueckii* ssp. *bulgaricus* is a strictly homofermentative species (Driessen et al., 1981, Thunell and Sandine, 1985). CO_2 formation is due to the activity of urease synthesized by *Streptococcus thermophilus*, which breaks down milk urea to CO_2 and NH_3 (Eck and Gillis, 1997; Juillard et. al., 1998). The scientific literature has argued that the CO_2 produced by *Streptococcus thermophilus* as a result of urea hydrolysis stimulates the development of *Lactobacillus delbrueckii* ssp. *bulgaricus* during co-culturing (Driessen et al., 1981; Louaileche et al., 1993; Louaileche et. al., 1996).

Streptococcus thermophilus stimulates the growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* in several ways – by reducing the active acidity of milk, by changing the redox potential of the medium, the consumption of the dissolved oxygen in milk and by CO_2 formation, thus crearing the anaerobic conditions necessary for *Lactobacillus delbrueckii* ssp. *bulgaricus* growth.

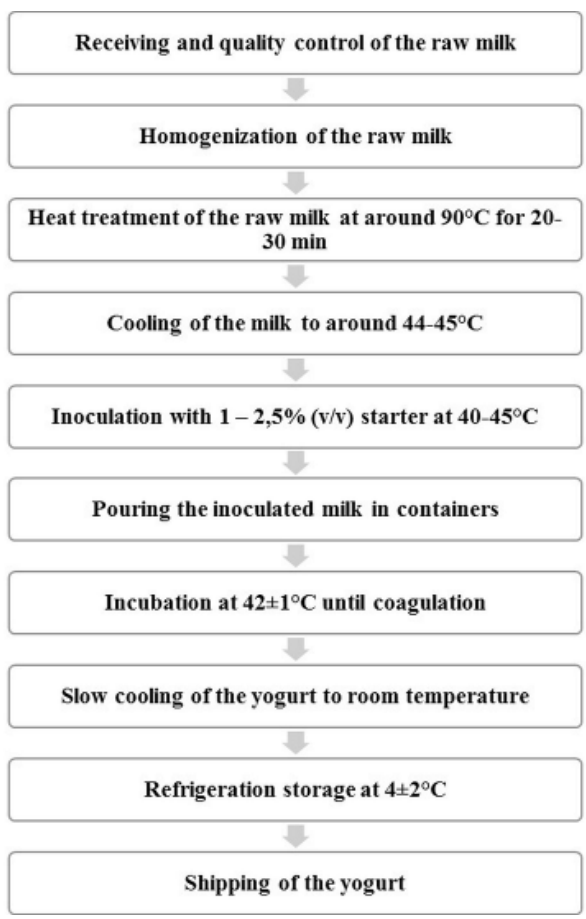

Figure 1. Scheme of production of traditional Bulgarian yogurt.

The complex and specific qualities of Bulgarian yogurt are due to the biological characteristics of both species in the composition of the starter (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*), the ratio between them and the biochemical and microbiological activities during their co-culturing. Difficulties in developing and maintaining yogurt starters arise from the differences in the generation times and the optimum growth temperatures of the two starter species (Simova, 2007; Driessen et al., 1981).

Thus, the development of a lasting symbiotic association between *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* is a difficult and time-consuming process (Simova, 2007; Driessen et al., 1981).

On one hand, starter lactic acid bacteria induce metabolic and flavor changes in dairy products; on the other hand, they aim at reducing the health risks by extending the shelf life of the product without changing its flavor.

Yogurt starters are obtained by culturing of pure cultures of the two starter species in a pH-controlled conditions and their subsequent blending, or by co-cultivation of the two starter species (Beal et al., 1994). Yogurt starters are traditionally obtained through periodic fermentation and the accumulation of some end metabolic products may limit cell growth. The use of continuous systems can overcome this limitation (Doleyres et al., 2004; Lacroix et al., 2004).

The continuous method of culturing of microorganisms has undeniable advantages compared to batch culturing in terms of mass production of starters. This leads to the successful overcoming of a number of difficulties associated with the development of mixed bacterial cultures; providing high productivity and automatic control of the process, which is significantly cost-effective; creating conditions for obtaining standardized starters with uniform properties and biochemical activity (Tamime and Robertson, 2003).

2. Health Benefits of Yogurt Consumption

Regular consumption of yogurt slows aging; prevents stomach mucosa atrophy, thereby improving the absorption of calcium; reduces the production of toxic substances by putrefactive bacteria and their subsequent resorption; lactic acid stimulates pancreatic action, which improves digestion; the milk nucleic acids affect smooth muscles and lead to lower blood pressure (Georgieva and Danova, 2013).

Biologically active peptides that inhibit blood clot formation in blood vessels are produced during yogurt fermentation as a result of the mutual growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Chomakov, 2007).

The high proteolytic activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* on β-casein results in the production of functional peptides, which lower blood pressure (Chomakov, 2007; Georgieva and Danova, 2013). The greatest amount of these peptides is produced during the mutual growth of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, as well as by the protocooperation of *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus* and *Lactococcus lactis* ssp. *lactis* biovar diacetylactis. Bulgarian yogurt enhances organism immunity.

Bulgarian yogurt is characterized by strong antitumor activity, which is due to *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*. Lactobacilli degrade quickly carcinogenic nitrosamines, which prevents tumor formation. Moreover

Streptococcus thermophilus synthesizes enzymes that regulate the differentiation of colon epithelial cells, which prevents colon cancer formation (Chomakov, 2007; Georgieva and Danova, 2013). Antitumor activity of Bulgarian yogurt is also due to the antimicrobial activity of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* against putrefactive and toxin-producing microorganisms. The high antioxidant activity of the symbiotic culture of *Lactobacillus delbrueckii* ssp. *bulgaricus* and Streptococcus *thermophilus* is very important for the antitumor activity of Bulgarian yogurt. Substances that increase the antioxidative properties of the milk are formed during their mutual growth in milk. The highest antioxidant activity was observed in starter YF-L 902 among the collection of starters: Starter XT-312 - *Lactococcus lactis, Lactococcus cremoris, Leuconostoc mesenteroides* ssp. *cremoris*, *Leuconostoc pseudomesenteroides, Lactococcus diacetylactis*; Starter LAF-3 - *Debaromyces hansenii*; Starter ST-Body-4 - *Streptococcus thermophilus*; Starter YF-L 902 - *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Streptococcus thermophilus*; and biomass of *Bifidobacterium* sp. (Visokogorskiy et al., 2011).

3. Probiotics – Definition, Properties and Health Claims

Probiotics are 'live microorganisms, which when administered in adequate amounts, confer a health benefit upon the host' (FAO/WHO, 2002). Several generic health advantages are attributed to probiotics, including antimutagenic and anticarcinogenic effects, immune system stimulation (immune modulation), antiinfection properties, improvement of gut function (Shah, 2006), effect on the metabolism of lipids, serum cholesterol reduction, alleviation of lactose intolerance symptoms, calcium resorption stimulation and nutritional enhancements (Ishibashi and Shimamura 1993; Shah 2007; Mortazavian et al. 2010; Korbekandi et al. 2011; Sanders et al. 2013). A number of studies have shown that probiotic bacteria synthesize antimicrobial substances, modulate the host immune system, inhibit the growth of *Helicobacter pylori*, lower lactose intolerance, and lower cholesterol level, have antimutagenic and anticancer action and prevent autoimmune diseases. They are also applied in the treatment of various types of diarrhea, gastro-intestinal diseases, Crohn's disease and hernia (Ishibashi and Shimamura 1993; Shah 2007; Mortazavian et al. 2010; Korbekandi et al. 2011; Sanders et al. 2013). The beneficial health effects and therapeutic applications of probiotics are summarized in Table 1.

The microorganisms that are associated with maintaining the balance of the gastro-intestinal microflora are lactobacilli and bifidobacteria.

Not all species and strains of lactobacilli and bifidobacteria could act as regulators of the gastro-intestinal microflora, but only those who are able to survive and grow under the different conditions of the digestive tract. As probiotic properties are strain specific, these effects cannot be generalized for bacteria contained in commercially available yogurt. This requires the mandatory selection of strains of lactobacilli and bifidobacteria with probiotic properties (Figure 2) (Denkova and Krastanov, 2012; Goldin and Golbach, 1992; Ouwehand et al., 2002; Kashtan, 1990; Segal, 1995; Erkkila and Petaja, 2000; Selle and Klaenhammer, 2013).

Table 1. Beneficial health effects and therapeutic applications of probiotics (Granato et al., 2010)

Beneficial effects	Therapeutic applications
Maintenance of balanced intestinal microflora	Prevention of urogenital infection
Enhancement of the immune system	Alleviation of constipation
Reduction of lactose-intolerance	Protection against traveller's diarrhea
Reduction of serum cholesterol levels	Prevention of infantile diarrhea
Anticarcinogenic activity	Reduction of antibiotic-induced diarrhea
Reduction of toxic compounds	Reduction of rotavirus diarrhea
Detoxification of carcinogens	Prevention of hypercholesterolaemia
Improved nutritional value of food – increase of nutrient bioavailability	Protection against colon/bladder cancer
	Prevention of osteoporosis
Reduction of intestinal pathogens	
Vitamin B production	
Improvement in absorption of Fe, Ca and Mg	

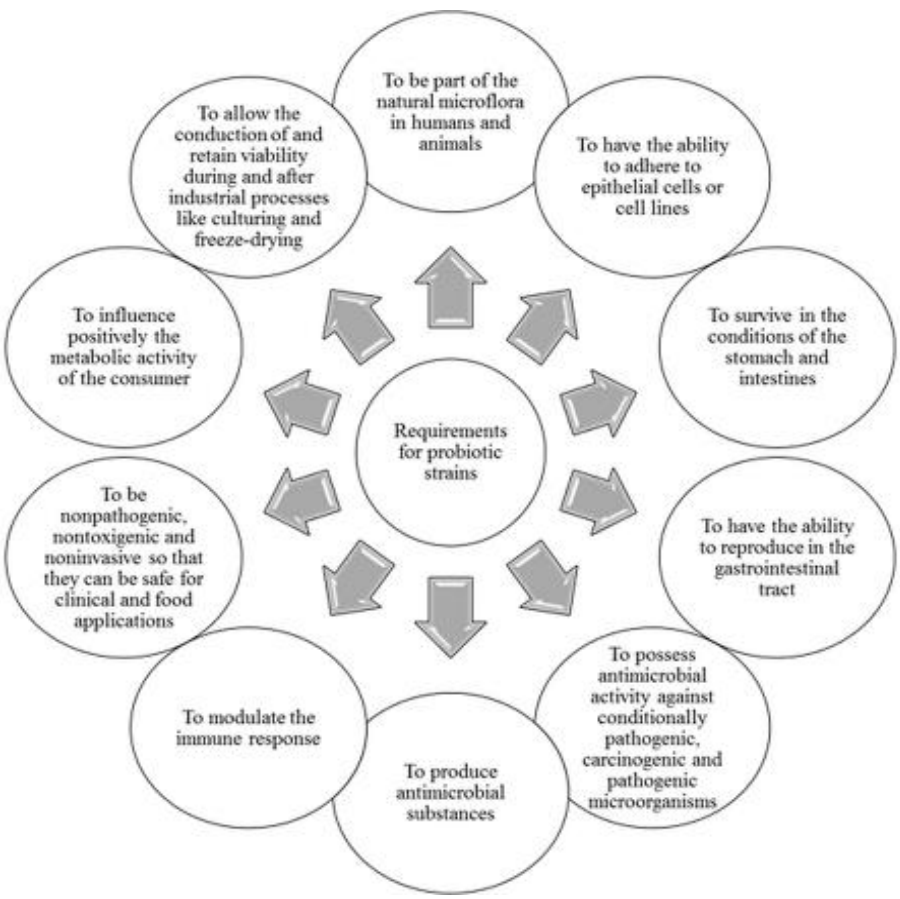

Figure 2. Requirements for probiotic strains.

According to Donald and Brow, 1993 and Wolfson, 1999 in order to be able to prevail in the balance of gastro-intestinal microbial association the concentration of viable beneficial probiotic bacteria should exceed 10^9 per gram probiotic preparation. To achieve this value, a

better understanding of the factors of cultivation, concentration, drying and storage is required (Denkova and Krastanov, 2012).

4. Probiotic Fermented Milks

Probiotic fermented milks are considered the most popular probiotic food products. Fermentation is a critical stage in obtaining these products. It is performed by probiotic organisms alone or by the support of adjunct cultures to probiotics (Mohammadi et al., 2012).

Probiotic microorganisms can be added to fermented milks by different methods. They can be added as nonfermenting microorganisms after the fermentation and cooling are complete aiming at being delivered to the gastro-intestinal tract (nonstarter probiotics); or can be added as starter cultures to ferment milk base (starter probiotics). In the latter method, complex relationships between probiotic microorganisms and milk ingredients as well as between probiotic microorganisms and support/adjunct cultures could occur. The most popular way is to add the probiotic microorganisms together with the adjunct starter cultures, since the adequate fermentation in milk rarely occurs by culturing of probiotics alone (Korbekandi et al., 2011; Tamime et al., 2005; Mohammadi et al., 2012).

Biorelationships among starter cultures may be synergistic or antagonistic. Because probiotic bacteria are unable to adequately ferment milk and produce fermented milk products with satisfactory sensory properties, it is a common practice to add traditional yogurt starter cultures together with probiotic strains, which is called “support culture” or “adjunct culture.” The slow growth of probiotic microorganisms in milk might lead to overgrowth of undesirable microorganisms, and the strains that do not grow well could produce unpleasant flavors (Tamime et al., 2005; Shihata and Shah, 2002). The coagulation time in the production of fermented milks with the use of probiotic cultures alone might be prolonged to 8–24 h in comparison with those produced with traditional yogurt starter (3–4 h) (Østlie et al., 2003; Saarela et al., 2000). Therefore, one of the most significant aspects in fermented functional food production is the interactions between the starter strains and the added probiotic bacteria (Saccaro et al., 2009).

Yogurt sterter bacteria can render both synergistic and antagonistic relationship with probiotic bacteria depending on the starter strains, the probiotic strains, and the compositional and process conditions (Oliveira et al., 2001). The starter microorganisms might improve the growth conditions for the probiotics by producing substances that stimulate probiotic growth or by reducing some inhibitors (such as oxygen pressure, especially for bifidobacteria). The survival of probiotics might be influenced by the metabolites produced by the starter microorganisms, including lactic acid, bacteriocins, hydrogen peroxide, and even volatile compounds (Mortazavian et al., 2011). *Lactobacillus delbrueckii* ssp. *bulgaricus* sharply produces lactic acid during fermentation (sharp acidification) and slowly continues lactic acid formation during refrigeration storage (postacidification).

The discussed phenomena lead to the unpleasant and undesirable sourness of yogurt as well as loss in probiotic viability (Tamime et al., 2005). Therefore, the main principle in determining culture composition for fermented milk production is the use of compatible strains of probiotics together with yogurt starter cultures (Mohammadi et al., 2012).

A number of factors influence probiotic viability in fermented milk products: biorelationships among starter cultures; probiotic bacterial strain; sequential fermentation,

separate fermentation and post-fermentation incubation; inoculation level/rate; growth factors and promoters; protective factors (buffering capacity and solid matrix); hydrogen peroxide; storage temperature and time; food additives; incubation temperature; dissolved oxygen and redox-potential; pH and titratable acidity; microencapsulation (Mohammadi et al., 2012).

When probiotics are used together with the starter culture in the production of fermented milks, several characteristics of the probiotic strains are under special attention including safety; fermentability (acidification-ability) in milk and fermentation time; cell viability in the product (retention of high concentrations of probiotic and starter microorganism cells during storage); formation of satisfactory sensory attributes; cost-effectiveness (reasonable starter and probiotic development and implementation cost); specific and confirmed health claims (Mohammadi et al., 2012).

Probiotic lactobacilli and, in particular, bifidobacteria grow poorly in milk because of the lack of nonprotein nitrogen (free amino acids and small peptides) and some vitamins, as well as to their low β-galactosidase activity and lack of proteolytic activity (Mohammadi et al., 2011). An appropriate and common method to compensate for their slow growth is to fortify milk with different growth factors (consumed by probiotics as nutrients) and/or growth promoters (which improve viability of probiotics but are not direct nutrients) such as casein, whey protein hydrolysates, L-cysteine, yeast extract, glucose, vitamins, minerals, and antioxidant. These additives have significant beneficial effects on probiotic microorganism survival (Mohammadi et al., 2011). Protein derivatives promote probiotic survival because of their nutritional value for the cells, the reduction of the redox potential of the media as well as the increase in the buffering capacity of the media (which results in a smaller decrease in pH) (Mohammadi et al., 2012).

The buffering capacity of a product can considerably influence probiotic cell viability. Increasing the buffering capacity of milk would stimulate the growth and activity of probiotics in fermented milks. It would lead to higher viability of probiotics not only in dairy fermented products, but also in the gastro-intestinal tract. Also, the pH of the products with higher buffering capacity declines slowly during fermentation and refrigeration storage which would also increase the survival rate of probiotic cells (Mohammadi et al., 2012).

Worldwide, the increased consumer awareness regarding the effect of food on health has caused an increasing trend for consumption of probiotic products which have beneficial effects on the human organism (Granato et al. 2010; Massoudi et al., 2015). Probiotic strains are added to develop foods with additional health benefits.

Lactobacillus sp. and *Bifidobacterium* sp. are the most common bacterial species used as probiotics for the production of fermented milks and other dairy products (Hill et al. 2014; Shakerian et al. 2014). The efficacy of probiotic food products is determined by the viability of the included probiotic bacteria, the number of viable and active cells per gram of probiotic food products at the moment of consumption (Tamime et al. 2005; Mortazavian and Sohrabvandi 2006; Massoudi et al., 2015). The cell viability of probiotic bacteria in a fermented food matrix is a prerequisite to guarantee the claimed health effects (Galdeano and Perdigon, 2004). Therefore, it is important to ensure a high survival rate of the probiotic bacteria both during production and over the product's shelf life (Del Bono et al. 2015). The minimum necessary concentration of probiotic bacteria to execute their inherent beneficial effects has been generally accepted to be 10^6 viable cells/g product at the moment of consumption (Gomes and Malcata 1999; Sarvari et al. 2014). Furthermore, many authors suggest that ingestion of 10^8–10^9 viable cells per day is needed to develop beneficial effects

for humans (Saxelin 1997; Beheshtipur et al. 2012; Massoudi et al., 2015). Maintaining a high level of viable probiotic cell count in yogurts throughout the shelf life, however, is not a simple task. Many factors influence the viability of probiotics in yogurts: strain variation, acid accumulation, interaction with starter cultures, level of dissolved oxygen and hydrogen peroxide (H_2O_2), and storage conditions (Talwalkar and Kailasapathy, 2003; Ng et al., 2011). Also many microbiological and technological factors induce changes in the functionality of probiotic bacteria in food, and in particular in fermented milks; differences in functionality might be observed for the same strain along different commercial lots of the same product (Del Bono et al. 2015; Massoudi et al., 2015).

To avoid the negative effects of environmental factors and to maintain the viability of microbial cells in a manner suitable to allow release of the cells in the right place in the gastro-intestinal tract is conducted microencapsulation in suitable matrices or cryoprotectant media (Ozer et al., 2005).

Microencapsulation of probiotics is the process of probiotic cell entrapment using coating materials (mainly, suitable hydrocolloids) for the mean of segregating and preserving them from the detrimental conditions of the surrounding environment, in a way that results in appropriate cell release in the right place in the gastro-intestinal tract (Krasaekoopt et al., 2003; Mortazavian et al., 2007; Pico and Lacroix, 2003). Microencapsulation preserves probiotic cells from detrimental environmental factors such as low pH and high acidity (Wenrong and Griffiths, 2000), bile salts (Lee and Heo, 2000), cold shocks induced by the process conditions such as deep freezing and freeze-drying (Shah, N. P., Ravula, 2000) molecular oxygen in case of obligatory anaerobic microorganisms (Sunohara et al., 1995), heat shocks caused by process conditions such as spray drying, bacteriophages (Gharsallaoui et al., 2007), and chemical antimicrobial agents (Sultana et al., 2000). Moreover, microencapsulation offers other advantages such as improvement and stabilization of sensory attributes (Gomes and Malcata, 1999) and immobilization of the cells for their homogeneous distribution throughout the product (Krasaekoopt et al., 2003; Mohammadi et al., 2012). Research demonstrates that microencapsulation increases probiotic survival by more than 2logN during refrigeration storage in comparison with free probiotic cells in yogurt.

Bifidobacteria are part of the normal intestinal microflora; thus they are important as a component of yogurt starters, because of their inherent properties and their biological role in the human body. Bifidobacteria grow in the colon, producing lactic acid, acetic acid and bacteriocins, thereof changing the conditions in the colon and pushing pathogenic and toxic microorganisms out of the biological niche. Thus, they remove toxins that lead to inflammation of colon mucosa, but at a later stage - to benign tumor formation. Inulin or oligofructose as “bifidogenic factor,” called prebiotics, are added to some bio-yogurts with bifidobacteria. “Prebiotics” are nonviable and nondigestible (or minimally digestible) food ingredients which are metabolized selectively by beneficial intestinal bifidobacteria and enhance their growth and/or activity.

Bifidobacteria are difficult to propagate in food due to their oxygen sensitivity and low acid tolerance, but the addition of prebiotics to dairy foods may lead to better results to ensure the presence of high concentrations of bifidobacteria during the shelf life of dairy products (Leahy et al., 2005).

Contrary to starter microorganisms, bifidobacteria do not have a significant role either in the acidification of milk, or in the formation of texture and/or flavour, but they play a health-promoting role in the gastro-intestinal tract of the consumer (Leahy et al., 2005). The

foodstuff is regarded as a vector to carry selected probiotic *Bifidobacterium* strains into the lower part of human intestine (Meile et al., 2008).

Fermented foods with probiotic properties are being produced from different food substrates (Farnworth, 2003). Most of these products are of dairy origin and include fresh milk (Klaver et al., 1993), fermented milk (Tamime et al., 1995), beverages, cheese (Gomes et al., 1995), cottage cheese (Blanchette et al., 1995), powdered milk, cookies, health foods, ice cream (Hekmat and McMohan, 1992), and dairy desserts (Laroia and Martin, 1991; Lourens-Hattingh and Viljoen, 2001).

Dairy products have a distinct role in probiotic delivery to the human gastro-intestinal tract, as these products provide probiotic bacteria with a suitable protective environment in which their growth and viability are promoted (Saarela, 2009).

5. Bio-Yogurt Fortified with Plant Protein

Nowadays, yogurt produced from cows' milk is commonly consumed in both developing and industrialized countries. However, the demand for alternatives to cows' milk is growing due to a number of problems like allergenicity, desire for vegetarian alternatives, etc., and therefore interest in a soy-based yogurt has developed. Probiotic milk-based yogurts are now being marketed, but consequently it is necessary for probiotic bacteria to be incorporated into soy-based yogurt-type fermentations (Farnworth et al., 2007).

Soy milk is the water extract of soy beans that is rich in proteins. In comparison with milk, soy milk has higher aspartate, arginine and folate content (Holland et al. 1995). Fermentation of soy milk with various microorganisms, especially lactic acid bacteria, could decrease the beany, grassy or soy flavour which in turn increases both the acceptability and the nutritional value of the resulting products (Liu and Lin 2000; Abdolmaleki et al., 2015).

Soy milk is obtained by extraction of soybeans. It has a specific taste and is rich in nutrients, proteins, vitamins B and E, Fe, Ca, Mn. Soy milk does not contain lactose and is particularly suitable for people with lactose intolerance and cow milk protein intolerance. It does not contain toxins and inhibiting substances. Soy milk contains flavones and isoflavones (phytoestrogens). In nature, isoflavones usually occur as glycosides and, once deconjugated by the intestinal microflora, isoflavones can be adsorbed into the blood (Awaisheh et al., 2005) and exert their protective function.

MATERIALS AND METHODS

1. Microorganisms

1.1. Microorganisms Used in the Development and Selection of Symbiotic Starter Cultures of Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus

Lactobacillus delbrueckii ssp. *bulgaricus*: *Lactobacillus delbrueckii* ssp. *bulgaricus* B1; *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1; *Lactobacillus delbrueckii* ssp. *bulgaricus*

M3; *Lactobacillus delbrueckii* ssp. *bulgaricus* MZ_2; *Lactobacillus delbrueckii* ssp. *bulgaricus* 26-12.

Streptococcus thermophilus: *Streptococcus thermophilus* B1; *Streptococcus thermophilus* TMZ2I; *Streptococcus thermophilus* T_3H_{8X}; *Streptococcus thermophilus* T3; *Streptococcus thermophilus* ZH8; *Streptococcus thermophilus* 8.

1.2. Microorganisms Used in the Development Bio-Yogurt Including Selected Probiotic Strains

From *Lactobacillus* sp.: *Lactobacillus acidophilus* 5R; *Lactobacillus acidophilus* A2; *Lactobacillus acidophilus* Ac; *Lactobacillus acidophilus* 2; *Lactobacillus acidophilus* A; *Lactobacillus gasseri* Bio; *Lactobacillus delbrueckii* ssp. *bulgaricus* GB-15; *Lactobacillus delbrueckii* ssp. *bulgaricus* GB; *Lactobacillus delbrueckii* ssp. *bulgaricus* LB M; *Lactobacillus delbrueckii* ssp. *bulgaricus* 144.

From *Bifidobacterium* sp.: *Bifidobacterium bifidum* BB1; *Bifidobacterium bifidum* BB2; *Bifidobacterium infantis* BI13; *Bifidobacterium infantis* BI15; *Bifidobacterium longum* BL11; *Bifidobacterium breve* BB18.

1.3. Microorganisms Used in the Development Bio-Yogurt Fortified with Plant Protein

Symbiotic starter MZ_2, containing *Lactobacillus delbrueckii* ssp. *bulgaricus* MZ_2 and *Streptococcus thermophilus* TMZ2I

From *Lactobacillus* sp.: *Lactobacillus acidophilus* 5R; *Lactobacillus delbrueckii* ssp. *bulgaricus* Y1.

From *Bifidobacterium* sp.: *Bifidobacterium bifidum* BB1; *Bifidobacterium infantis* BI13; *Bifidobacterium longum* BL11; *Bifidobacterium breve* BB18.

All strains are part of the cultural collection of the Department of "Microbiology" at the University of Food Technologies, Plovdiv, Bulgaria.

2. Media

2.1. MRS-Broth - composition (g/dm^3): peptone from casein - 10; yeast extract - 4; meat extract - 8; glucose - 20; K_2HPO_4 - 2; sodium acetate - 5; diammonium citrate - 2; $MgSO_4$ - 0.2; $MnSO_4$ - 0.04; Tween 80 - 1 cm^3/dm^3. pH was adjusted to 6,5. Sterilization - 15 minutes at 121 ºC.

2.2. MRS-Agar - composition (g/dm^3): MRS-broth + 2% agar. Sterilization - 15 minutes at 121 ºC.

2.3. Sterile Skimmed Milk with Titratable Acidity of 16-18º T - composition (g/dm^3): skimmed milk powder (Scharlau) - 100. Sterilization - 15 minutes at 121 ºC.

2.4. Saline Solution - composition (g/dm^3): NaCl - 5. Sterilization - 20 minutes at 121ºC.

2.5. *LAPTg10 – Broth* - composition (g/dm^3): peptone - 15; yeast extract - 10; tryptone - 10; glucose - 10. pH was adjusted to 6,6 – 6,8 and Tween 80 - 1cm^3/dm^3 – is added. Sterilization - 20 minutes at 121ºC.

***2.6. LAPTg10 – Agar* -** composition (g/dm^3): LAPTg10-broth + 2% agar. Sterilization - 20 minutes at 121ºC.

***2.7. Bifidobacterium sp. Agar Medium* -** composition (g/dm^3): peptone - 10, yeast extract – 10, lactose - 10, $MnSO_4$ – 1, casein hydrolyzate - 8, NaCl - 3.2, CH_3COONa – 1, agar - 20. pH was adjusted to 6.6 - 6.8. Sterilization - 20 minutes at 121°C.

2.8. Soy Milk

Soy milk was obtained using unit "soy cow" CK20 ("soeva krava" CK20) by soaking and grinding of whole soybeans of the variety "Pavlikeni 121" with water and conducting water-vapor extraction with the formation of liquid mass and separation of soy milk from the solution through a filter press.

3. Physiological and Biochemical Methods

3.1. Determination of the Titratable Acidity

The Thorner method was used to determine the acidification-ability of microorganisms. One °T (Thorner degree) is equal to 1 cm^3 0.1 N NaOH, spent on the neutralization of an equivalent amount of organic acids contained in 100 cm^3 of culture medium. The method is based on the titration of the sample with 0.1 N NaOH. For that purpose 10 cm^3 of each test sample were mixed with 20 cm^3 of distilled water. The mixture was titrated with 0,1 N NaOH using phenolphthalein as an indicator until the appearance of pale pink color that persisted over one minute.

3.2. Determination of the Number of Viable Microorganism Cells

Appropriate tenfold dilutions of each sample in saline solution were prepared. The last 3 tenfold dilutions were spread plated on the appropriate solid medium (MRS-agar or LAPTg10-agar – for the enumeration of lactic acid bacteria) or pour plated (*Bifidobacterium* sp. agar medium - for the enumeration of bifidobacteria). The inoculated plates or tubes were cultured 3 days at optimum temperature for the development of the corresponding microorganisms - 37±1°C until the formation of countable single colonies.

4. Preparation of Traditional Yogurt Starter Combinations

24-hour cultures of the selected strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* developed in skimmed milk were mixed in a ratio of *Lactobacillus delbrueckii* ssp. *bulgaricus*: *Streptococcus thermophilus* = 1 : 2. The following starter combinations were prepared (Figure 3):

1. *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* B1;

2. *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* B1;
3. *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* B1;
4. *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I;
5. *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* TMZ2I;
6. *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* TMZ2I;

Sterile skimmed milk was inoculated with 2% inoculum of the corresponding combination and the inoculated milk was incubated at the appropriate temperature until coagulation. The study was conducted at 3 temperature regimes of incubation: 37±1°C, 41±1°C and 44±1°C.

The changes in the concentration of viable cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*; the ratio between the concentrations of viable cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*; the titratable acidity and the coagulation time were monitored by daily passaging of each combinations for 4 consequtive days.

The yogurts obtained after the fourth day of passaging were stored in a refrigerator at 4±2°C for 10 days (10-day pause), then again daily passaging of each combination for 4 consequtive days was performed. The changes in the concentration of viable cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*; the ratio between the concentrations of viable cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*; the titratable acidity and the coagulation time were monitored. The scheme of the experiment is given in Figure 3.

5. Preparation of Bio-Yogurt Including Selected Probiotic Strains

Sterile skimmed milk was inoculated with 1% inoculum of the corresponding symbiotic starter and/or suspension of the probiotic strain(s). The concentration of viable probiotic cells in the final product should be above 10^9cfu/cm^3. The inoculated milk was incubated at 41±1 °C until coagulation.

The changes in the concentration of viable cells of *Lactobacillus* sp. and *Streptococcus* sp.; *Bifidobacterium* sp., the ratio between the concentrations of viable cells of *Lactobacillus* sp. and *Streptococcus* sp.; and the titratable acidity of the resulting yogurts during refrigeration storage (4±2°C) for 30 days were monitored.

6. Preparation of Bio-Yogurt Fortified with Plant Protein

Sterile soy milk was inoculated with 1% inoculum of the corresponding symbiotic starter and/or suspension of the probiotic strain(s). The concentration of viable probiotic cells in the final product should be above 10^9cfu/cm^3. The inoculated milk was incubated at 41±1 °C until coagulation.

The changes in the concentration of viable cells of *Lactobacillus* sp. and *Streptococcus* sp.; *Bifidobacterium* sp., the ratio between the concentrations of viable cells of *Lactobacillus* sp. and *Streptococcus* sp.; and the titratable acidity of the resulting yogurts during refrigeration storage (4±2°C) for 20 days were monitored.

7. Bioreactor and Culturing Conditions

The culturing of lactic acid bacteria was conducted in a laboratory bioreactor with geometric volume of 2 dm^3 and working volume of 1,5 dm^3. The management and monitoring of basic parameters of fermentation were carried out by a control device "Sartorius A2," which included a control loop the stirring rate, temperature, pH, etc.

The culturing of the lactic acid bacteria was carried out in the following order: the apparatus was washed, then filled with a 0.3% neomycin solution for cold sterilization for 24 h. Then the apparatus was washed several times with sterile saline solution or distilled water, after which it was ready to carry out the fermentation process.

The sterile culture medium was loaded into the apparatus using a peristaltic pump. Culturing was performed in reconstituted skimmed milk.

8. Processing of Results

Data from triplicate experiments were processed with software MS Office Excel 2010, using statistical functions to determine standard deviation and maximum error of assessment at levels of significance $\alpha < 0,05$.

RESULTS AND DISCUSSION

1. Development and Selection of Symbiotic Starter Cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*

The development of starters for fermented milks and cheeses with high nutritional and physiological value, safety and quality is an important factor in improving consumer's health status. The use of symbiotic combinations of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains, selected and developed to effectively impact milk substances is essential for the realization of technological processes and obtaining high-quality dairy products.

As a result of years of research a number of strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* were isolated from spontaneously fermented milk, vegetables, sourdough and identified by molecular genetic methods (Table 2).

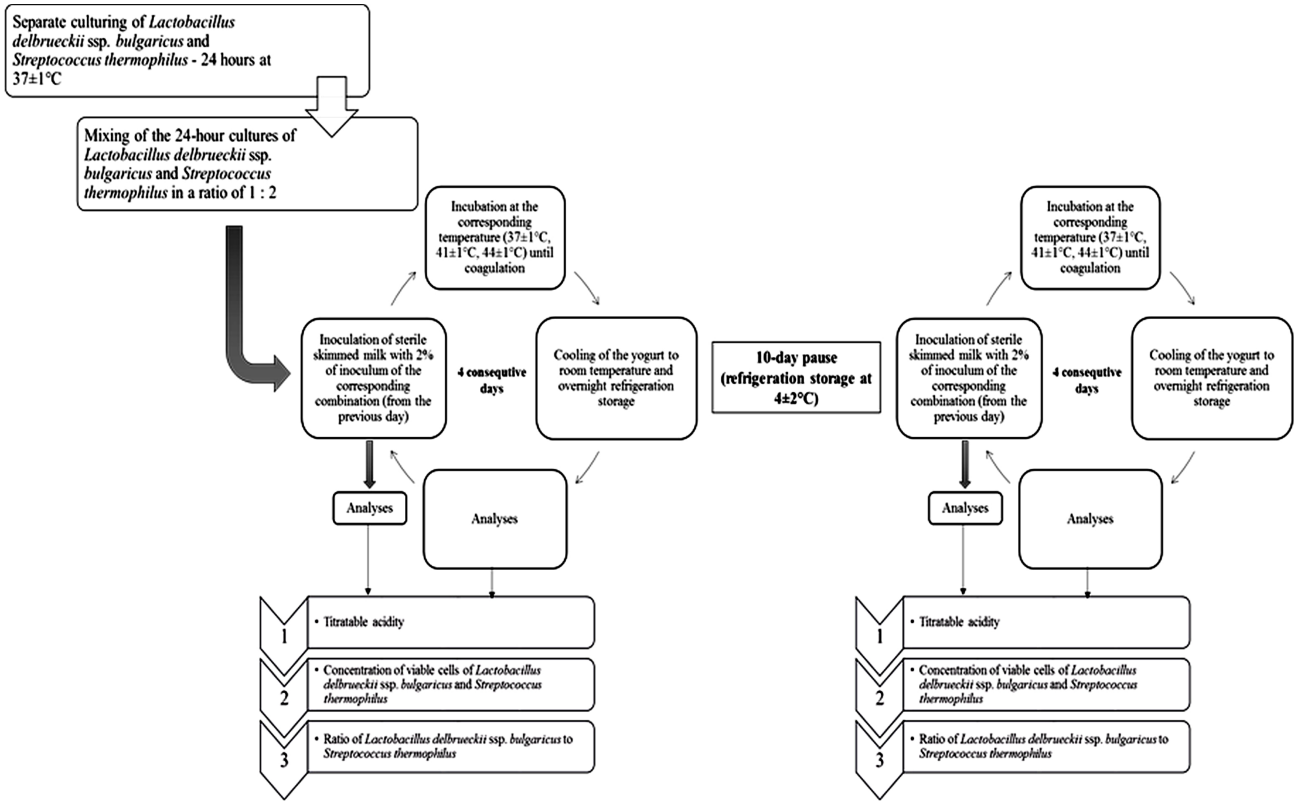

Figure 3. Scheme for the development and selection of symbiotic starter cultures of *Lactobacillus delbrueckii* ssp. *bulgaricus* и *Streptococcus thermophilus.*

Table 2.

Lactobacillus delbrueckii ssp. *bulgaricus*	*Streptococcus thermophilus*
Lactobacillus delbrueckii ssp. *bulgaricus* B1	*Streptococcus thermophilus* B1
Lactobacillus delbrueckii ssp. *bulgaricus* TAB1	*Streptococcus thermophilus* TMZ2I
Lactobacillus delbrueckii ssp. *bulgaricus* M3	*Streptococcus thermophilus* T_3H_{8X}
Lactobacillus delbrueckii ssp. *bulgaricus* MZ_2	*Streptococcus thermophilus* T3
Lactobacillus delbrueckii ssp. *bulgaricus* 26-12	*Streptococcus thermophilus* ZH8
	Streptococcus thermophilus 8

Among them were selected strains that were used for the development of symbiotic yogurt starters.

Production of various fermented foods is based on the participation of lactic acid bacteria introduced as starters during the technological process. The quality of the starters depends on the physiological, biochemical and technological properties of the microorganism strains included in their composition. The inclusion of probiotic strains as components of the starters enhances the health effect of the resulting fermented foods. The participation of probiotic lactic acid bacteria strains leads to increased health benefits when consuming dairy products produced with probiotic cultures. Only certain strains of lactobacilli have probiotic properties, which requires systematic studies on the probiotic potential of newly isolated lactobacilli strains and selection of those that feature probiotic properties for inclusion in the composition of the starters for different types of functional foods.

1.1. Selection of Lactic Acid Bacteria Strains for Use in the Composition of Yogurt Starters

Four strains of *Lactobacillus delbrueckii* ssp. *bulgaricus* - *Lactobacillus delbrueckii* ssp. *bulgaricus* B1, *Lactobacillus delbrueckii* ssp. *bulgaricus* M3, *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB2, were studied in the present work. The four strains of *Lactobacillus delbrueckii* ssp. bulgaricus possess probiotic properties, but the research on their probiotic potential was not the subject of the present work (Teneva et al., 2015a, Teneva et al., 2015b).

The acidification-ability, the accumulation of high concentrations of viable cells and the coagulation time during the separate culturing of each of the *Lactobacillus delbrueckii* ssp. *bulgaricus* strains in skimmed milk were studied in a series of experiments (Table 3). The studied lactobacilli group was heterogeneous in terms of cell growth and acidification-ability. Three strains were characterized by increased acidformation and reproductive ability - *Lactobacillus delbrueckii* ssp. *bulgaricus* B1, *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1, *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 (Table 3). These 3 *Lactobacillus delbrueckii* ssp. *bulgaricus* strains coagulated the skimmed milk for about 9 hours with accumulation of high concentrations of viable cells - 10^{12}cfu/cm^3 and titratable acidity of 72 to 89 °T (Table 3). Thus these three strains were selected for the further research.

Streptococcus thermophilus strains are also included in the composition of yogurt starters along with *Lactobacillus delbrueckii* ssp. *bulgaricus*. Three *Streptococcus thermophilus*

strains - *Streptococcus thermophilus* B1, *Streptococcus thermophilus* T_3H_{8X} and *Streptococcus thermophilus* TMZ2I were included in the present study (Table 3). This group was characterized by a higher heterogeneity in terms of the tested properties – acidification and reproductive ability. The content of viable cells after milk coagulation ranged within 10^{10}-10^{13}cfu/cm^3, and the coagulation time was in the range of 6 to 9.2 hours. Differences were also observed in the titratable acidity of the cultures – 53 - 80 °T (Table 3). The strains *Streptococcus thermophilus* B1 and *Streptococcus thermophilus* TMZ2I were selected for further research based on the results for the titratable acidity, the coagulation time and the accumulation of high concentrations of viable cells (Table 3).

Table 3. Growth and acidification-ability of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains during separate culturing in skimmed milk

Strain	Titratable acidity, ºT		N, cfu/cm^3		Coagulation time, h
	Before coagulation	After coagulation	Before coagulation	After coagulation	
L.d. ssp. *bulgaricus* B1	19	89	$1{,}5.10^7$	8.10^{12}	9
L.d. ssp. *bulgaricus* TAB1	21	80	$1{,}2.10^7$	1.10^{12}	9,20
L.d. ssp. *bulgaricus* TAB2	18	54	$2{,}3.10^7$	1.10^{10}	Above 10
L.d. ssp. *bulgaricus* M3	21	72	$1{,}6.10^7$	4.10^{12}	9
St.thermophilus B1	21	80	8.10^6	$1{,}8.10^{13}$	6,10
St.thermophilus T_3H_{8x}	18	53	1.10^6	1.10^{10}	9,20
St.thermophilus TMZ2I	18	60	5.10^6	8.10^{10}	9,20

1.2. Study of the Co-Culturing of Combinations of Selected Lactobacillus delbrueckii ssp. bulgaricus and Streptococcus thermophilus Strains

Six combinations, each consisting of one of *Lactobacillus delbrueckii* ssp. *bulgaricus* strain and one *Streptococcus thermophilus* strain, were composed using the selected *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains. All of them were examined for the presence of symbiotic growth. The changes in the titratable acidity, the coagulation time, the concentration of viable cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*, and the ratio of *Lactobacillus delbrueckii* ssp. *bulgaricus* to *Streptococcus thermophilus* during culturing at a temperature of 37 ± 1°C, 41 ± 1°C or 44 ± 1°C were monitored.

The results of the co-cultivation of the six combinations of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* at 37 ± 1°C are presented in Figure 4.

All strains in the six combinations grew together in skimmed milk at a temperature of culturing of 37 ± 1°C, but the cocci prevailed in all combinations. Therefore, the ratio of *Lactobacillus delbrueckii* ssp. *bulgaricus* : *Streptococcus thermophilus* was within the range of 1:7.5 to 1:12, i.e., the resulting fermented foods were streptococcal. This in turn led to a lower titratable acidity (52-72 °T) and sweet taste of the resulting yogurts (Figure 4).

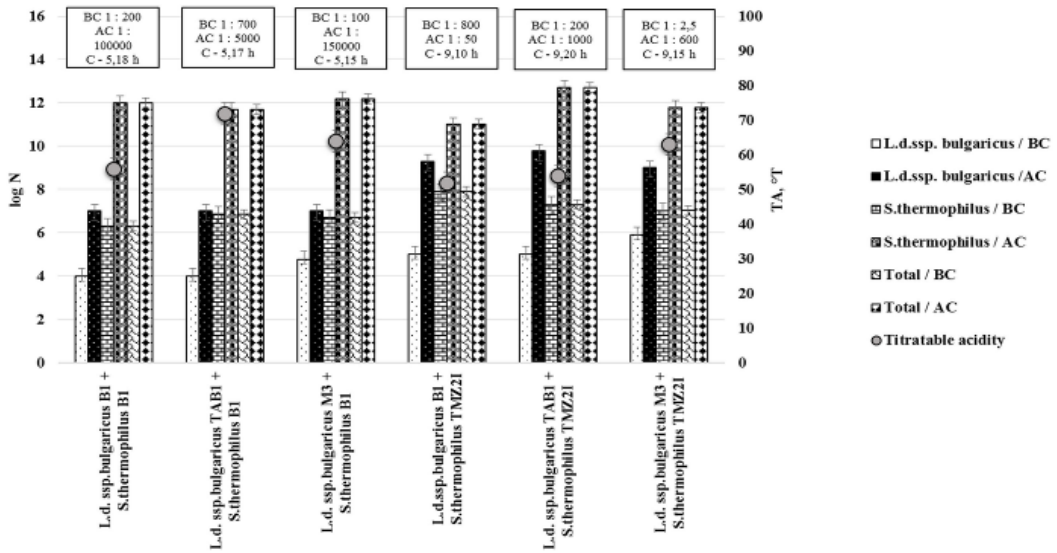

Figure 4. Growth and acidification-ability of the six combinations of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* during co-culturing in skimmed milk at 37 ± 1°C.

BC - before coagulation; AC - after coagulation; C – coagulation time.

Different trend was observed in the culturing of the six combinations at 41±1°C. Symbiosis was not established in culturing in skimmed milk between the strains in the combinations of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* B1; *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* B1; *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* B1; *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* TMZ2I. The streptococci gradually prevailed over lactobacilli (Figure 5 - Figure 8), thus the titratable acidity gradually decreased and the coagulation time was up to 4,5 hours.

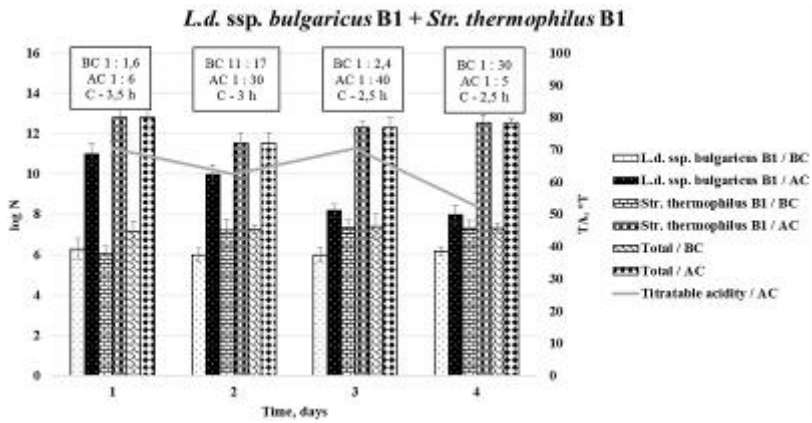

Figure 5a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

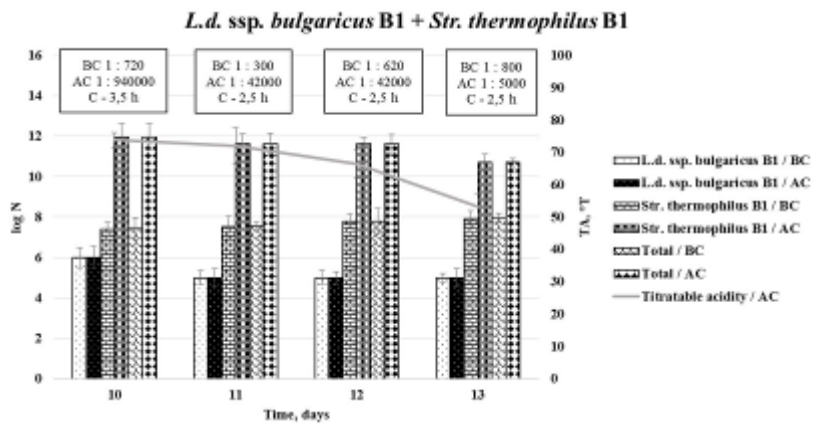

Figure 5b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days after the 10-day pause.

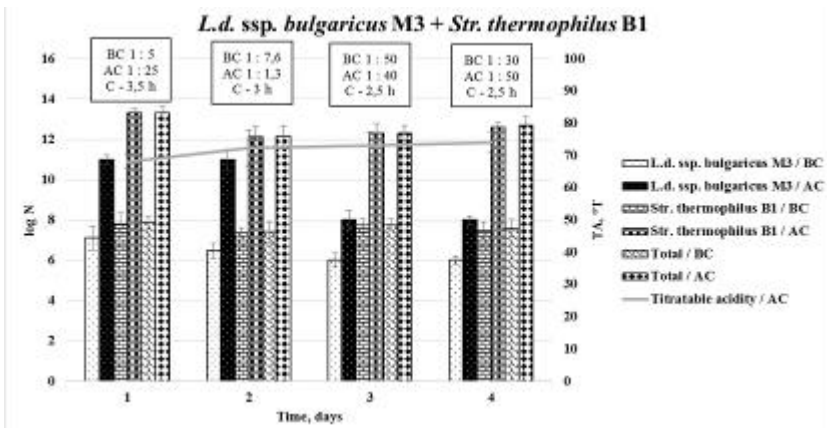

Figure 6a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

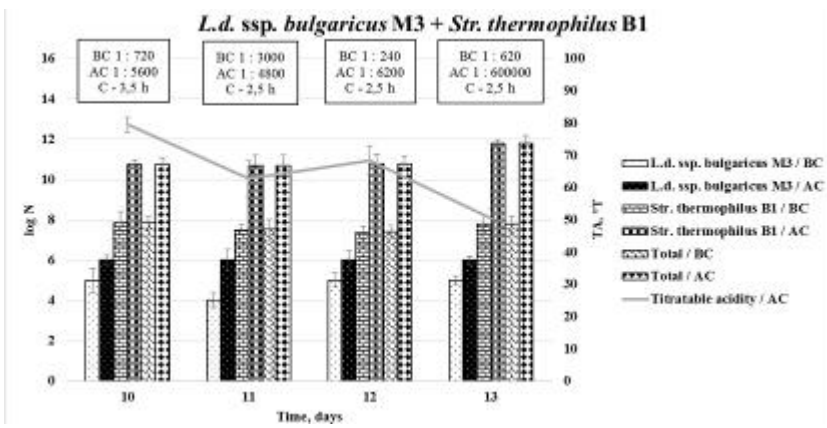

Figure 6b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days after the 10-day pause.

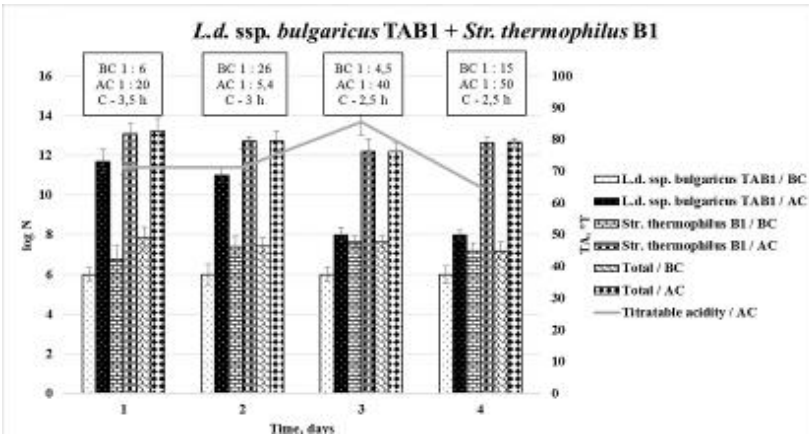

Figure 7a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

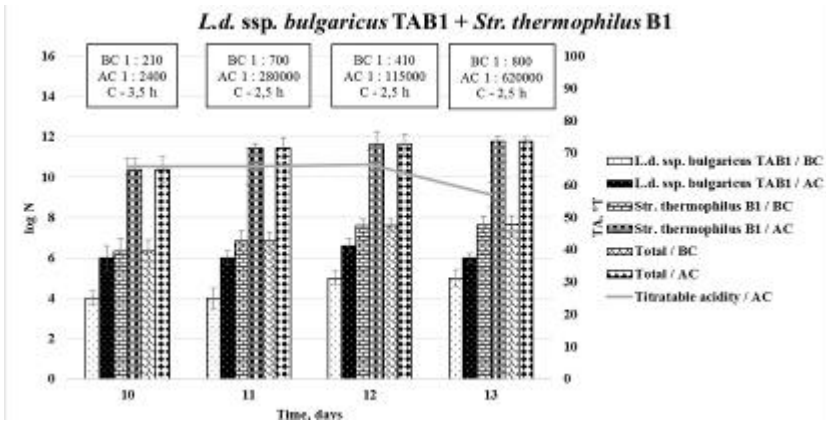

Figure 7b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days after the 10-day pause.

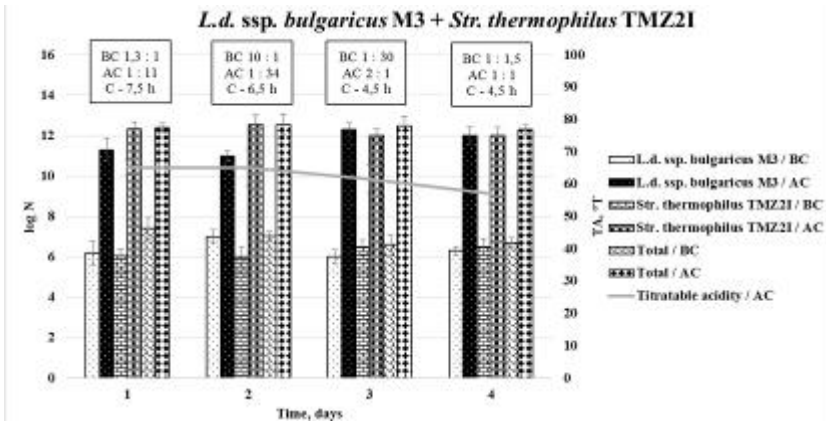

Figure 8a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

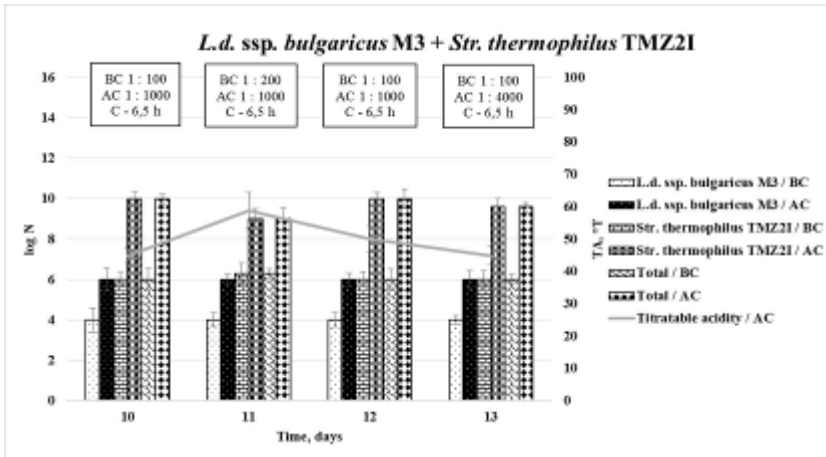

Figure 8b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days after the 10-day pause.

In the other 2 combinations, *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I; *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* TMZ2I, symbiosis was achieved on the fourth day from the start of the process (Figure 9a and Figure 10a).

During the first period of passaging these two combinations tended to stabilize in terms of titratable acidity, number of viable cells of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* as well as ratio between *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* (Figure 9a and Figure 10a). After the 10-day pause the stable symbiosis between the strains in the composition of these two starters was maintained. The resulting yogurts were characterized by high concentrations of viable cells (10^9 - 10^{10}cfu/cm^3), moderate titratable acidity (50-65 °T) and ratio between *Lactobacillus delbrueckii* ssp. *bulgaricus:Streptococcus thermophilus* ranging from 1:1 to 1:2 (Figure 9b and Figure 10b).

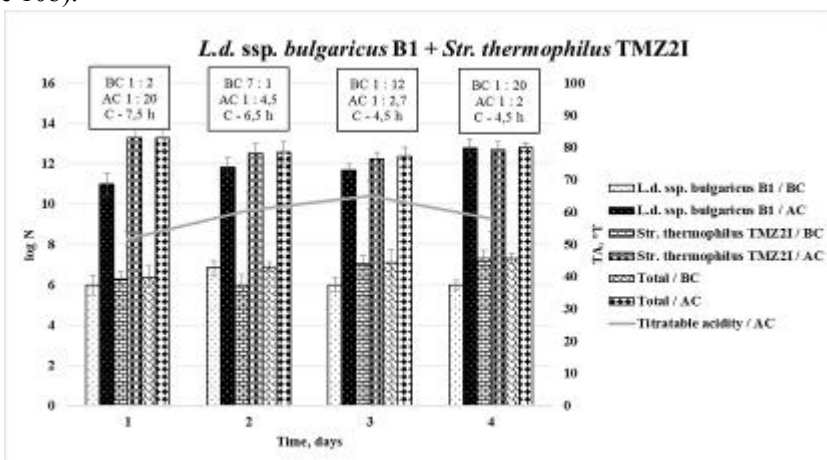

Figure 9a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

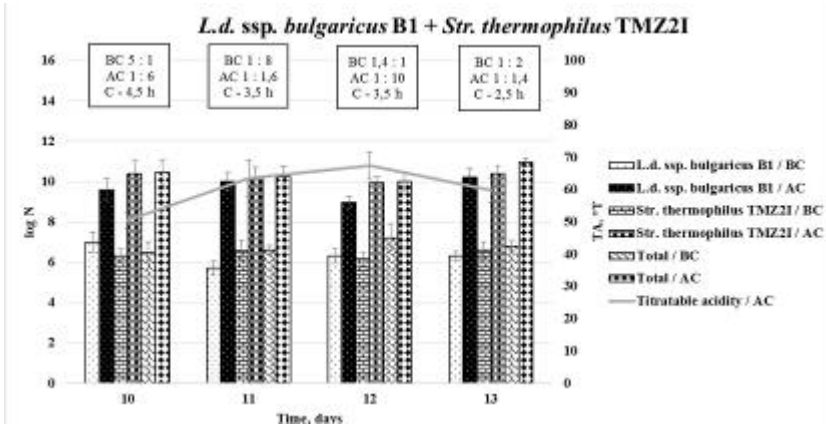

Figure 9b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days after the 10-day pause.

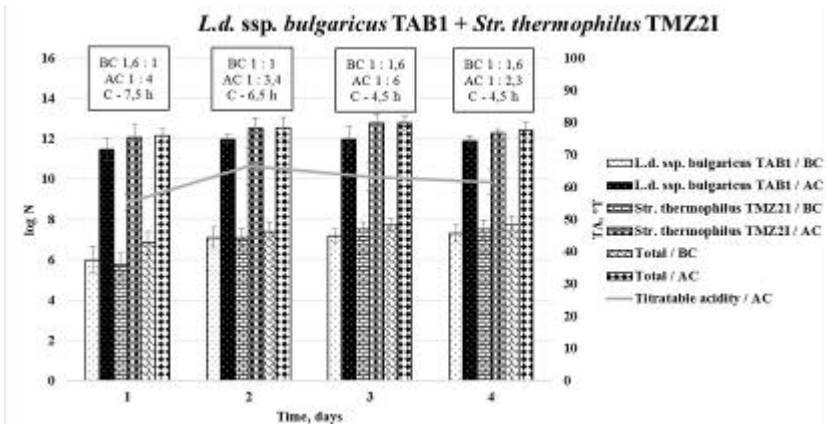

Figure 10a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

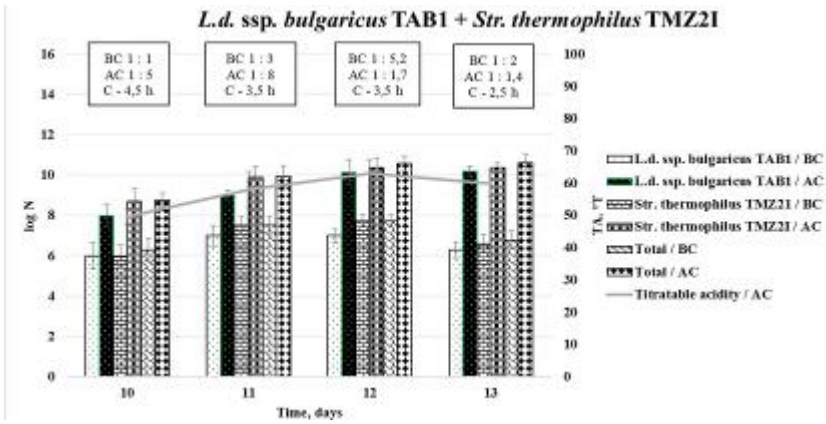

Figure 10b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 41±1°C at daily passaging for 4 consequtive days after the 10-day pause.

The combinations *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* B1; *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* B1 showed no symbiotic growth during their co-culturing in skimmed milk at a temperature of 44±1°C (Figure 11 and Figure 13). Streptococci had a dominant role, taking the upper hand over lactobacilli and the ratio was in their favor (over 3-4logN). Acidformation was weaker, but the coagulation time was 2.5 hours (Figure 11 and Figure 13).

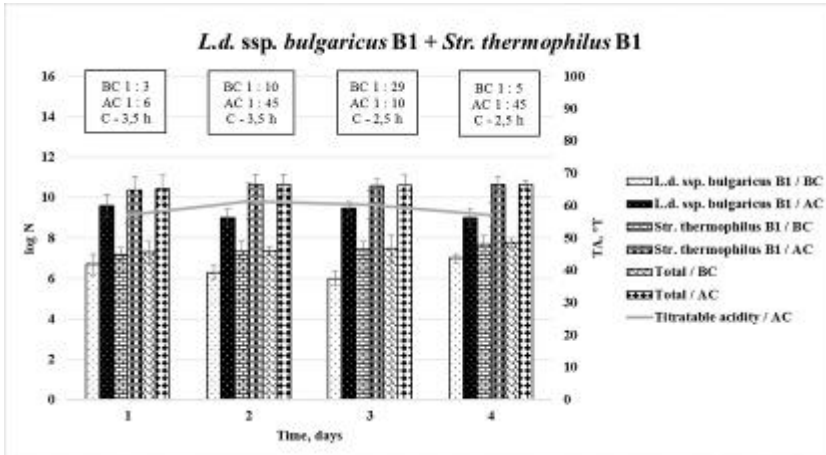

Figure 11a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

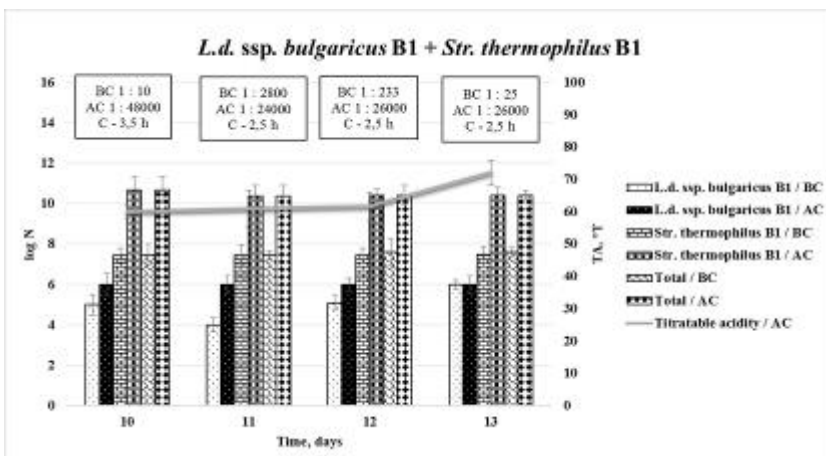

Figure 11b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days after the 10-day pause.

In the combinations *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* B1; *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* TMZ2I stabilization of the starter was observed up to the fourth day from the start of the daily passaging (Figure 12a and Figure 14a). The concentration of viable cells was over 10^{10}cfu/cm^3, the titratable acidity was 60-70°T and the ratio between *Lactobacillus*

delbrueckii ssp. *bulgaricus* and *Streptococcus thermophilus* was 1:1. After the 10-day pause the synergy between the two strains in each of the two combinations was violated. The streptococci gradually prevailed, the acidification slowed down and the coagulation time reached 4.5 hours (Figure 12b and Figure 14b). The discussed combinations are considered unsuitable for the preparation of symbiotic starters and showed the need for compulsory study of the strains for compatibility and symbiotic growth during the development of yogurt starters. The data showed that achieving symbiosis is a strain-specific, not species-specific property.

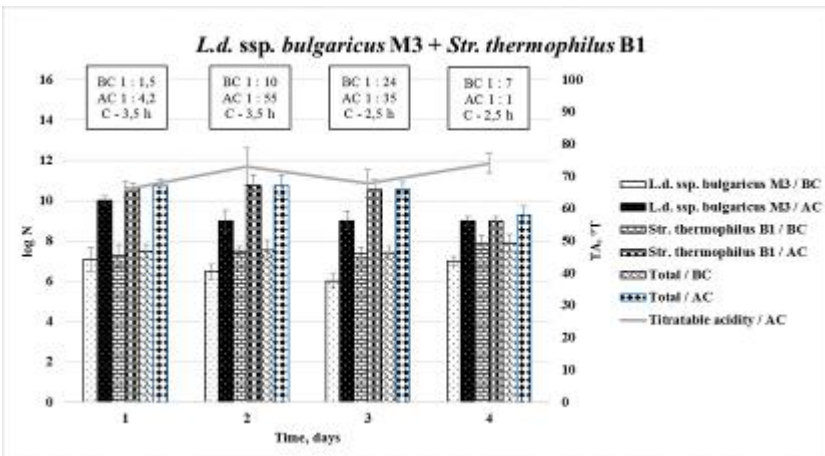

Figure 12a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

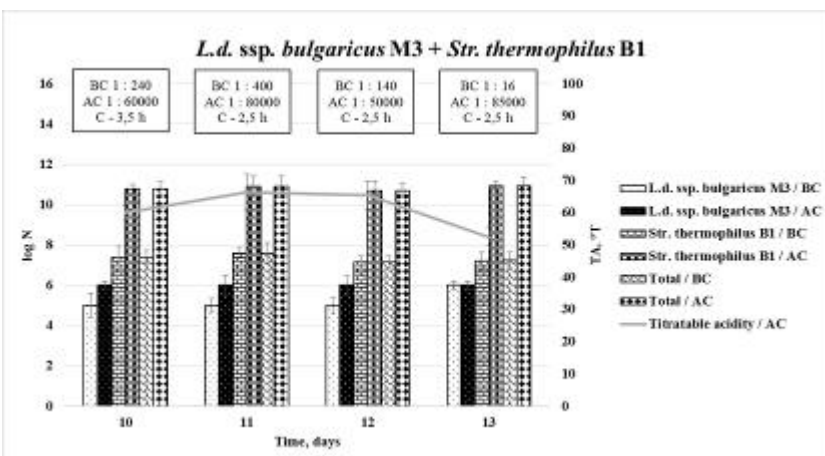

Figure 12b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days after the 10-day pause.

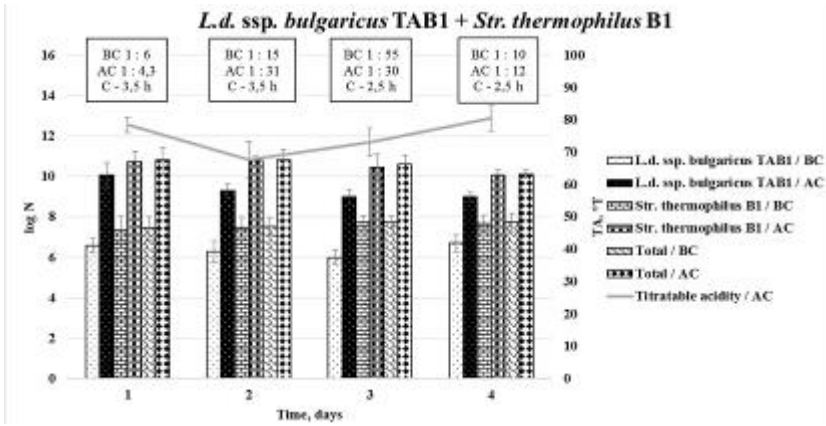

Figure 13a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

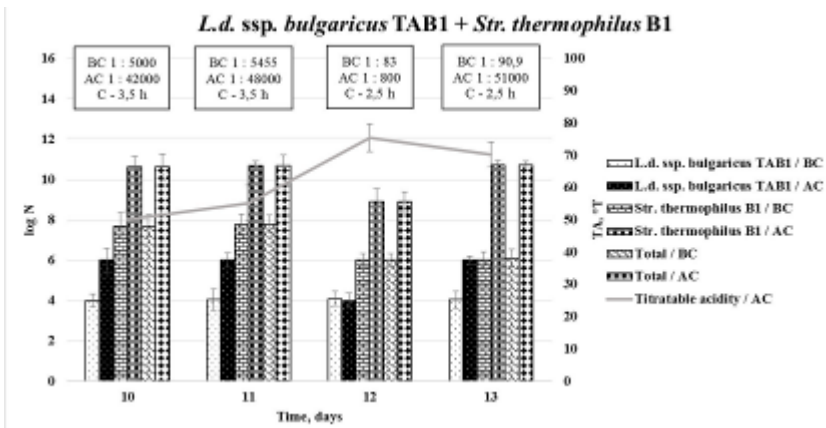

Figure 13b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* B1 during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days after the 10-day pause.

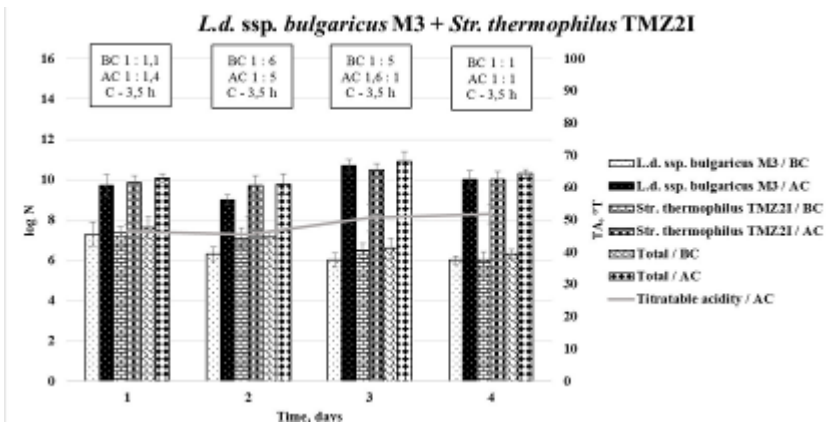

Figure 14a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

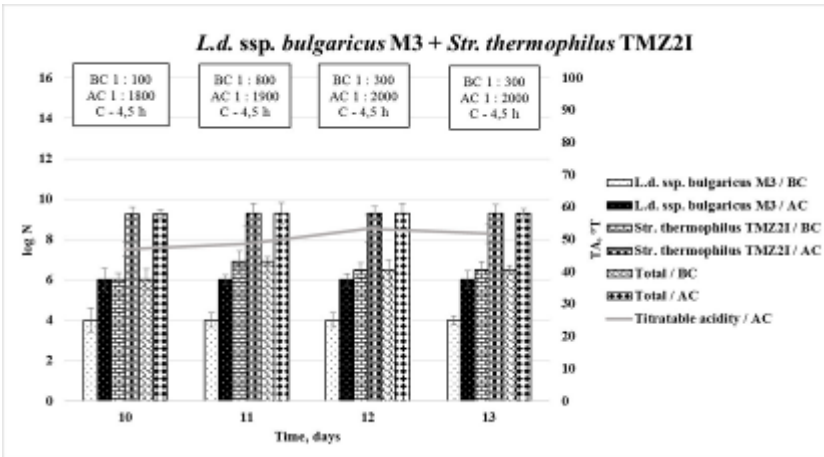

Figure 14b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days after the 10-day pause.

The results of the co-culturing of the combinations *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* TMZ2I; *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I at 44±1°C are presented in Figure 15 and Figure 16. In both combinations stable symbiosis was achieved up to the fourth day of the daily passaging (Figure 15a and Figure 16a) and it was maintained after the 10-day pause (Figure 15b and Figure 16b). The concentration of viable cells was over 10^{11}cfu/cm^3, the titratable acidity was 60-70°T and the ratio of *Lactobacillus delbrueckii* ssp. *bulgaricus* to *Streptococcus thermophilus* was in the range of 1:2 to 1:1.

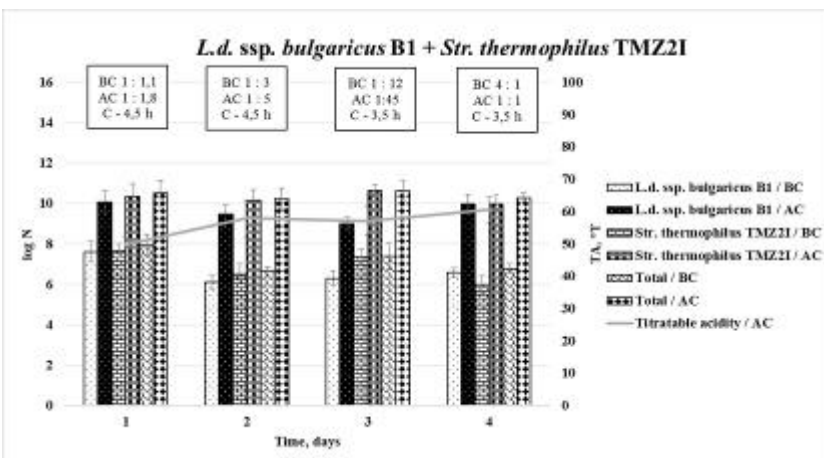

Figure 15a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

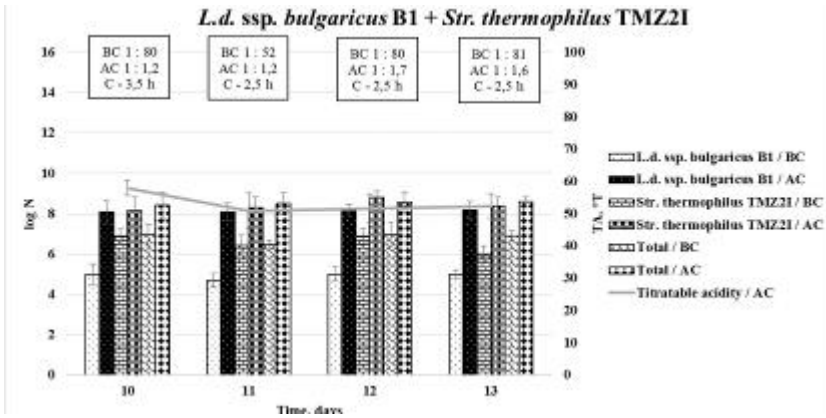

Figure 15b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days after the 10-day pause.

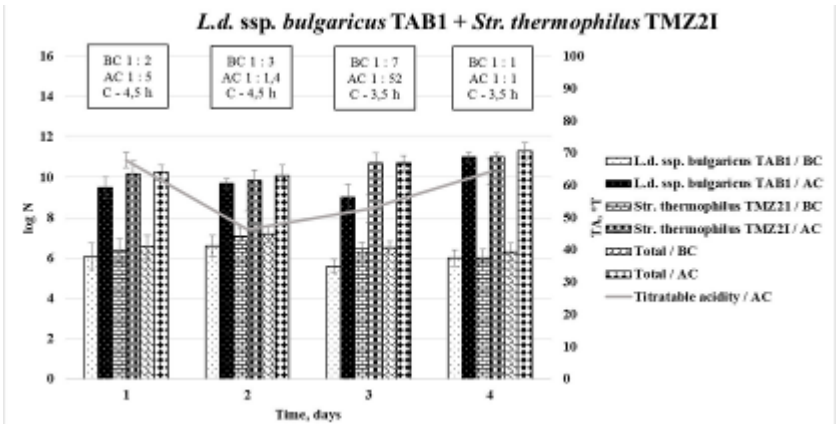

Figure 16a. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days right after the mixing of the strains.

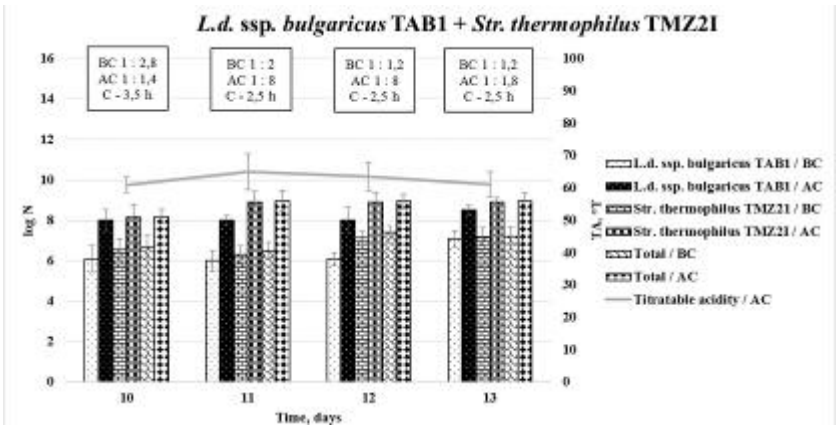

Figure 16b. Growth and acidification-ability of the combination of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I during co-culturing in skimmed milk at a temperature of 44±1°C at daily passaging for 4 consequtive days after the 10-day pause.

In the comninations containing *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 no symbiotic development was observed. At all three temperatures the streptococci prevailed over the lactobacilli. Probably, the *Lactobacillus delbrueckii* ssp. *bulgaricus* M3 cells are sensitive to streptococcal metabolic activity or need additional growth factors in the medium.

The conducted research on the development of yogurt starters demonstrates the importance of achieving symbiosis between the *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains in order to produce a quality product. The symbiosis between *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* largely determines the flavor complex of dairy foods as well. The stability of the starters during their incubation at different growth temperatures (41±1°C and 44±1°C) reveals the possibility of their application in the manufacture of various fermented dairy foods.

On the basis of these studies were selected 2 symbiotic starters for fermented dairy foods: *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* TMZ2I; *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I.

1.3. Dynamics of Batch Cultivation of Lactobacillus delbrueckii ssp. bulgaricus TAB1 and Streptococcus thermophilus TMZ2I

The dynamics of growth, acid formation and the coagulation time during separate batch culturing and co-culturing of the strains from the composition of the starter *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I in a laboratory bioreactor with mechanical stirring were examined.

The results obtained in the separate batch culturing of *Streptococcus thermophilus* TMZ2I and *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 are shown in Figure 17, Figure 18, Figure 19 and Figure 20.

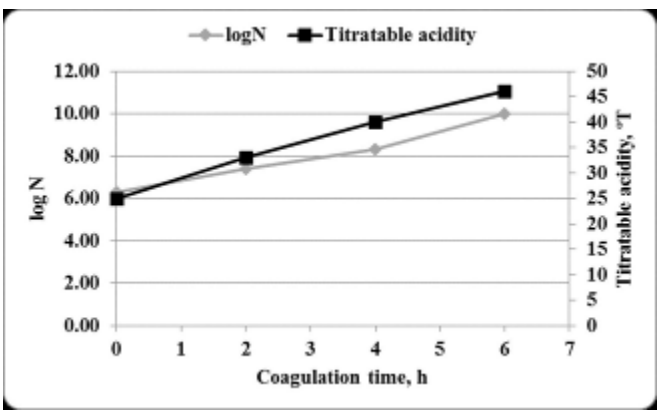

Figure 17. Dynamics of change of the concentration of viable cells and the titratable acidity during batch culturing of *Streptococcus thermophilus* TMZ2I in the laboratory bioreactor.

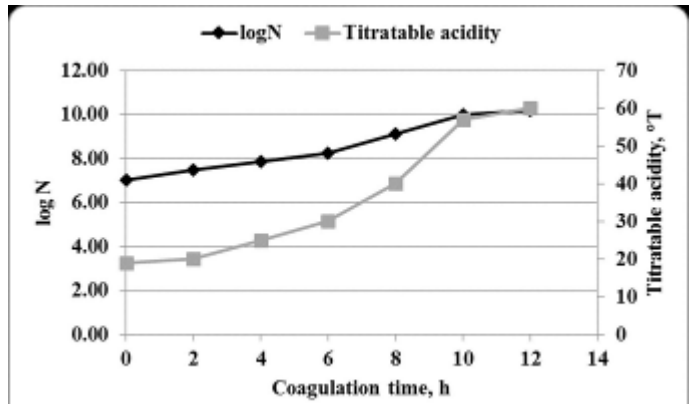

Figure 18. Dynamics of change of the concentration of viable cells and the titratable acidity during batch culturing of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 in the laboratory bioreactor.

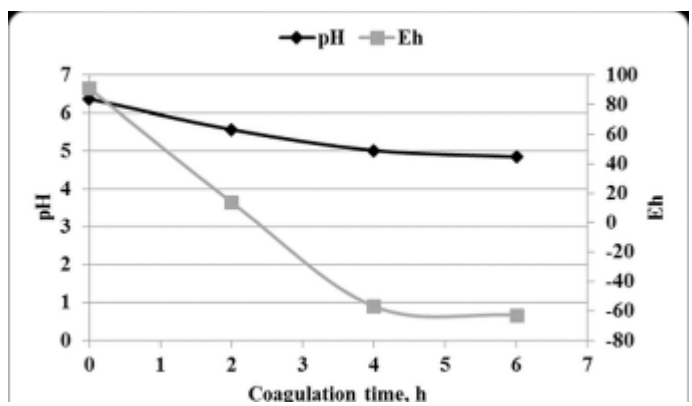

Figure 19. Dynamics of change of the pH and the redox potential during the batch culturing of *Streptococcus thermophilus* TMZ2I in the laboratory bioreactor.

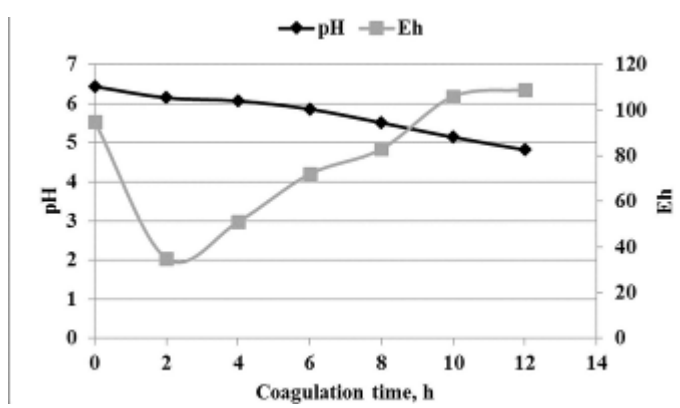

Figure 20. Dynamics of change of the pH and the oxidation-reduction potential during the batch culturing of *Lactobacillus delbrueckii* spp. *bulgaricus* TAB1 in the laboratory bioreactor.

In the batch culturing of *Streptococcus thermophilus* TMZ2I the lag-phase was significantly shorter (about 1,5 h) in comparison with that in the batch culturing of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 (about 5 h). Coagulation of the milk fermented with *Streptococcus thermophilus* TMZ2I occurred on the sixth hour from the beginning of the process, while achieving a high concentration of viable cells - over 10^{10}cfu/cm^3. The titratable acidity increased with a constant rate throughout the whole process. In the batch culturing of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 milk coagulation occurred at the 12th hour at a concentration of active cells, comparable to that of *Streptococcus thermophilus* TMZ2I (about 10^{10}cfu/cm^3).

During the lag-phase of the separate culturing of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 the titratable acidity was maintained relatively constant, then began its gradual increase and at the moment of coagulation it was about 65ºT.

The dynamics of change of pH and the redox potential during the separate batch culturing of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I was monitored as well (Figure 19 and Figure 20).

During the separate batch culturing of both tested strains an expected trend of decrease in the pH values was observed. More intensive decrease of pH in the culturing of *Streptococcus thermophilus* TMZ2I was observed up to the fourth hour from the beginning of the process, and then it remained relatively constant until the coagulation of the milk, when the pH reached 4.85. In the separate batch culturing of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 the pH reduction was constant up to the moment of coagulation, when the pH reached 4,81. Major differences in the values of the redox potential were obtained for both strains. In the culturing of *Streptococcus thermophilus* TMZ2I the redox potential decreased sharply up to the fourth hour and remained constant until the end of the process, taking value of -63 mV. In the culturing of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 the redox potential decreased during the lag-phase. With the introduction of the culture in the exponential growth phase, the redox potential began increasing and from the 10th hour until the time of coagulation of the milk it remained constant - +109mV.

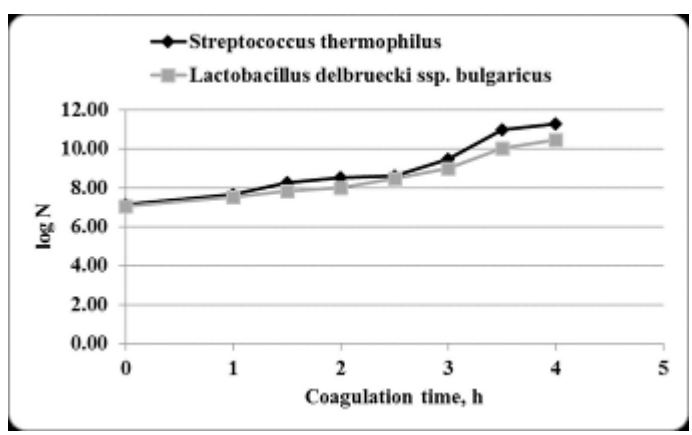

Figure 21. Dynamics of the changes in the concentrations of viable cells during batch co-culturing of *Streptococcus thermophilus* TMZ2I and *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 in the laboratory bioreactor.

A batch process of co-culturing of *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I in a bioreactor with mechanical stirring was performed. The dynamics of the mutual growth, the coagulation time and the changes in the titratable acidity, determined by the metabolic activity of the two strains in the composition of the starter, were monitored (Figure 21 and Figure 22).

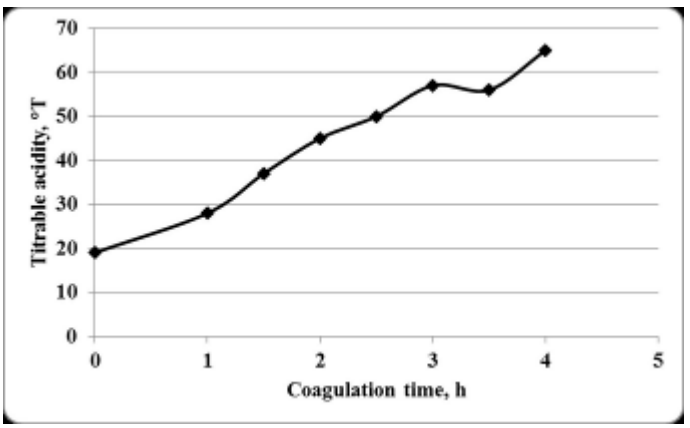

Figure 22. Dynamics of the changes in the titratable acidity during batch co-culturing of *Streptococcus thermophilus* TMZ2I and *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 in the laboratory bioreactor.

The changes in the pH and the redox potential during the batch co-culturing of *Streptococcus thermophilus* TMZ2I and *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 were also traced (Figure 23).

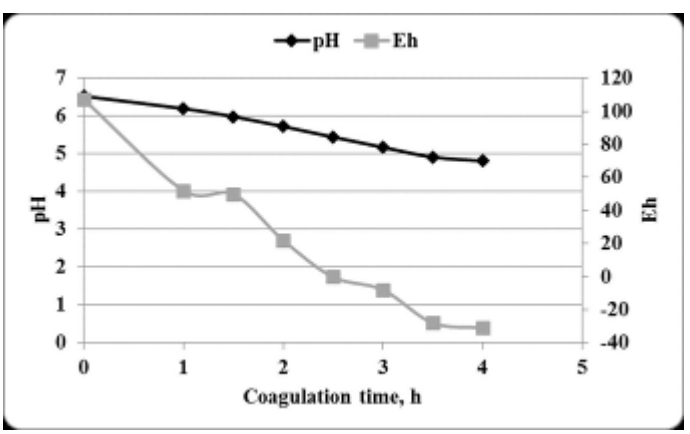

Figure 23. Dynamics of the changes in the pH and the redox potential during batch co-culturing of *Streptococcus thermophilus* TMZ2I and *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 in the laboratory bioreactor.

In a mixed population *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I coagulated the milk much quicker (for about 4 h) compared to the coagulation time during their separate culturing. The symbiotic interaction between the two strains can clearly be observed in the significant shortening of the lag phase in comparison to that in the separate culturing of *Lactobacillus delbruekii* ssp. *bulgaricus* TAB1 (duration of

the lag phase about 1,5 h). This was due to the ability of *Streptococcus thermophilus* TMZ2I to absorb the dissolved oxygen and thus to provide anaerobic environment for *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 growth. This was confirmed by the continuous decrease in the redox potential values, which were around -31 mV at the end of the process. During the batch co-culturing both strains in the composition of the starter reached high concentrations of active cells after the coagulation of the milk. For *Streptococcus thermophilus* TMZ2I the concentration of active cells was about 10^{11}cfu/cm^3, while for *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 it was about 10^{10}cfu/cm^3.

The symbiotic starters MZ_2 (including *Lactobacillus delbrueckii* ssp. *bulgaricus* MZ_2 and *Streptococcus thermophilus* TMZ2I) and 1Cm (including *Lactobacillus delbrueckii* ssp. *bulgaricus* MZ_2, *Streptococcus thermophilus* TMZ2I and *Streptococcus thermophilus* T3) were developed and selected as a result of analogous studies (Denkova et al., 2009). The fermentation at 37±1°C, at 41±1°C and at 44±1°C using these symbiotic starters resulted in obtaining high quality dairy products, allowing their use in the production of both yogurt as well as in the production of soft or hard cheeses.

2. Development of Bio-Yogurt Including Selected Probiotic Strains

From spontaneously fermented foods (yogurt, vegetables, sourdough) and from human faeces were isolated and identified lactobacilli and bifidobacteria strains with probiotic potential - high antimicrobial activity against pathogens causing foodborne diseases; resistance to the action of gastric juice and different concentrations of bile salts; good adhesion ability to the intestinal mucosa; retaining high concentrations of active cells in the conduction of industrial processes - culturing with obtaining of preparations with high concentrations of viable cells, which are retained in the process of lyophilization and storage (Table 4).

Table 4. Lactobacilli and bifidobacteria with probiotic potential

Lactobacillus	*Bifidobacterium*
Lactobacillus acidophilus 5R	*Bifidobacterium bifidum* BB1
Lactobacillus acidophilus A2	*Bifidobacterium bifidum* BB2
Lactobacillus acidophilus Ac	*Bifidobacterium infantis* BI13
Lactobacillus acidophilus 2	*Bifidobacterium infantis* BI15
Lactobacillus acidophilus A	*Bifidobacterium longum* BL11
Lactobacillus gasseri Bio	*Bifidobacterium breve* BB18
Lactobacillus delbrueckii ssp. *bulgaricus* GB-15	
Lactobacillus delbrueckii ssp. *bulgaricus* GB	
Lactobacillus delbrueckii ssp. *bulgaricus* LB M	
Lactobacillus delbrueckii ssp. *bulgaricus* 144	

Probiotic bacteria can be added to yogurt together with the yogurt starter before fermentation or to the final product right before packaging.

Yogurt has long been recognized as a product with health benefits. New multi-strain yogurt starters with the inclusion of probiotic strains of *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and others to the conventional yogurt starter aimed at increasing of its physiological action because *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* survive poorly during their passage through the digestive tract are being developed (Gilliland, 1979). The bacteria used in the manufacture of traditional yogurt - *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* – do not belong to the composition of the normal intestinal flora and they are not resistant to the low pH and the presence of pepsin and bile (Gilliland, 1979). Their positive health effect is due to the formation of fermentation metabolites or substances with antimicrobial action through which they inhibit the growth of pathogenic bacteria and improve digestion (Gilliland, 1979).

The probiotic yogurts developed by our scientific team include high concentrations of living cells of probiotic strains of *Lactobacillus acidophilus* (acidophilic milk - *Lactobacillus acidophilus* 5R), *Lactobacillus gasseri*, bifidobacteria (bifid milk - *Bifidobacterium* complex including *Bifidobacterium longum* BL11, *Bifidobacterium infantis* BI15 and *Bifidobacterium breve* BB18), *and Lactobacillus delbrueckii* ssp. *bulgaricus* GB-15. Therefore, bio-yogurt is yogurt which contains high concentration of living cells of probiotic microorganism in the presence of which the beneficial effect of yogurt consumption is enhanced. According Rybka and Kailasapathy, 1995 in order to achieve this effect, the concentration of viable cells of probiotic bacteria in the final product should be no less than 10^9cfu/cm^3.

Technology for the preparation of bio-yogurt with a high concentration of viable cells (over 10^9cfu/cm^3) of the probiotic strain *Lactobacillus delbrueckii* ssp. *bulgaricus* GB-15 and the traditional yogurt starter MZ_2, which are retained in the course of storage at 4±2°C for 30 days has been developed (Figure 24).

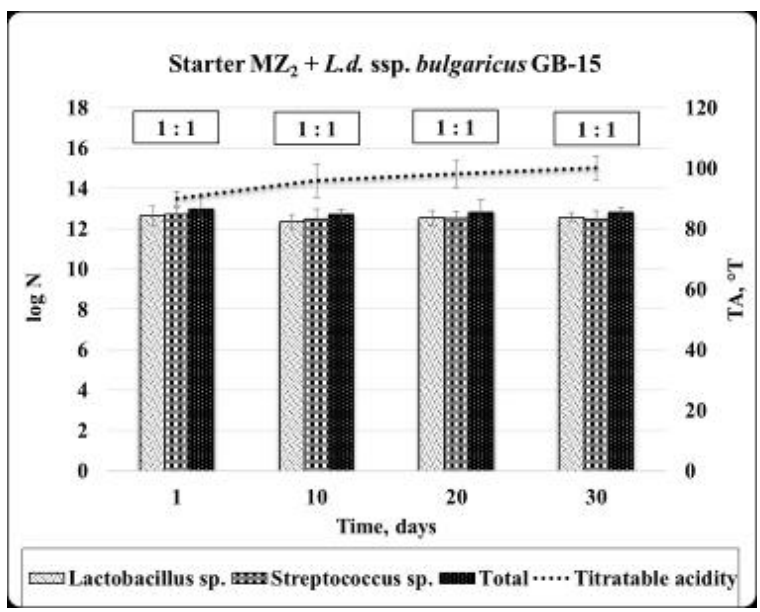

Figure 24. Bio-yogurt with the symbiotic yogurt starter MZ_2 and the probiotic strain *Lactobacillus delbrueckii* ssp. *bulgaricus* GB-15 in the course of storage at 4 ± 2 ° C for 30 days.

Lactobacillus gasseri is a prolific autochthonous microorganism that colonizes the gastro-intestinal tract, oral cavity, and vagina in humans (Selle and Klaenhammer, 2013).

The widespread colonization of mucosal niches by *L. gasseri* indicates its prevalence as a commensal in healthy individuals. The absence of any association with a health detriment affirms its safety. In fact, *L. gasseri* is commonly found in infants and is one of the predominant species involved in early colonization of the gastro-intestinal tract and is persistent throughout adulthood (Selle and Klaenhammer, 2013).

Bio-yogurt with high concentration of viable *Lactobacillus* sp. cells (more than 10^9cfu/cm^3) was produced using the probiotic strain *Lactobacillus gasseri* Bio and the traditional yogurt starter MZ_2. The high concentration of viable cells was retained in the course of storage at 4 ± 2 ° C for 30 days (Figure 25).

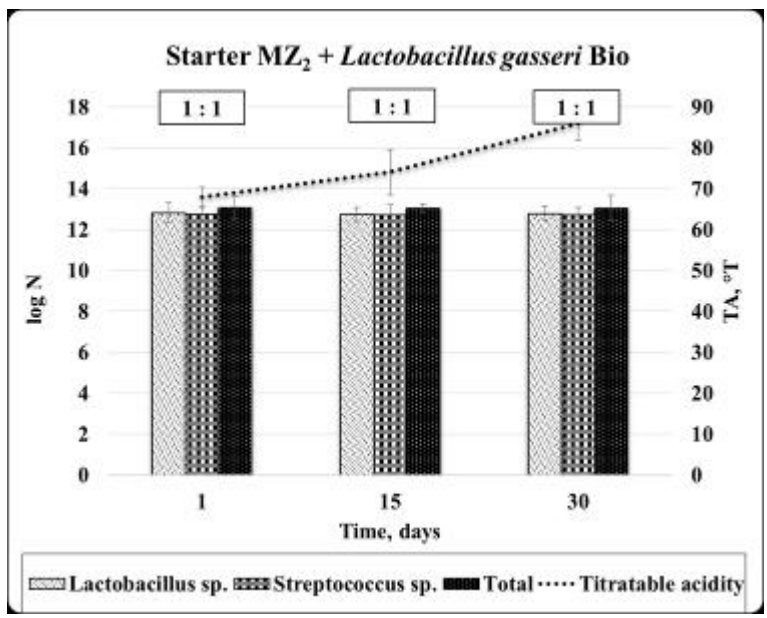

Figure 25. Bio-yogurt with the probiotic strain *Lactobacillus gasseri* Bio and the symbiotic yogurt starter MZ_2 in the course of storage at 4±2°C for 30 days.

The group of *Lactobacillus acidophilus* includes slime-producing and nonslime-producing strains. Yogurt produced as a result of fermentation of *Lactobacillus acidophilus* strains alone has pronounced citric acid taste and / or slime texture. Therefore only nonslime-producing probiotic *Lactobacillus acidophilus* strains can be used in yogurt preparation together with traditional yogurt starter. Bio-yogurt with high concentration of viable lactobacilli cells (more than 10^{12}cfu/cm^3) with the probiotic strain *Lactobacillus acidophilus* 5R and the symbiotic yogurt starter MZ_2, was produced. The high concentration of viable lactobacilli cells was retained in the course of storage at 4 ± 2 ° C for 30 days was obtained (Figure 26).

Bifidobacteria fermentation alone is a lengthy process (over 12 hours) and the resulting end product has a specific flavor complex, which differs from the inherent flavor of traditional yogurt. Bifid yogurt with introduction of a complex of probiotic bifidobacteria - *Bifidobacterium longum* BL11, *Bifidobacterium infantis* BI15 and *Bifidobacterium breve*

BB18, and the symbiotic yogurt starter MZ_2 was prepared. In the final product the concentration of viable bifidobacteria cells was above 10^9cfu/cm^3, and it was retained during storage at 4±2°C for 60 days (Figure 27).

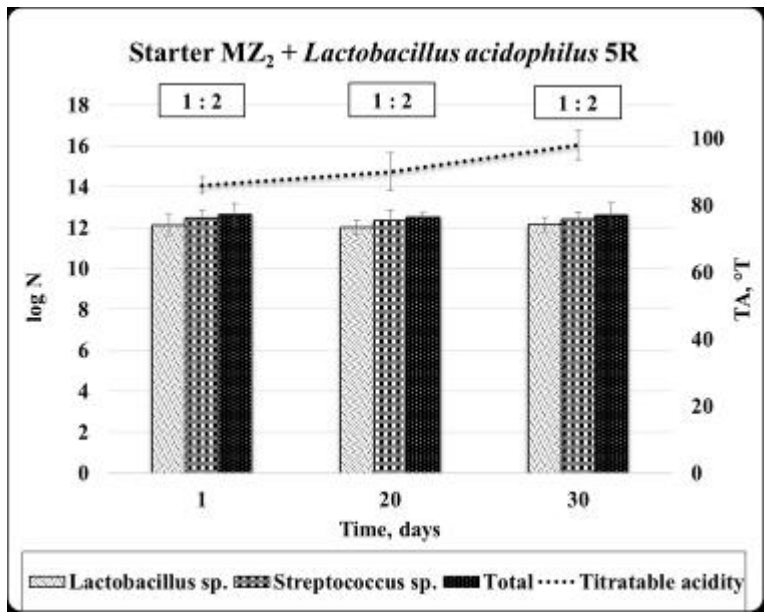

Figure 26. Bio-yogurt with the probiotic strain *Lactobacillus acidophilus* 5R and the symbiotic yogurt starter MZ_2 in the course of storage at 4±2°C for 30 days.

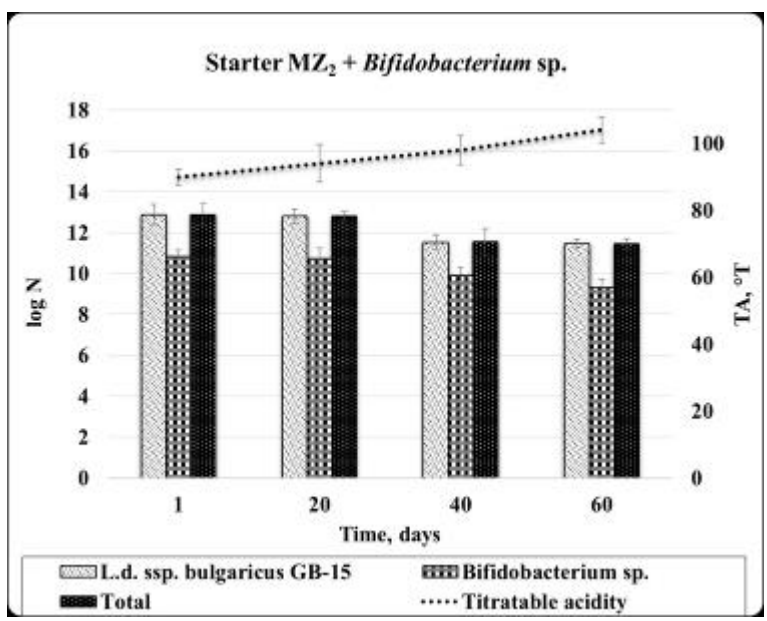

Figure 27. Bio-yogurt with probiotic bifidobacteria strains and the symbiotic yogurt starter MZ_2 during storage at 4±2°C for 60 days.

The retaining of the viability of the lactobacilli and bifidobacteria strains with probiotic properties in bio-yogurt is essential to provide their inherent therapeutic effect. It should be noted that the incorporation of bifidobacteria in dairy foods is difficult as they grow poorly in milk and require anaerobic environment, low redox potential and adding bifidogenic factors.

Organoleptic assessment of the obtained yogurts with probiotic bacteria was conducted according to indicators in Majchrzak et al., 2010. All probiotic yogurts received high scores from the organoleptic assessment (unpublished data).

3. Development of Bio-Yogurt Fortified with Plant Protein

Probiotic bacteria may be included in the preparation of soy yogurts. Unfortunately, most of the studies on the growth of probiotic bacteria in soy extracts are made with pure cultures. The information about the growth of mixed cultures on soybean substrates is scarce (Farnworth et al., 2007).

Bifidobacteria have α-galactosidase activity that allows them to utilize sugars such as raffinose and stachyose, and sufficient proteolytic activity to support their growth in soy milk (Farnworth et al., 2007).

Soy milk is used directly as raw milk for the production of fermented nondairy foods. One symbiotic starter for soy milk, probiotic *Lactobacillus* sp. strains and a probiotic bifidobacteria complex were included in our experimental studies. The probiotic strains could be applied either alone or together with the symbiotic yogurt starter in order to obtain soy yogurts, but the concentration of the probiotic strain(s) in the final products should be above 10^9cfu/cm^3. Five soy yogurt variants were prepared and stored at refrigeration conditions (4±2°C) for 20 days: soy yogurt with symbiotic starter MZ_2 (Figure 28); soy yogurt with symbiotic starter MZ_2 and the probiotic strain *Lactobacillus delbrueckii* spp. *bulgaricus* Y1 (Figure 29); soy yogurt with the probiotic strain *Lactobacillus delbrueckii* spp. *bulgaricus* Y1 (Figure 30); soy yogurt with the probiotic strain *Lactobacillus acidophilus* 5R (Figure 31); soy yogurt with the probiotic bifidobacteria complex – *Bifidobacterium longum* BL11, *Bifidobacterium infantis* BI13, *Bifidobacterium bifidum* BB1 and *Bifidobacterium breve* BB18 (Figure 32).

Upon storage of the soy yogurt variants for 20 days under refrigeration conditions the concentration of living cells was maintained and the titratable acidity rose slightly up to the 10th day of storage (Figure 28, Figure 31 and Figure 32). During further storage of the soy yogurts no significant change in the concentration of active cells and in the titratable acidity was observed (Figure 28 – Figure 32). All the soy yogurt variants retained high concentrations of viable cells of the strains included in the composition of the starter (above 10^{11}cfu/cm^3) as well as of the added probiotic strain(s) and moderate titratable acidity ranging from 54 °T to 82 °T at the 20th day of storage (Figure 28, Figure 29, Figure 30, Figure 31, Figure 32).

Besides the valuable qualities of soy milk the intake of the prepared soy yogurts would deliver to the body the necessary amount of probiotic lactic acid bacteria to normalize and maintain the balance of the microflora in the gastro-intestinal tract.

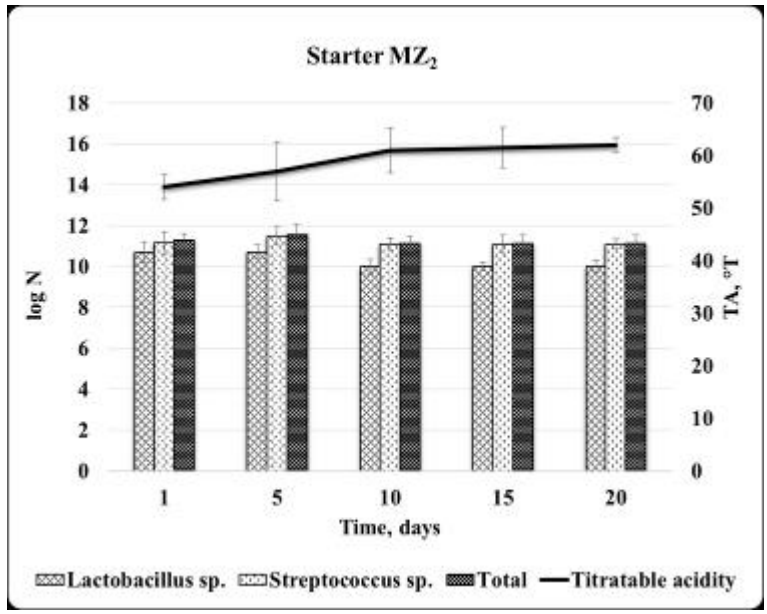

Figure 28. Soy yogurt with symbiotic starter MZ_2 during storage at 4±2°C for 20 days.

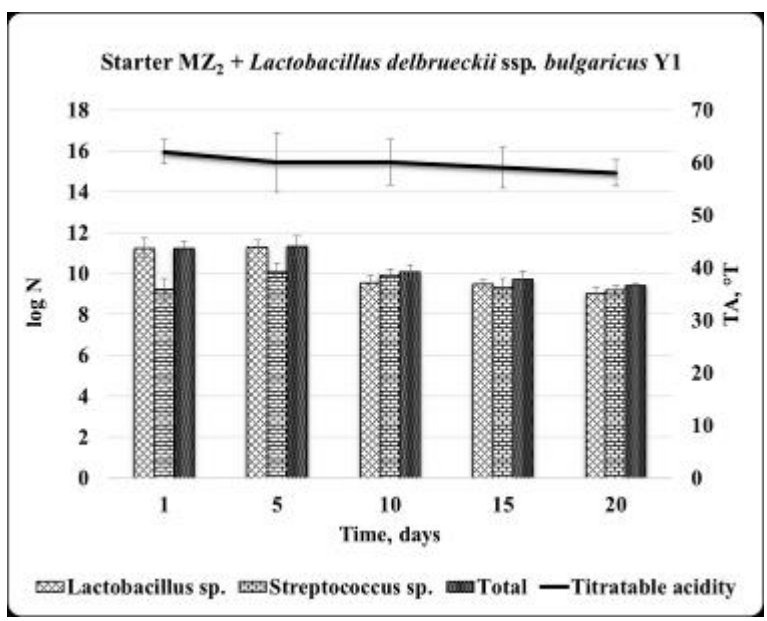

Figure 29. Soy yogurt with symbiotic starter MZ_2 and the probiotic strain *Lactobacillus delbrueckii* ssp. *bulgaricus* Y1 during storage at 4±2°C for 20 days.

The soy yogurt prepared with the symbiotic yogurt starter (Figure 28) differed in terms of organoleptic features from the acidophilic (Figure 31) and the bifid (Figure 32) soy yogurt as it had yogurt taste and slight soy flavor. In the acidophilic and bifid soy yogurt were accumulated metabolic products as a result of the metabolism of acidophilic bacteria or bifidobacteria and these soy yogurts were characterized by pleasant yogurt taste without soy flavor.

With their pleasant yogurt taste, with the high concentration of viable cells of acidophilic bacteria and bifidobacteria, with the ability to be stored for a long time the obtained soy products are suitable food for adults and children with digestive system disorders, cardiovascular diseases and others as well as for children suffering from lactose intolerance. So soy yogurts successfully replace dairy yogurts and implement remedial and preventive function as well.

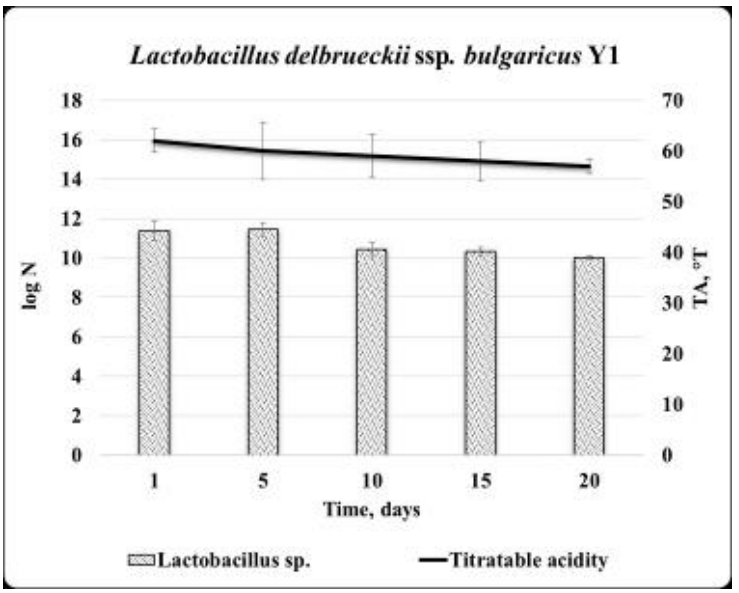

Figure 30. Soy yogurt with the probiotic strain *Lactobacillus delbrueckii* ssp. *bulgaricus* Y1 during storage at 4±2°C for 20 days.

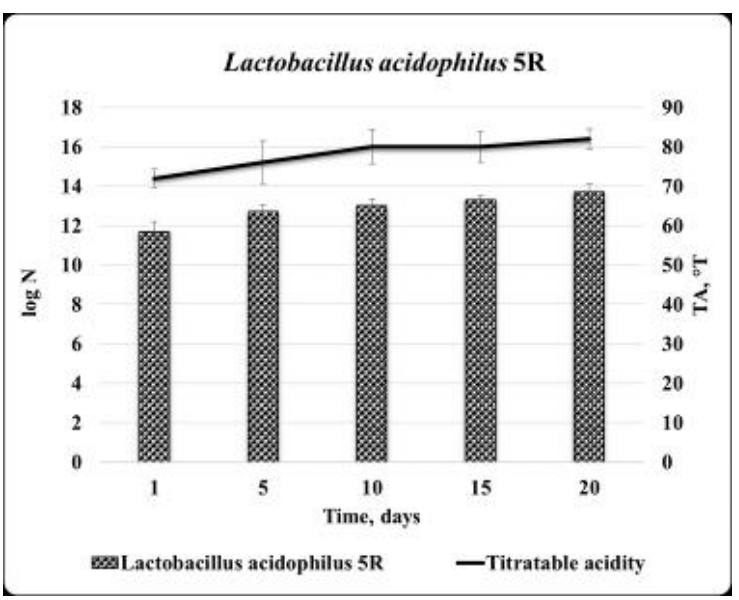

Figure 31. Soy yogurt with the probiotic strain *Lactobacillus acidophilus* 5R during storage at 4±2°C for 20 days.

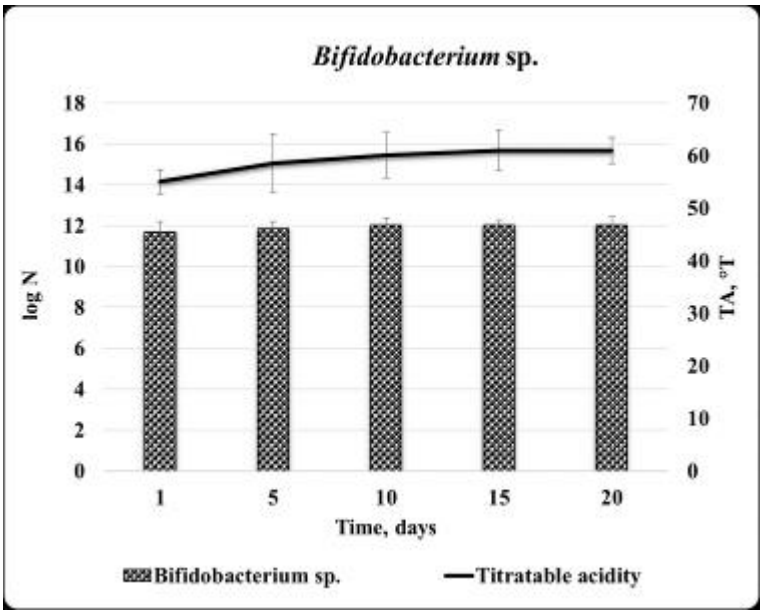

Figure 32. Soy yogurt with the probiotic bifidobacteria complex during storage at 4±2°C for 20 days.

CONCLUSION

The selection of *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* strains and the development of symbiotic starters for different dairy products with high nutritional and physiological value, safety and quality is an important factor in improving consumer's health status. The developed symbiotic starters *Lactobacillus delbrueckii* ssp. *bulgaricus* B1 and *Streptococcus thermophilus* TMZ2I; *Lactobacillus delbrueckii* ssp. *bulgaricus* TAB1 and *Streptococcus thermophilus* TMZ2I; Starter MZ_2 (including *Lactobacillus delbrueckii* ssp. *bulgaricus* MZ_2 and *Streptococcus thermophilus* TMZ2I) and Starter 1Cm (including *Lactobacillus delbrueckii* ssp. *bulgaricus* MZ_2, *Streptococcus thermophilus* TMZ2I and *Streptococcus thermophilus* T3) allow the realization of technological processes and obtaining high-quality yogurt and other dairy products with inherent health beneficial effects.

The addition of selected probiotic lactobacilli and bifidobacteria strains and complexes to yogurt either before or after yogurt fermentation enhances the positive effects of yogurt consumption on human health because the obtained probiotic yogurts contain high concentrations of viable probiotic cells which exert their preventive and therapeutic role after consumption.

Plant milks (soy milk) are a very good alternative to animal proteins when it comes to yogurt production. They are also characterized by a number of additional inherent plant health benefits. Besides, they offer a solution for a number of conditions associated with lactose intolerance and milk protein allergies. The obtained probiotic soy variants have good organoleptic profile, no soy flavor and high concentrations of viable probiotic cells.

All the developed dairy and non-dairy yogurts carry significant amounts of beneficial microflora, which when intaken with food, would perform its due preventive role in the gastro-intestinal tract.

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BIOGRAPHICAL SKETCH*ES*

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Publications Last 3 Years:

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Chapter 8

THE CONVERGENCE AND DIVERGENCE OF CULTURAL VALUES IN FUNCTIONAL FOOD CONSUMPTION: THE EXPERIENCES OF THE MALAYSIAN MARKETPLACE

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ABSTRACT

Food with curative properties has attracted interest among many consumers around the world due to the increasing attention paid to food-related health issues, such as food's potential ability to prevent diseases and improve consumers' mental state or quality of life. Most of the extant studies on functional food examine the role of functional food in developed Western nations which might not suitable for developing countries. Thus, the role of culture and value systems in influencing functional food consumption is poorly understood in developing countries. In Asia, foods with functional properties have been an important part of Asian culture for centuries, even though the term 'functional food' is not commonly used. For many years, Asian countries have supplemented their diets with naturally occurring substances with health-giving and curative properties. The knowledge of functional foods is often passed from generation to generation through oral traditions. In Malaysia functional food is a type of culturally-based food that is unique to ethnic group. Over time, though, pervasive cultural changes brought about by intergroup communications, economic development and greater social interaction promotes a certain level of cultural convergence in functional food consumption. In this chapter we will discuss the role of ethnicity and the dynamics of cultural and value changes in traditional and emerging economies, explore the convergence of cultural values in functional food

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consumption in multicultural societies, and outline lessons for businesses that compete in functional food markets.
Keywords: functional food, Malaysia, cultural value convergence

INTRODUCTION

Food has significant societal, historical and religious roles, in addition to nutritional value. It represents cultures and customs; provides opportunities for sharing, giving and social interaction; and offers nutrition, pleasure and satisfaction. Technically, all foods are functional, as they fulfil a basic human need and provide nutritive value. However, the term 'functional food' implies an additional physiological benefit beyond meeting basic nutritional needs. Functional food has been recognised as a separate category in the food market (Bech-Larsen et al., 2001; Poulsen, 1999; Saher et al., 2004; Tee et al., 2004; A. Y.-T. Wong et al., 2015) and it is one of the world's most intensive areas of food product innovation (Bigliardi and Galati, 2013; Bistrom and Nordstrom, 2002).

For centuries, there has been a deep-rooted belief that foods and herbs have health-giving and curative properties. This type of food has been an important part of Asian culture for centuries, even though the term 'functional foods' is not commonly used as they are referred to as 'health foods.' In Malaysia, each ethnicity's food beliefs and knowledge comes from oral tradition, family history, personal experience and reading (Hassan, 2011c). Traditional knowledge is defined as a cumulative, collective body of knowledge, experience, and values held by societies with a history of subsistence, while oral tradition (by speaking) is the transmission of knowledge from one generation to the next.

Functional food is generally agreed to be food that provides health benefits beyond basic nutritional requirements. The term functional food was first introduced in Japan in the mid-1980s to distinguish these products from medicine. In Japan, functional food refers to processed foods containing ingredients that aid specific bodily functions, in addition to being nutritious (Arai, 2002; Martirosyan and Singh, 2015). Japan is the first and only country with a specific regulatory approval process for functional foods.

Although, there is no universally accepted definition of functional food but a simple definition has proposed by The International Food Information Council (IFIC) of functional food as any "modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains" (International Food Information Council Foundation, 1998). The well accepted definition is that functional food as "a concept, which covers foods that are enriched with various kinds of (naturally occurring), components/substances (e.g., vitamins, minerals or probiotic cultures) or modified in a way so that the product provides an additional physiological benefit that may prevent disease or promote health" by Bech-Larsen et al. (2001: p. 1).

To achieve a certain degree of conceptual equivalence, in the context of this book chapter we believe that "food and medicine have the same origin(s) and are both intended to maintain human health; substances in functional food can have medicinal value but medicine itself is not a functional food. Medicinal products have to be prescribed by physicians, either traditional (unqualified) Malaysian medical practitioners like *sinsei, bomoh* or *dukun,* or qualified medical practitioners. Functional food can be procured like any other food but has health-enhancing properties" (Hassan, 2011b).

FUNCTIONAL FOOD IN MULTICULTURAL SOCIETY – MALAYSIA PERSPECTIVE

Food with curative properties has attracted interest among many consumers around the world, probably due to the increasing attention paid to food-related health issues, such as food's potential ability to prevent diseases and improve consumers' mental state or quality of life. Foods with functional properties have been an important part of Asian culture for centuries. However, the role of how local culture and value systems in influencing the consumption of food with health benefits is little understood and has been largely neglected in academic research on developing countries. Furthermore, the term 'functional food' is not commonly used 'health food' is the usual terminology in most Asian countries (Tee et al., 2004). When it comes to functional food, there are some relevant studies, but most focus on societies with a dominant culture, particularly the United States and certain European countries. The United States has a predominantly Anglo-Saxon culture; its subcultures remain on the periphery. This monocultural scenario is not relevant to countries like Malaysia, which has no single dominant culture. In Malaysia, consumers can be clearly slotted into separate compartments according to their specific ethnic group. One cannot simply study the majority of inhabitants—the Malays—and then generalize the findings to other Malaysian cultural groups.

In Malaysia, interest in functional foods is gaining momentum and some products and processes have been patented and commercialized (Ahmad, 1996). However, functional food is still a relatively new concept in Malaysia (Salleh et al., 2015). There is an increasing array of foods on the Malaysian health food market claiming to boost vitality, reverse ageing or cure and prevent specific diseases. Some of these functional foods are culturally-based (traditional) and others are modified, fortified or totally new foods (modern). The modern functional food products are easier and more convenient to consume than traditional functional foods.

For many years, Asian countries such as Malaysians have supplemented their diets with naturally occurring substances. Malaysia has 8,000 species of flowering plants and about 6,000 of these have medicinal value (Muhamad, 1991). Of these, 1,200 are used in traditional medicine. Each of the three ethnic groups in Malaysia (Malay, Chinese and Indian) has its own beliefs about food and wellbeing and each has its own popular food with functional properties.

In Malaysia, food products are controlled under the Malaysian Food Regulations 1985. There are no specific regulations for health foods or functional foods (K. Y. Tan et al., 2015; Tee et al., 2004), although a new regulation on nutrition labelling and claims was introduced on March 31, 2003. The Malaysian government has appointed the Drug Control Authority (DCA) and the National Pharmaceutical Control Bureau to formulate a separate regulation for dietary supplements (Tee et al., 2004). In addition, expert committees have been established to evaluate the health claim applications and provide consultancy services to the local food authorities (K. Y. Tan et al., 2015). They define dietary supplements as "products intended to supplement the diet, taken orally in forms such as pills, capsules, tablets, liquids or powders and not represented as conventional foods" (Tee et al., 2004: p. 10).

Although, the studies on functional food been carried out but the role of culture and value systems in influencing functional food consumption still remains poorly understood in

developing countries especially in multicultural society. In this chapter we will discuss the role of ethnicity in Malaysia and the dynamics of cultural and value changes in traditional and emerging economies, and outline lessons for businesses that compete in traditional functional food markets.

MALAYSIAN CONSUMERS – THE MALAY, CHINESE AND INDIAN

This section examines the convergence of values among consumers of different ethnic backgrounds in Malaysia. The suggestion that there exists some cultural convergence or commonalities that influence the consumption of functional food is vital to this chapter; as such the issue is examined in depth.

Malaysia is a Southeast Asian country occupying parts of the Malay Peninsula and the island of Borne, consist of cultural mosaic of races and religions. The three main ethnic groups in Peninsular Malaysia are Malay, Chinese and Indian. The Malay (*Bumiputera)* constitute 67.4 percent of the population, the Chinese 24.6 percent, and the Indians 7.3 percent, according to the 2010 Malaysia Population and Housing Census (Department of Statistics Malaysia, 2015). There are also several indigenous *Orang Asli* tribes—original people—who live in rural areas and in the forests of Peninsular Malaysia, and in Borneo. These tribes are not included in this study because of their limited consumer role.

The Malays originated in different parts of Peninsula Malaysia and archipelagic Southeast Asia. This ethnic group has been resident in Malaysia the longest of the three main groups and they are known as *bumiputera*, meaning 'sons of the soil.' The Malays receive more privileges from the government than the Chinese and Indian ethnic groups that arrived in the country during the period of British rule. The Malays constitute just over one half of the nation's population and are the dominant ethnic group of the country, both culturally and politically. To be considered Malay one must be Muslim, speak the Malay language and observe and practice the traditions of Malay culture (Colonial Office, 1957).

Traditional Malay culinary styles, food beliefs and medicines have been greatly influenced by ancient traders from Indonesia, India, the Middle East and China. According to Hirschman (1987), there is evidence of extensive contact between the Malays, Indians and Chinese lasting more than a thousand years (Lamb, 1964; Purcell, 1948, 1967). The Straits of Malacca were an early trading route for merchants between China, India and Arabia. These merchants not only traded goods, but also exchanged cultural values and medical beliefs (Laderman, 1983). Malays classify food on its internal 'hotness,' 'coldness' and '*bisa*' (*allergy food*); beliefs that mirror ancient Chinese and Indian traditions. Malays have used herbs and plant roots from the rainforest as traditional dietary supplements for generations (Ahmad, 1996; Anonymous, 2005). Some of the popular Malay foods for boosting vitality and preventing aging, cancer, diabetes, and hypertension are *mengkudu/noni* juice (*morinda citrifolia)* (Bhatia et al., 2015; Nandhasri et al., 2005; Wang et al., 2002), *petai* (*parkia speciosa*) (Seyedreihani et al., 2016; Wong et al., 2006), *pegaga* (*centella asiatica*) (Mohd Ilham et al., 1998), and *tongkat ali* (*eurycoma longifolia*) (Mohd Ilham et al., 1998; Rehman et al., 2016). They also incorporate functional ingredients in their cooking. Fresh herbs like *daun kemangi* (a type of basil), *daun kesum* (polygonum or laksa leaf), nutmeg, *kunyit* (turmeric) and *bunga kantan* (wild ginger buds) are used extensively in Malay dishes.

Traditional spices like cumin and coriander are used in conjunction with the Indian and Chinese spices of pepper, cardamom, star anise and fenugreek. Most of these herbs and spices are also believed to have some medicinal value (Alsarhan et al., 2014; Rehman et al., 2016).

The Chinese first arrived in Malaysia in the 15th century, when the Ming princess Hang Li Po and her entourage established a thriving community at Malacca that gave rise to the *babas* and *nyonyas* of today. The mass immigration of Chinese began in the 19th century as labour for British tin mines. Ethnic Chinese constitute about 26.0 percent of the total Malaysian population and have managed to preserve a distinct identity and culture (Freedman, 2001). Their sub-identities can be differentiated by dialect, principally Hokkien, Cantonese and Teochew (C. B. Tan, 2000). Most ethnic Chinese are multilingual and speak Malay, English and Mandarin, in addition to their native dialect. Many Chinese speak and write Malay fluently because that language has been the medium of instruction in secondary schools since independence. Despite their Malay education, Chinese-Malaysians have distinct accents, diets, mannerisms and lifestyles.

The most widely held health belief in Chinese medicine is the *yin/yang* theory that also appears in Chinese cuisine (Hinrichs and Barnes, 2013; Ho, 1985). *Yin* and *yang* represent the negative and positive principles of nature, as well as hotness and coldness. Fundamental to this theory is the notion that good eating habits are essential to maintaining good health (Ho, 1985; Weng and Chen, 1996; Williams, 2016). Health is believed to be a balance of positive (*yang*) and negative (*yin*) energy (*chi*) in the body, a belief that derives from Traditional Chinese Medicine (TCM). An unbalanced diet will cause discomfort and illness; a perfect equilibrium of *yin* and *yang* is crucial to maintaining harmony and balance. All things, including body organs, disease and food are categorized as *yin* or *yang* (Chan-Yip and Kirmayer, 1998; Hinrichs and Barnes, 2013). TCM views the human body as an integrated whole and any medical treatment must be considered holistically and based on symptoms and signs (Weng and Chen, 1996; Williams, 2016). There are four parts to TCM nutrition: food as diet, food as tonic, food as medicine and food as abstention. In TCM, it believe that food and medicine are equally essential in treating and preventing diseases (Weng and Chen, 1996). Furthermore, "Chinese medical practitioners consider that food and medicine come from the same source, (and) are based on the same basic theories and have the same uses" (Weng and Chen, 1996: p. 11). Chinese functional foods that are believed to have antioxidant affects and prevent cancer include ginseng (A. S. Wong et al., 2015; Yi et al., 1999), soyabean (*Glycine max*) (Yi et al., 1999), green tea (Fujiki et al., 1996), mushrooms (Chang, 1996; Kozarski et al., 2015), hawthorn fruit (*Crataegus pinnatifida*) (Arai, 2002; Yi et al., 1999), and Chinese wolfberry (He et al., 2012; Yi et al., 1999).

India is believed to be have been influencing the Malay Peninsula as long ago as the sixth to seventh centuries BC (Netto, 1961), but the first Indian trade centres were established at Kedah and Perak in the early 4th century. These early traders came from the Pallavas lands along the Caromandel coast. Trade between Malaysia and the area to Kedah and Penang is still active today. Indian traders used the Pallava alphabet, wrote in Sanskrit and were followers of Brahma, Shiva and Vishnu. In the 4th century, these traders left Brahmin sacrificial posts at Kutai in east Borneo, *Saiva* inscriptions in Champa and Cambodia, and Buddhist inscriptions in Kedah and Wellesley province (Winstedt, 1935). There were three distinct migrations of Indians into Malaysia (Netto, 1961). The first phase began in antiquity and ended with the Portuguese conquest of Malacca in 1511; the second phase ended with the

arrival of British in Penang in 1786. The third phase, modern Indian immigration, began in 1833 when Indians were brought into the country to work at sugar and rubber estates.

Indians believe in the traditional Indian system of Ayurvedic medicine. Ayurveda is an intricate system of healing practices that originated in India thousands of years ago (Agarwal and Kapil, 2016; Alagiakrishnan and Chopra, 2001; Bhungalia et al., 2004). The term Ayurveda consists of two Sanskrit words: *ayu*, which means life, and *veda*, meaning knowledge. In Ayurveda, health is influenced by the connectedness of the body, mind and spirit (Agarwal and Kapil, 2016). Each individual is made up of three *doshas*: *vata*, *pitta* and *kapha* and each *dosha* represents a certain bodily activity. In Ayurveda, each individual has a different ratio of *doshas* and these must be understand and balanced in order to achieve good health. Ayurvedic practitioners believe that food plays an important role in balancing the *doshas.* Plants are also key Ayurvedic remedy and at least 1,400 plant species are used in traditional Ayurvedic medicine (Bhungalia et al., 2004). The Ayurveda practice is ingrained in Malaysian Indian cooking. For example, turmeric (*Curcuma longa*), a plant native to South India, is a common ingredient in Indian food. Turmeric is believed to have phytochemical and antioxidant properties that help suppress multiple myeloma and blood cancer (Krishnaswamy, 1996). Moreover, several studies have recognized turmeric as anticancer, anti-diabetic, antioxidant, antimicrobial and others (Yadav et al., 2013). Other Indian spices and herbs are thought to boost energy and provide health benefits, for examples, brain, mental, joint and muscle health (Robson and Robson-Garth, 2015).

CULTURAL CONVERGENCE AND DIVERGENCE IN FUNCTIONAL FOOD CONSUMPTION

The concept of convergence theory is that the nations of the world become more similar because of economic development, modernization and interaction through commerce and trade. Much of the similarity is driven by industrialization notion (Kerr et al., 1960). This is the view of modernization theory, which argues that, with time and economic development, different societies will become more similar to each other (Eisenstadt, 1965). Peacock, Hoover and Killian (1988) stated that "convergence is equivalent to reduction in inequality" on the one hand and, on the other, that "divergence is an increase in inequality" (p. 842). These definitions are intended to contextualize inequality between nations undergoing international economic development. This research considers convergence in terms of functional food consumption and health-related values. The argument is that some functional food consumption values are converging into more general common values, regardless of ethnicity and traditional values. If that is the case, the three different ethnic groups in Malaysia should become more similar after a period of rapid economic development.

Convergence happens when nations achieve similar levels of economic development, they become more alike in terms of social status and life (Coughlin, 2001). Malaysia is a rare example of a plural society in which different ethnic groups accommodate each other without losing their own identity. They speak their native language and practice their own cultures. However, with time and space, social interaction is likely to drive a certain degree of cultural convergence. Ethnic groups in Malaysia are willing to adopt the practices of other cultures, as

long as these practices are not harmful and are not against their fundamental values and beliefs.

Social changes can change value systems (Cote and Levine, 2014; Hassan, 2011a; Hassan et al., 2009). One aspect of social change is economic development. One controversial idea is that economic development is associated with shifts away from absolute norms and values toward values that are increasingly rational, tolerant, trusting and participatory (Inglehart and Baker, 2000). There is also much evidence from previous research (Bronfenbrenner, 1986; Hoff-Ginsberg and Tardiff, 1995; Super and Harkness, 1986) that value systems within an ethnic group vary because of differences in income, occupation, living conditions and exposure (length and intensity) to another culture.

According to Giddens (1986), social interaction comprises the glances, non-verbal communication and verbal communication that occurs in daily social encounters. It is important to study daily social interaction because the routine of daily life involves us in face to face interactions with others and helps us understand the larger social system (Chuah, 2001). Malaysia is a multi-ethnic nation in which each ethnic group maintains a distinct identity and culture (Freedman, 2001). There have been numerous attempts by the Malaysian government to develop a national identity by integrating and uniting the multi-ethnic groups into one nation (Abraham, 1999; Federation of Malaya, 1956; Federation of Malaysia, 1985; Joseph and Holden, 2001).

Table 1. Differences between Malays, Chinese and Indians in Malaysia

Characteristic	Malay	Chinese	Indian
Ethnic Composition	65.1% of total population	26.0% of total population	7.7% of total population
Language	Mostly bilingual: Malay and English	Mostly trilingual: Malay, English and their native dialect (Cantonese, Mandarin, etc).	Mostly trilingual: Malay, English and Tamil.
Religious Beliefs	Mainly Muslim	Mainly Buddhist, Confucian and Taoist with a minority of Christian and Muslim	Mainly Hindu with a minority of Christian and Muslim
Cultural Values	Collectivist rather than individualistic. Value belonging, respect for elders, spirituality/faith in God, humility, face/self-respect, tact/indirectness, generosity, sensitivity to feelings, politeness and close relationships.	Collectivist rather than individualistic. Hard work/diligence, pragmatism, perseverance, education, wealth/prosperity, family, face, harmony and risk-taking.	Collectivist rather than individualistic. Value face, fear of God, sense of belonging, brotherhood, family, hard work, filial piety, karma and loyalty.
Culture and Tradition	Celebrate two main festivals: *Hari Raya Aidil Fitri* and *Hari Raya Haji*.	Celebrate Chinese New Year and Moon Cake festival.	Celebrate two main festivals: *Deepavali* and *Thaipusam*.
Food Belief and Behaviour	Foods are classified as cold, hot and bisa.	Based on ying-yang theory (positive and negative energy).	Foods are classified as cold and hot.
Health Beliefs and Practices	Traditional Malay medicine.	Traditional Chinese medicine.	Traditional Indian Ayurveda.

Source: Developed for the research reported in this chapter

Available evidence shows that cultural practices can change or converge over time. This occurs because of changes in social structure, mainly due to urbanization, modernisation, industrialization and globalisation (Mun et al., 2015). After 50 years of independence, Malaysia is a rapidly developing country; its society is becoming more integrated as social structures and lifestyles change. Over time, continuous economic development changes cultures and societies. Economic growth leads to modernization, urbanization, industrialization and globalization and drives structural change and cultural convergence. In Malaysia, these effects became apparent after the government introduced a new economic policy (NEP) in the aftermath of the May 1969 race riots. The violence occurred in response to economic imbalance among Malay, Chinese and Indian ethnic groups. The two main objectives of the NEP were to "eradicate poverty regardless of race" and "restructure(e) society to eliminate the identification of race with economic function" (Jomo 2004, p. 3). The NEP significantly contributed to economic growth, urbanization, industrialization and modernization in Malaysia. It created employment opportunities, improved educational levels, increased income, encouraged lifestyle changes, and led to the emergence of a middle class. However, cultural and social change is narrowing the gap between ethnic groups, at least so far as the consumption of functional food is concerned. These social changes stem from government policies, economic development and increased interaction between members of the different ethnic groups. As a result, the values surrounding the consumption of functional food are becoming increasingly homogeneous. Ultimately, the extent to which cultural convergence homogenizes the consumption of functional food is an empirical measure, and this research is primarily concerned with gauging it.

Social interaction among ethnic groups promotes cultural convergence over time (Hassan et al., 2009; Mun et al., 2015). Living in close proximity is an important part of fostering interaction and breaking down solitudes between different ethnic groups (C. B. Tan, 1982). The Malaysian environment fosters strong social interaction in schools, colleges and the workplace. New housing estates comprised of Malays, Chinese and Indians are an important way of promoting inter-ethnic relationships. Constant interaction ensures more mutual understanding and respect. In contrast, a lack of interpersonal interaction tends to reinforce stereotypes and hostility at the group level (C. B. Tan, 1982). Good social interaction promotes acceptance of functional food from other cultures. For example, in Malaysia it has showed that Indian or Chinese participants who grew up in Malay neighbourhoods or worked side by side with Malays had no problem adopting Malay culturally-based functional food (Hassan et al., 2009). Similarly, Malays who interacted with Chinese or Indian neighbours and colleagues accepted other culturally-based functional food, as long it was *Halal.* They would try Chinese or Indian functional foods as long they did not contain animal-based ingredients or alcohol (Hassan et al., 2009). There is also evidence that Malays, Chinese and Indians adopted each other's cooking styles. This may be because most culturally-based food ingredients from the three ethnic groups can be easily obtained from grocery stores and supermarkets.

Culturally-based food behaviour regarding functional food consumption can converge over time. To a certain degree, cultural practices change and converge over time. Lee and Tse (1994) stated that some consumption behaviour is ingrained in ethnic identity and other behaviour is not. In this chapter, cultural value convergence means that the cultures of the three ethnic groups (Malay, Chinese and Indian) tend to become more alike due to increasing social interactions. Health-related values are a good example of convergence. Malay, Chinese

and Indian consumers have a similar motive for choosing to consume food with curative properties—maintaining ideal health and preventing or curing illness (Hassan, 2011a). To some extent, this shared goal increases the similarity and equality of these consumers. Thus, cultural convergence may occur in the consumption of functional food, so that consumers will choose health-related foods that are culturally-based, interculturally-based or modern.

According to Hassan (2011a), the convergence of consumption can be observed in health related values in Malaysia. Malay, Chinese and Indian consumers have a similar motive for choosing to consume food with curative properties – maintaining ideal health and preventing or curing illness. To some extent, this shared goal increases the similarity and equality of these consumers. Thus, cultural convergence occurs in the consumption of functional food because consumers are willing to consume health-related foods that are culturally-based or inter-culturally based (Hassan, 2011a, 2011b).

MARKET OPPORTUNITY OF FUNCTIONAL FOOD IN MALAYSIAN MARKET

Rapid improvements in the socio-cultural and economic life of Malaysia in the last five decades have made consumers more educated and economically stable. To some extent, cultures converge, new identities arise and people's identities shift over time. However, the underlying culture still defines the emerging cultures. Malaysian consumers are willing to accept traditional and modernized functional food from other cultures, because of the perceived health benefits of these foods. Cultural convergence is creating opportunities for consumers to experiment with functional food alternatives from other cultural groups. Cultural convergence can be regarded as a filter that removes some the 'oddness' from the functional foods of different cultures. In other words, cultural convergence makes some previously culturally-specific functional food more palatable to consumers from other cultures. In Malaysia, this convergence has to follow the dominant values of the Malay–Muslim majority. Cultural convergence is more likely to happen if functional food products are acceptable to the Malays and their value systems.

Thus, the success of the functional food business depends on being innovative, offering safe products and satisfying consumer needs. One of these needs is for foods that offer health benefits. Thus, an effective approach for changing consumption behaviour is advertising the health-related aspects of food and providing useful health information on food labels. Also, socio-demographic factors can be used to tailor health intervention and marketing campaigns to specific population sub-groups.

Food industry practitioners must offer products that meet consumer needs and demands. New research and product development must be based on scientific discoveries to offer novel products based on ethnicity. Market-oriented functional foods must be innovative, branded and well-communicated. These new kinds of products may demand new modes of distribution, marketing, advertising, and so forth. In sum, industry practitioners need to be alert and open to new ideas.

RECOMMENDATIONS FOR THE FUNCTIONAL FOOD PRACTITIONER

Market oriented functional food products. There is an opportunity for food manufacturers to create market-oriented functional food products targeted at specific consumer groups. The factors identified in this study can be used as a measure for developing customer value co-created functional food products. Customer value co-creation is a customer-centric approach that produces an overall experience that is more responsive to customer needs. Customer-based value co-created functional food products will enable food practitioners to develop tailored holistic products that maximize consumer needs and experiences. Tailored functional food products will help satisfy consumer demands for health, convenience and sensory pleasure. These demands arise in the cultural and personal values of each ethnic group. These market-oriented functional foods will gain consumer acceptance and maximise consumer satisfaction.

Functional food innovation. Product research, development and marketing are crucial for the long-lasting market success of functional food products. Food producers should work closely with food scientists and nutritionists to create functional food products that meet the needs of the three ethnic groups. This will give food producers the opportunity to commercialize the traditional functional foods of each ethnic group into modern products that are marketable to a wider range of consumers. Modernized traditional functional foods that are healthy, tasty, convenient and tailored to consumers' basic needs will be locally and globally marketable. There is already an example of this type of product in the Malaysian food market: instant coffee with ginseng *(tongkat ali)*. This Chinese and Malay functional food is a well-accepted drink believed to boost energy and revitalise tired minds in Malaysian market place.

Functional food branding. Branding is an important aspect of a marketing strategy. Consumers are more willing to accept familiar and reliable brands. Brand awareness appears to minimize customer perceptions of risk. Consumers are more likely to trust (and perceive less risk with) a brand with a good track record, than an unknown brand. Consumers are also more likely to believe a message that is endorsed by credible sources (Schmidt et al., 1997). Therefore, food producers can collaborate with government agencies and other organizations when promoting functional food. For instance, manufacturers can have the health claims of their products endorsed by the Heart Foundation or other health-related agencies.

Communicating functional food. In Malaysia, consumers can usually be delineated by their ethnic group. This study suggests that a better communication strategy should be formulated to increase the favourable perception of functional food as a whole. The current rigid ethnic-based strategy must give way to a more flexible strategy. Besides that, the term 'functional food' is quite new in the Malaysian food market. Consumers have little knowledge of the health effects of the specific functional foods of other ethnic groups, which suggests that more sophisticated communication activities are needed. Therefore, functional food practitioners should manufacture and market food products that are acceptable to all ethnic groups. This must be done carefully, to make the food appealing to people from all cultural groups, while still retaining the food's unique ethnic characteristics. This may require cooperation with the government and a good communication strategy will be crucial. The communication should be aimed at educating and delivering a truthful and clear health message which emphasizes consumer benefits, safety and quality (Bruhn, 1999).

Distributing functional food. Health food supplements are available from pharmacies, health food stores, supermarkets, direct-selling agents and the internet. In Malaysia, the two main distribution channels for health food products are pharmacies and licensed direct-selling companies. Indeed, there are around 520 licensed direct-selling companies in Malaysia (Malaysian Dietary Supplement Association, 2006). One-on-one marketing or word of mouth is the best way to sell functional food products because consumers feel more confident with this approach. Other functional foods, can easily be found in supermarkets or grocery stores. Food producers should consider all these distribution alternatives for their functional food products.

CONCLUSION

This chapter focused on the unique aspects of Malaysian society and explained the issues surrounding the concept of cultural convergence in functional food consumption. Culturally based values embedded in each ethnic groups underpin consumer preferences by affecting all aspects of food behaviour, food choice and food consumption. Culture is a learned trait, a group phenomenon that can be transmitted from one generation to the next. The rapid economic development is changing Malaysia's social structure in terms of income, occupation and living conditions. These changes increase the extent to which the three ethnic groups in Malaysia are exposed to each other's cultures. These changes can revolutionize value systems and lifestyles. In the presence of socialization, this transmission process would encourage. Cultural values encompass historical, traditional and spiritual antecedents of functional food consumption, in that they represent the influences taught to a consumer as part of his or her upbringing in a society. The knowledge functional food is embedded in the local knowledge of the Malay, Chinese and Indian ethnic groups in Malaysia. Evidence shows that after more fifty years of independent, the Malays, Chinese and Indians do share information on traditional functional foods among themselves. Functional foods potentially offer consumers many health benefits. Regardless of their ethnicity, consumers in multicultural communities share their knowledge of functional food via oral tradition, word of mouth and mass media. The use of functional foods to prevent or cure disease reflects a cultural change among the three ethnic groups in Malaysia. The demand for traditional functional food has increased as consumers understand that health-related food provides benefits beyond basic nutrition.

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