Functional Foods and Beverages

In vitro Assessment of Nutritional, Sensory, and Safety Properties

Edited by
Nicolas Bordenave and Mario G. Ferruzzi

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Preface

Food functionality is a wide concept that encompasses nutritional/health functionality, food safety and toxicology, as well as broad aspects of visual and organoleptic properties of food. The evaluation of all these individual aspects have been widely covered in many books and review articles over the years. So, why have a book on *in vitro* systems for testing aspects food functionality?

As you will read in this book, *in vitro* techniques bridge the gap between standard analytical techniques (chemical and biochemical) and *in vivo* human testing, which remains the ultimate translational goal for evaluation of the functionality of food. Although well established, this domain is constantly evolving toward closer and higher throughput prediction of *in vivo* properties and outcomes. *In vitro* testing facilitates high throughput assessment of food properties in a cost-effective manner without practical and ethical challenges of human testing. By establishing tight control of testing conditions, these approaches also allow for detailed mechanistic insights to be developed on food functionalities and therefore a better understanding of interactions between food and human physiology. Nevertheless, *in vitro* models, as with all model systems, have their own limitations. Research and development efforts are continuously progressing to refine these methods, their predictive power, and their applicability to diverse systems and conditions. *In vitro* testing of food functionality is therefore a field of its own and with this in mind, is deserving to be the main subject of its own text. The ambition of this book is to establish the current state-of-the-art of *in vitro* models, from their physiological basis to their conception, their uses, and finally their future.

Chapter 1 reviews the concepts of functional foods and food functionalities, highlighting the necessity of evaluating such functionalities. In the next section (Chapters 2 and 3), Chapter 2 overviews the physiology of the gastrointestinal tract, presenting features that constitute the basis of *in vitro* models for evaluating food's nutritional, toxicological and allergenic properties. Chapter 3 covers the physiology of sensory perception of food, taste and texture. In the final section (Chapters 4 to 9), Chapter 4 overviews the *in vitro* models of host–microbial interactions within the gastrointestinal tract as well as the gastrointestinal model themselves. Chapters 5 and 6 address the *in vitro* models for the digestion and absorption of macronutrients, micronutrients and phytonutrients. Chapters 7 and 8 address the *in vitro* evaluation of specific food hazards, namely toxicants and allergens. Finally, Chapter 9 presents the challenges of linking *in vitro* analysis of taste, aroma and flavor to their actual perception.

We hope that this book will be useful to food scientists, graduate students, professors and professionals, in academia, government research or food industry R&D, who are working hard to deliver safe products of increasingly high quality to consumers.

Nicolas Bordenave
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1

Overview of Functional Foods

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1.1 Overview of Functional Foods

1.1.1 Foods and Nutrients are Linked to Health and Disease

The Centers for Disease Control and Prevention (CDC) indicates that a healthy lifestyle, including healthy foods, is one strategy to prevent chronic disease (CDC, 2012). Epidemiological studies have shown a diet rich in fruits and vegetables can reduce the risk of inflammatory and age-related chronic diseases, including many cancers, cardiovascular disease, and inflammation (Barbaresko *et al*., 2013; Esposito and Giugliano, 2006; Heggie *et al*., 2003; Hu, 2003).

Foods, especially plant foods, contain non-nutrient bioactive compounds that have potential to synergistically and positively impact health. The primary classes are phenolic compounds, carotenoids, alkaloids, nitrogen-containing compounds, organosulfur compounds, and phytosterols (Liu, 2004, 2013a,

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2013b). More than 5000 bioactive components have been identified in plant foods (Liu, 2004, 2013a), but it is thought that more than 25,000 bioactive components are actually present. Most of these components are metabolized to different compounds during and after digestion. Considering these 25,000 bioactives and all of their metabolites, it would be unrealistic to conclude there is a single compound which serves as a "silver bullet" for health promotion. Instead, it is the combination of many dietary compounds consumed from a variety of whole foods that likely confers the greatest health benefits (Liu, 2004). Undoubtedly, there is still much research required in order to fully understand the role of bioactive dietary compounds and their metabolites in human health.

1.1.2 Definition of Functional Foods

Defining functional foods can be difficult. There is no U.S. Food and Drug Administration (FDA) definition of functional foods, and all foods can be considered "functional" because all cause some physiological response. The Academy of Nutrition and Dietetics (AND) defines functional foods as "whole foods along with fortified, enriched, or enhanced foods that have a potentially beneficial effect on health when consumed as part of a varied diet on a regular basis at effective levels" (Crowe and Francis, 2013). Similarly, the Institute of Food Technologists (IFT) defines functional foods as "foods and food components that provide a health benefit beyond basic nutrition (for the intended population)" (IFT, 2005). Thus, functional foods can encompass fresh foods, such as tomatoes and broccoli, along with processed or cooked foods, such as tomato juice and broccoli soup. Functional foods also include foods that naturally contain nonnutrient bioactive components, such as flax seeds, as well as foods fortified with bioactive components, such as various nutrition bars.

1.1.3 Functional Foods Market

As reviewed by E. Sloan, the *Nutrition Business Journal* (2013) reports that worldwide sales of functional foods were $118 billion in 2012. With an increase of 7% from 2011 to 2012, the

United States is the largest market for functional foods (sales of $43.9 billion), followed by Japan ($22 billion), the United Kingdom ($8.1 billion), and Germany ($6.4 billion) (Sloan, 2014). Also reviewed by E. Sloan, the Multi-Sponsor Surveys' 2012 Gallup Study of Nutrient Knowledge and Consumption reports that 60% of adults in the U.S. consume functional foods or beverages at least occasionally (Sloan, 2014). These statistics confirm that not only is the study of functional foods valuable for consumer health, but also that there is interest by the food and nutrition industries to develop new products for consumers that truly improve health (Pricewaterhouse Coopers, 2009).

1.1.4 How Functional Foods are Studied

Large epidemiological studies are usually used to discover a potential association between a food or group of foods and a health condition. Due to wide variability in various characteristics of epidemiological cohorts (e.g. diet and other environmental exposures, race and other genetic factors), randomized, controlled, human clinical intervention studies are used to identify cause and effect relationships between a specific food and a health condition. These randomized controlled trials are considered the "gold standard", mandatory to develop health claims, and usually required to develop dietary recommendations. While it is recognized that cellular or other *in vitro* models will never perfectly replicate the complex system of the human body, *in vitro* methods are an essential piece of the puzzle. They can be used to understand the identity and quantity of bioactive components in foods and their metabolites once the food is consumed. In addition, *in vitro* models are used to study mechanisms of action as well as absorption and metabolism. Because even small human clinical intervention studies are very expensive and time intensive, *in vitro* preclinical models are often used to validate epidemiological data, predict the outcome of a human or animal study, justify execution of human clinical trials, and predict human sensory perception of functional foods.

In vitro methodologies are typically used throughout a "crops to the clinic" approach to functional foods research, from growing the plant, producing a food product, analyzing the bioactive components, and predicting the bioavailability and biological

activity of the bioactives, all with the goal of justifying use of the food in a human clinical study (Ferruzzi *et al*., 2012). These aspects are discussed below.

When growing a plant to be used as a functional ingredient in a food product, *in vitro* methodologies are used to understand genetic and molecular pathways which influence the levels of bioactive components in a plant. For example, genetic mapping techniques can be developed to identify plant varieties that contain higher levels of a specific bioactive component or a form of the bioactive component which is more biologically active or more bioavailable (Battino *et al*., 2009; Kuzina *et al*., 2011). In addition, growing conditions such as temperature, light, and soil nutrients can be modulated to optimize levels of a particular bioactive component (Bumgarner *et al*., 2012). Processing conditions, inclusion of other ingredients, and storage conditions can also impact the stability and biological activity of bioactive compounds, and thus can be monitored using *in vitro* analytical methods to identify and quantify bioactive compounds in food.

The techniques of high performance liquid chromatography (HPLC) in combination with photodiode array (PDA) and/or mass spectrometry (MS) are used for analysis of bioactive compounds in foods. PDA is sufficient to quantify compounds that are adequately detected with UV-Vis absorption, while MS, based on a compound's unique mass-to-charge ratio (m/z), is essential for compounds that require greater selectivity or are at lower concentrations and require greater sensitivity. Tandem MS (MS/MS) and accurate mass measurements provide further confidence in quantitation and identification, respectively. These methods are used to identify and quantify a range of bioactive compounds and their metabolites to help answer a variety of research questions. As above, analytical methods are essential to study the impact of different plant varieties, growing conditions, maturity levels, and plant disease on the type and amount of bioactive components in plants, and the impact of processing, storage, and the presence of other ingredients on the bioactive levels of a functional food product, ultimately predicting the potential health benefits. Bioactive identification and quantification is also critical when evaluating the stability and metabolism of a compound during simulated (*in vitro*) digestion and absorption, in addition to after consumption by animals or humans.

In vitro methods can be used to simulate the bioaccessibility and bioavailability of a functional food or a specific bioactive compound before advancing to a human clinical study. Bioactive components or their active metabolites must reach the target tissue in order to have a health benefit. Thus, bioactives must be released from the food, must remain stable during oral, gastric, and intestinal digestion, and must be delivered to the target tissue (Rein *et al*., 2013). Digestive stability and bioaccessibility can be predicted using cell-free methods, while absorption and transport across cells can be investigated using Caco-2 intestinal cell methods, saving valuable time and research funds. Many factors can influence stability, digestion, and absorption, such as the chemical properties of the bioactive component, the food source and its matrix, interaction with other components in the food, pH, and temperature (Failla *et al*., 2008; Rein *et al*., 2013). Newer multi-compartmental models are also being developed that connect cultures of different cell types (e.g. intestine, liver, and adipose tissue) in order to study metabolism (Vinci *et al*., 2012).

Biological activity can also be predicted using *in vitro* models. Antioxidant activity is one of the most common *in vitro* screening assessments, but bioactive components have many synergistic mechanisms of action that go beyond antioxidant activity (Liu, 2004). Assessing multiple mechanisms provides a more complete picture of the potential biological activity of a bioactive or food. Therefore, the health benefits of a food or ingredient should not be based on a single antioxidant assay, and it is important that a multi-faceted approach be taken before drawing conclusions. The appropriate *in vitro* model will be dependent on the disease or health condition that is being targeted. For example, when evaluating a food for its potential benefit in reducing risk of cardiovascular disease, *in vitro* markers might include models of platelet function (collagen-induced platelet aggregation, TRAP-induced P-selectin expression as a marker of platelet activation) (Ostertag *et al*., 2011), inhibition of LDL carbamylation (Ghaffari and Shanaki, 2010), models of carotid injury (Sheu *et al*., 2013), oxidative stress-induced cardiomyocyte injury (Li *et al*., 2013), and hemolysis assays (Li *et al*., 2013).

It is important to note the limitations of *in vitro* methods. It is impossible to replicate the conditions of the human body. For example, with *in vitro* experiments, there is no homeostasis, cell

studies usually only include one type of cell grown in a monolayer, and experiments are usually optimized for maximum cell growth, all conditions which do not occur in the human body (Hartung and Daston, 2009). Thus, *in vitro* studies are only predictive of potential biological activity. In order to validate findings from *in vitro* experiments, animal and human clinical trials are required.

1.2 Functional Foods and their Regulatory Aspects

Around the world, most commercially available products consumed are categorized as food or drugs, with drugs intending to cure, prevent, treat, or mitigate disease, while food is generally consumed for taste, aroma, or nutritive value (Nutrilab v. Schweiker, 1983). Although functional foods may provide benefit beyond standard nutritive value, they must adhere to food regulations. The primary objective of regulatory authorities is to protect the public by ensuring food safety and preventing misleading or false product claims. Many countries do not have specific functional food regulations and often manage these through pre-market evaluation of health benefit/disease risk reduction claims. Japan has one of the most developed regulatory frameworks for functional foods. In Japan, functional foods are officially recognized under a specific "food for specified health use (FOSHU)" which permits claims related to reduction of disease risk (Shimizu, 2012). Japanese regulatory authorities review these foods before they can be placed on the market. The application must include significant scientific evidence that demonstrates the benefit of the health claim, safety, and physical and chemical characterization.

The approach to establishing safety of a functional food does not differ from other foods. Similar to other regulatory bodies (such as the European Union and Canada), the US FDA has published guidance on the studies necessary to support the safety of a new food ingredient (Center for Food Safety and Applied Nutrition, 2007). This guidance helps develop data to demonstrate that a food ingredient is safe for the specific use at a specific use level, including for use as a functional food.

1.3 Nanotechnologies in Functional Foods

Nanotechnology is increasingly being used in functional food products. Nanotechnology is defined by the U.S. National Nanotechnology Initiative as "the understanding and control of matter at dimensions between approximately 1 and 100 nanometers (nm), where unique phenomena enable novel applications not feasible when working with bulk materials or even with single atoms or molecules" (U.S. National Nanotechnology Initiative, 2014). One application of nanotechnology in functional foods and nutraceuticals is to protect a functional ingredient from degradation during production, storage, or digestion (e.g. acidity of stomach) (Ranjan *et al*., 2014). For example, George Weston Foods (North Ryde, New South Wales, Australia) incorporates tuna fish oil into bread to enhance the bread's health benefits, but to avoid off odors and flavors, the fish oil is encapsulated within nanoparticles and is released only in the acidic environment of the stomach rather than in the food product (Neethirajan and Jayas, 2011). Nanotechnology is also being used to control the release rate of a functional ingredient, improve bioavailability of a compound, or target delivery to a specific cell type or tissue (Ranjan *et al*., 2014). For instance, nanotechnology has been used to encapsulate probiotic bacteria to protect it from harsh stomach conditions, allowing controlled release of the bacteria in the neutral environment of the intestine (Neethirajan and Jayas, 2011). Several different types of delivery systems are being evaluated for use in food products, including:

- **Micelles:** Lipid-soluble bioactives, e.g. limonene, lycopene, lutein, omega-3 fatty acids, and essential oils, have very low bioavailability, limiting their use in functional foods (H. Chen, Weiss, and Shahidi, 2006; McClements and Xiao, 2012). Because of their polar head groups and nonpolar tail groups, micelles are being evaluated for encapsulation of nonpolar functional food components to allow incorporation into beverages (H. Chen *et al*., 2006). This strategy has been used in the pharmaceutical industry to more effectively deliver poorly water-soluble drugs (McClements and Xiao, 2012).

- **Liposomes and cubosomes** are bi- or tri-phasic structures used to encapsulate water-soluble compounds within a hydrophilic component and conversely, to encapsulate lipid-soluble compounds within a lipophilic component. Liposomes and cubosomes are currently being used or evaluated for their ability to encapsulate the proteins lactoferrin and nisin Z to increase the shelf life of dairy products, encapsulate phosvitin (naturally found in egg yolk) to inhibit lipid oxidation in dairy products and ground pork, and encapsulate vitamin C to maintain its activity during long refrigerated storage (H. Chen *et al.*, 2006). Cubosomes can be altered with pH and temperature changes, and can thus be used to control the release of functional compounds (H. Chen *et al.*, 2006).
- **Nanoemulsions:** Because these emulsions are so fine, they are clear to the eye rather than opaque (B. Chen *et al.*, 2013; H. Chen *et al.*, 2006). Nanoemulsions are thermodynamically more stable than regular emulsions, and therefore do not separate over time (Ranjan *et al.*, 2014). Because of these properties, nanoemulsions are commonly used in parenteral nutrition formulations (H. Chen *et al.*, 2006), to add fat-soluble bioactive components to clear beverages (B. Chen *et al.*, 2013), and to obtain a creamy mouthfeel with limited lipid levels (H. Chen *et al.*, 2006).
- **Biopolymeric nanoparticles** are nanopolymers that are linked to form solid particles. A variety of different types of compounds can be encapsulated with biopolymeric nanoparticles, and their use is becoming more popular in functional ingredients. Examples include chitosan (derived from crustacean shells) and the synthetic polymers polylactic acid, polyglycolic acid, and combinations of lactide, galactide, and caprolactone (H. Chen *et al.*, 2006).

New tools also are incorporating biological and chemical ligands that can direct functional compounds within nanoparticles to a specific cell type (H. Chen *et al.*, 2006). The ability to deliver a functional component to a targeted cell or tissue site increases effectiveness and efficiency, allows the compound to be incorporated into the product at lower levels, and therefore can result in fewer adverse effects. For example, if salt could be incorporated in such a way that it is directed only to taste buds

that detect salt, the amount used in the food could be greatly decreased. In the future, there is potential to use nanotechnology to release a bioactive compound only in response to a specific biological trigger, such as a biochemical or genetic marker, leading to possibility of personalized nutrition (H. Chen *et al.*, 2006). Use of nanotechnology in food applications is still in the early stages, and much research is needed to ensure safety, including how nanoparticles are absorbed in the gastrointestinal tract, where nanoparticles are distributed in human body, how long they remain, what concentration they reach, and if the nanoparticles affect unintended biological activity (McClements and Xiao, 2012; Ranjan *et al.*, 2014). As with any new technology, consumers must be educated in order to maintain confidence in the technology and products using the it (Ranjan *et al.*, 2014).

1.4 Sensory Functionalities of Foods

Consumers have expectations regarding the appearance, aroma, flavor, taste and texture of food products and, as such, these sensory properties are key drivers to product acceptance. As a consequence, food companies have traditionally invested heavily in the identification and optimization of important sensory attributes. Emerging evidence, however, suggests that consumers are becoming more savvy and are increasingly looking beyond the sensory attributes to other product characteristics that influence acceptance and choice (Ares, 2011; Wills *et al.*, 2012). In this regard, interest in functional foods has recently burgeoned as consumers are seeking to improve health and wellness by incorporating functional ingredients into their diets. Unfortunately, many bioactive compounds have negative taste and flavor properties and require focused efforts to improve their palatability (Sun-Waterhouse and Wadhwa, 2012). However, for many functional foods, the level of sacrifice that consumers are willing to make in taste and flavor in favor of functionality is unknown. Further research is needed to understand these tradeoffs and to determine their relative importance in different populations (e.g. healthy vs. diseased).

As with all food products, the sensory properties of functional foods should, as much as possible, be optimized to meet or surpass consumer expectations. Prior to executing expensive sensory and consumer testing, instrumental analyses can be completed to gain insight into various physical parameters of the product that influence sensory variables (Kilcast, 2013). Because the human senses are impacted by physiological and psychological factors, it is impossible to replicate human senses with an instrument, which can only provide a discrete measure of a specific property. However, instrumental analyses might be used to reduce the number of samples on which to conduct human sensory assessment. For each product, instrumental analyses should be verified with human sensory panels so that they can be used to predict future results. The superficial appearance and color of food are the first parameters of quality evaluated by consumers. Colorimetry can be used to measure factors contributing to the product's appearance including the chromaticity and radiance, surface reflectance, transmittance and/or translucency (Clydesdale, 1978). Rheological assessments, infrared spectroscopy, water activity measurement and texture analysis can provide understanding of food properties related to perceived texture attributes including viscosity, tenderness, crunchiness, or chewiness, respectively (L. Chen and Opara, 2013; Tunick, 2011). Quantification of volatile and nonvolatile compounds enables the detection and identification of chemical species contributing to flavor, odor, and taste. These techniques are particularly important in understanding matrix interactions (e.g. sequestration of hydrophobic compounds into lipids) that impact flavor release and product perception as well as in the identification of taints or other compounds contributing unique sensations. Moreover, real-time chemical analysis with techniques such as proton transfer reaction-mass spectrometry or selected-ion flow tube-mass spectrometry enable correlating concentration of volatile flavor compounds in exhaled air to perception of flavor attributes.

In summary, functional foods have much potential to positively impact human health. Both consumers and the food and nutrition industries are eager to take advantage of these foods with health benefits beyond those imparted by traditional nutrients. A "big picture" crops to the clinic approach to studying

functional foods is essential. *In vitro* methodologies are an important piece of this puzzle, and can be used to identify and quantify bioactive components in foods, identify potential mechanisms of action, and predict bioavailability and metabolism, and thus can be used to justify execution of a human clinical trial.

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2

The *In vivo* Foundations for *In vitro* Testing of Functional Foods: The Gastrointestinal System

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2.1 Introduction

Despite lacking universally accepted definitions, there is substantial interest amongst consumers and researchers in using food to augment or optimize health, the concept underlying functional foods (Weaver, 2014; Milner, 1999). A significant reason for this underlying interest is a shift in nutritional research over the past 30 years from focusing on nutritional deficiencies to nutritional optimization. This shift is partly due to accumulating evidence linking poor diet to chronic non-communicable diseases including diabetes, cardiovascular disease, and obesity along with an increasing prevalence of these conditions (Hasler *et al*., 2009; Cencic and Chingwaru, 2010). Further, recent advances in the "Omics" fields of metabolomics, nutrigenomics, proteomics, and microbiomics have created additional means by which functional foods can be studied. These fields highlight the possibility of progressing towards additional evidence-based, optimized nutrition (Rist *et al*., 2006).

Functional Foods and Beverages: In vitro Assessment of Nutritional, Sensory, and Safety Properties, First Edition. Edited by Nicolas Bordenave and Mario G. Ferruzzi.

Despite considerable interest in functional foods, credible science-based evidence linking functional foods to health benefits is lacking (Weaver, 2014). Although the FDA does not specifically define functional foods, the 1990 Nutrition Labeling and Education Act (NLEA) mandates a high standard of scientific evidence for obtaining FDA approval for health claims associated with foods (Williams, 2005). Randomized controlled trials, epidemiologic studies, and *in vivo* and *in vitro* studies are needed to identify functional foods and validate their associated health claims (Sohaimy, 2012; Weaver, 2014). Aside from identifying functional foods and their role in treatment of specific disease or for maintaining health, there is a need for mechanistic studies of functional foods. Fundamentally, mechanistic research of functional foods must answer several questions:

- How are the bioactive components within a functional food processed by the gastrointestinal system?
- What are the physiologic mechanisms and targets through which functional foods exert their beneficial effects?
- What are biomarkers of exposure and response to components within functional foods?
- What are appropriate doses of functional foods?
- What are the safety considerations of functional foods? (Milner, 1999).

Since functional foods require ingestion prior to conferring any health benefits, all functions of functional foods begin with the gastrointestinal tract. Additionally, the gastrointestinal environment extensively modifies many functional foods such that the bioactive compound of a functional food that reaches circulation differs from what was ingested (Foltz *et al.*, 2010). As such, understanding the gastrointestinal tract and its underlying physiology is prerequisite for answering the questions above. *In vitro* modeling of the gastrointestinal tract can further elucidate answers to these aforementioned questions (Salminen *et al.*, 1998; Pang *et al.*, 2012).

2.2 Overview of the Structure of the Gastrointestinal Tract

Defined simply, the gastrointestinal tract (GIT) is a hollow, muscular tube consisting of the mouth, esophagus, stomach, small intestine, large intestine, and anus. It is 20–30 feet long in its

entirety and has a substantial surface area due to its length and finger-like projections extending from the inner intestinal lining known as villi. The wall of the GIT is largely composed of four layers: mucosa, submucosa, muscularis (or muscularis propia), and serosa (or adventitia). Each layer is comprised of specialized tissues with specific functions.

2.2.1 Mucosa

The mucosa is the innermost layer of the GIT. It is composed of the epithelium, lamina propria, and the muscularis propria (Ellen Kahn, 2010). The epithelium provides a physical barrier to the external environment as the mucosa contacts the gastrointestinal lumen, the inner cavity of the GIT. Epithelium is particularly relevant to functional foods since it is the primary site responsible for digesting and absorbing these foods. There are multiple cell types within the epithelium that vary throughout the regions of the GIT. Notably, intestinal epithelial cells (IECs) and secretory cells, such as enteroendocrine cells, are primarily responsible for digesting and absorbing foods. The epithelial cells are specialized for the absorption of nutrients and exchange of water and electrolytes, while the enteroendocrine cells such as cholecystokinin (CCK) and glucagon-like-peptide 1 (GLP-1) secreting cells are responsible for promoting enzymatic breakdown of macronutrients and regulating satiety, respectively (Farré and Tack, 2013). Due to the predominance of hormone producing enteroendocrine cells within the epithelial layer, the GIT is the largest endocrine organ in the body (Chaudhri *et al*., 2006).

The lamina propria is the layer of connective tissue between the epithelium and the muscularis mucosa and supports the epithelial layer. It also contains immune cells such as plasma cells, macrophages, and lymphocytes (Shils and Shike, 2006). The muscularis mucosa is a thin layer of smooth muscle. Its contractions assist with the secretion of products contained in secretory cells within the epithelial layer (Ellen Kahn, 2010).

2.2.2 Submucosa

The submucosa is a layer of collagenous connective tissue situated between the muscularis mucosa and the muscularis propria and provides flexibility to the gastrointestinal tract. The

submucosa contains blood and lymphatic vessels that recover absorbed nutrients that have traversed the epithelial layer. The submucosa also contains Meissner's plexus, a nerve fiber plexus containing post-ganglionic sympathetic and parasympathetic neurons. Additionally, scattered lymphoid nodules known as Mucosa Associated Lymphoid Tissue (MALT) are located in the submucosa (Shils and Shike, 2006).

2.2.3 Muscularis (or Muscularis Propria) and Serosa (or Adventitia)

The muscularis is primarily responsible for the motility of the GIT. It contains muscle organized in two layers, an inner circular layer and an outer longitudinal layer. The muscular layers of the GIT are composed of smooth muscle with the exception of skeletal muscle in the upper esophagus and external anal sphincter. Auerbach's plexus, a nerve fiber plexus containing parasympathetic and sympathetic fibers, is localized between these two layers of smooth muscle (Shils and Shike, 2006). The serosa is the outermost layer of the GIT and is comprised of a thin layer of mesothelial cells. The adventitia is an outer layer of connective tissue. These outer layers contain nerves and blood vessels.

While all of these components of the GIT are critical to its function, a majority of the *in vitro* methods used to represent the GIT primarily model the mucosal layer, specifically the intestinal epithelial cells.

2.2.4 Additional Components of the Gastrointestinal Tract: Accessory Organs, Vasculature, Innervation, Gut-Associated Lymphoid Tissue, and Microbiome

Focusing on the GIT solely as a hollow tube does not fully encompass the scope of the GIT. Its accessory organs, vascular supply, innervation, immune function, and microbial colonization are all intrinsic to its proper function.

2.2.4.1 Accessory Organs of the GIT

The GIT is intimately linked with several accessory organs. These include salivary glands, the pancreas, and liver. Their exocrine secretions (bile, pancreatic enzymes) are vital to digestion.

These secretions access the lumen of the gastrointestinal tract via the salivary, pancreatic, and biliary ducts respectively.

2.2.4.2 Vasculature of the GIT: Blood and Lymphatic Supply

The GIT also depends on its blood and lymphatic supply. Blood and lymphatic vessels mediate transport of absorbed nutrients from the mucosa to the rest of the body. The venous drainage of the small intestine and colon enters the portal vein and then proceeds to the liver for metabolism or direct entry into the systemic circulation via the hepatic veins. The arterial blood supply provides nutrients and oxygen to the GIT through the celiac, superior mesenteric, and inferior mesenteric arteries.

2.2.4.3 GIT Innervation

The enteric nervous system (ENS) is the intrinsic nervous system of the GIT (Konturek *et al*., 2004). It is an aggregate of neurons, ganglia, and nerve fibers that supply the muscles, epithelial and enteroendocrine cells, and the blood vessels of the GIT (Konturek *et al*., 2004). As a whole, the ENS contains 400–600 million neurons, which is approximately equal to the number of neurons located in the spinal cord (Furness, 2012). The ENS consists of both sympathetic and parasympathetic pathways and connects to the central nervous system (CNS) via pre-vertebral ganglia, the vagus nerve, pelvic nerves, and sympathetic pathways (Furness, 2012). The gut–brain axis denotes the bidirectional interaction of the CNS and ENS.

2.2.4.4 Gut-Associated Lymphoid Tissue

The GIT is a site of mucosal immunity. The mucosal immune system within the GIT is a complex network of immune cells designated as gut-associated lymphoid tissues (GALT). GALT contains up to 70% of the body's immune cells. These cell populations are responsible for both innate and adaptive immune responses within the epithelium, lamina propria, and submucosa. GALT is compartmentalized in several anatomic structures including mesenteric lymph nodes, Peyer's patches, and lymphoid follicles (Goto and Ivanov, 2013).

2.2.4.5 Intestinal Microbiome

A crosstalk exists between the ENS, GALT, and the microbiome, which is the dynamic ecosystem of 100 trillion microbes (1×10^{14}) within the GIT (Han, 2014; Robinette and Colonna, 2014; Collins *et al.*, 2012). As such, the microbiome deserves distinct mention as a functional component of the GIT. Joshua Lederberg coined the termed microbiome to describe "the ecological community of commensal, symbiotic, and pathogenic microorganisms that literally share our body space and have been all but ignored as determinants of health and disease" (lederberg and McCray, 2001; Grice and Segre, 2012). The number of microbial cells within the microbiome exceeds the total number of human cells; in fact, the microbiome constitutes 90% of the cells within the entire human body (Savage, 1977). The human microbiome consists of greater than 50 phyla, with a predominance of Firmicutes, Bacteroides, Actinobacteria, and Proteobacteria (Cho and Blaser, 2012; Mondot *et al.*, 2013). Above all, the composition of the microbiome is altered by diet, which has implications in both health and disease (Albenberg and Wu, 2014; Mondot *et al.*, 2013). Although several studies report the impact of fermentable fibers (prebiotics) on microbiota composition, more studies are needed to evaluate the impact of different types of "functional foods" on microbiota composition and function (Cencic and Chingwaru, 2010).

2.3 Functions of the GIT and Associated *In vitro* Modeling

The functions of the GIT are the product of tightly regulated interactions between its constituents. While the GIT has many functions, most importantly, the GIT is the primary site of food processing. As such, the structure and function of the GIT is integral to the functionality of functional foods. The functions of the GIT include the absorption of nutrients; secretion; motility; regulation of immune responses; serving as a barrier to the external environment; and the storage, fermentation, and removal of fecal matter. The anatomy and physiology underlying these functions is remarkably complex. This complexity is amplified by dynamic changes in GIT structure and physiology in response to food. Admittedly, detailing every aspect of the

function of the gastrointestinal tract is beyond the scope this chapter. Johnson *et al*. provides an excellent detailed review of gastrointestinal physiology (Johnson *et al*., 2012). The subsequent text will briefly highlight the functions of the GIT in order to understand *in vitro* models of gastrointestinal physiology applicable to understanding functional foods (see Table 2.1).

2.3.1 Motility

Once swallowing commences, the GIT has the challenge of transporting swallowed substances through the entire length of the GIT while mixing ingested materials and gastrointestinal

Table 2.1 Functions of the gastrointestinal tract and their corresponding *in vitro* models

Function	*In vitro* model
Motility Storage and removal of fecal matter	Isolated intestinal segments Computer modeling *(in silico)* Tissue engineering Gut-on-a-chip
Barrier function secretion absorption	Intact tissue methods • Isolated intestinal perfusions • Ussing chambers • Everted gut sacs • Precision cut intestinal slices Monolayer cell cultures/cell line models • Co-cultures • Transwell cultures 3-dimensional cell cultures Organoids/enteroids Gut-on-a-chip
Immune response	Co-culture models (PBMCs, isolated PBMCs)
Fermentation	*In vitro* fermentation systems • Batch cultures • Continuous cultures • Artificial digestive systems

PBMC, peripheral blood mononuclear cell.

secretions to facilitate proper digestion and absorption. As such, motility is one of the fundamental functions of the GIT that can modulate functional food effects through its impact on food absorption. Indeed, abnormal GIT motility that could lead to changes in transit time is a component of a multitude of diseases of the GIT such as gastroparesis, chronic intestinal pseudo-obstruction, and constipation; these can affect food absorption and the resident time of the unabsorbed portion of food in the colon and thus fermentation rate. *In vitro* models to assess motility are detailed below.

2.3.1.1 The Foundations of GIT Motility: Smooth Muscle Cell Contractions (SMC) and ENS Regulation

The primary effector of GIT motility is the smooth muscle cells (SMCs) within the muscularis externa of the GIT (Bitar *et al.*, 2014). SMCs derive their contractile properties from the contractile cytoplasmic filaments, actin and myosin. These SMCs also possess several selective and nonselective membrane ion channels that create a resting membrane potential and allow for membrane depolarization. In particular, L-type Ca^{2+} channels are primarily responsible for the depolarization of the cell membrane that generates contractions. These channels generate an influx of calcium that depolarizes the cell membrane and generates contractions via a signaling cascade consisting of series of phosphorylations and dephosphorylations of various intracellular proteins. (Hansen, 2003; Koch, 2012). This process is known as electrical-contraction coupling. The depolarization of the cell membrane of SMCs can pass cell to cell through communication via gap junctions. These gap junctions and intermediate junctions providing mechanical cell-to-cell connections allow multiple SMCs to contract in tandem as a syncytium.

Coordinated contractions of SMCs can generate complex motility patterns such as tonic contractions, peristalsis, and the migrating motor complex. These patterns, in addition to others within the GIT, vary in direction, amplitude, distance, and with fed and fasting states. Gastrointestinal motility patterns are influenced and regulated by extrinsic (CNS) and intrinsic (ENS) innervation, in addition to input from interstitial cells, such as Interstitial Cells of Cajal (ICCs) and PDGFRα + cells. The ENS is markedly complex as it contains several types of neurons, which

provide efferent and afferent communication between the gut and the CNS. Furness provides an excellent review of the anatomy and physiology of the ENS (Furness, 2012). Similarly, ICCs typically serve as pacemaker cells responsible for generating slow waves. However, the GIT contains several subpopulations of ICCs with both distinct and overlapping functions. These subpopulations of ICCs were reviewed by Blair *et al.* (2014). PDGFRα + cells provide inhibition through the inhibitory actions of purines (Sanders *et al.*, 2014). Hansen reviewed the complex neurohormonal regulation of SMCs (Hansen, 2003).

2.3.1.2 *In vitro* Motility Modeling

Several models of *in vitro* motility exist. The models typically consist of isolating segments of the GIT, computer modeling, or tissue engineering. Gut-on-a-chip is recent technology with potential motility applications.

2.3.1.2.1 Isolating Segments of GIT

Most models involving isolation of GIT segments involve isolating intestinal segments from rats, guinea pigs, rabbits, or utilizing human intestinal biopsies. Bathing the segments in a salt-based physiologic solution such as Tyrode's solution augments the viability of the segments. Isometric force transducers and pressure transducers are utilized to determine the direct effects of drugs or bioactive components in functional foods on smooth muscle contraction in the isolated segments (Peddireddy, 2011). These studies are typically variations on classic techniques described by Magnus and Trendelenberg in the early 1900s (Percy, 1995). More recently, Hoffman *et al.* described an advanced technique involving the use video cameras known as the gastrointestinal motility monitor (GIMM). The technique allows for continuously monitors propulsion in isolated segments of intestine through use a computer, camera, organ baths, and custom software (Hoffman *et al.*, 2010).

2.3.1.2.2 Computer Modeling

The use of computer simulations to recapitulate the motility of the GIT is referred to as *in silico* (Harrison *et al.*, 2004). These computer models use physiologic-based data to generate computed approximations of intestinal physiology. These models

primarily use computational fluid dynamics with data derived from MRI studies. Cheng *et al*. provides an excellent review of models pertinent to gastric motility (Cheng *et al*., 2013).

2.3.1.2.3 Tissue Engineering

Tissue bioengineering applied to modeling GIT motility is a recent technique. It entails utilizing natural or synthetic materials to create scaffolds that mimic GIT organs on to which smooth muscle cells are attached. The bioengineered constructs are then innervated using transplanted enteric neural progenitor stem cells. These techniques have been reviewed by Bitar *et al*. (2014). These models can evaluate the effects of drugs and functional foods on the mechanical and electrical properties of smooth muscle cells.

2.3.1.2.4 Gut-on-a-chip

"Gut-on-a-chip" is essentially a micro-device containing two microfluidic compartments separated by a porous membrane coated with extracellular matrix and human cell lines.

It is a recent advance in *in vitro* cell-based modeling. Kim *et al*. have demonstrated that Caco-2 cells spontaneously develop three-dimensional intestinal villi with peristalsis like movements when fluid flows over the cells cultured in the device's microchannels (Kim *et al*., 2012). This technique has potential for assessing the effects of flow and motility on nutrient absorption.

2.3.2 Barrier Function, Secretion, and Absorption

The GIT has an approximate surface area of 200–400 m^2 due to its tubular shape, intestinal villi, and microvilli of the brush border. Subsequently, the GIT has the most exposure of any organ system to the external environment (Scaldaferri *et al*., 2012). The gastrointestinal epithelium lines the extensive surface area of the GIT and serves as the fundamental physical demarcation between the lumen's external environment and the body's internal environment. Through the provision of this physical demarcation, the intestinal epithelium serves as a gatekeeper, dictating the passage of some substances between the internal and external compartments, while preventing the passage of others.

Essentially, the intestinal epithelium is the primary actor in the barrier, secretory, and absorptive functions of the GIT. These functions are mediated by the intestinal epithelial cell membrane and two characteristics of intestinal epithelial cells (IECs): apical tight junctions that seal the paracellular pathway between IECs, and both apical and basolateral ion transport proteins. Recapitulating these characteristics is integral to *in vitro* models of gastrointestinal barrier function, secretion, and absorption.

2.3.2.1 Tight Junctions and the Barrier Function of the GIT

IECs and their interconnecting tight junctions are just one component of the complex, multilayered boundary known as the gastrointestinal barrier. The barrier includes both physical and chemical components that impede the traversal of luminal pathogens and antigens into the host. The first layer of the external barrier is a chemical barrier, consisting of gastric acid, bile salts, and digestive enzymes that provide an inhospitable environment for most ingested pathogens due to its pH and osmolarity. Additionally, the gut microbiota capable of colonizing this layer provides additional barrier function through inhibiting and/or limiting the colonization of other, potentially pathogenic microbes through competition (Bischoff *et al*., 2014; Scaldaferri *et al*., 2012). The next layers include an unstirred water layer and a mucus layer containing antimicrobial peptides (i.e. defensins, lysozymes, RegIIIγ, etc), secretory IgA, and the gel forming mucin, MUC2 (Salzman, 2010; Camilleri *et al*., 2012; Scaldaferri *et al*., 2012). Beneath the mucus layer is a glycocalyx layer consisting of glycosylated proteins such as carcinoembryonic antigen (CEA) and CEA-related cell adhesion molecules (CEACAM) (Ou *et al*., 2009). The gut epithelium serves as the final physical external barrier between the luminal and host environments. The gut epithelium is a 20 μm-thick monolayer of several types of intestinal epithelial cells (IECs) including enterocytes (in the small intestine) or colonocytes (in the colon), Paneth cells, enteroendocrine cells, M cells (primarily in the ileum), and G cells (in the stomach). The IECs form a cohesive barrier due to the presence of intercellular mechanical connections: tight junctions (TJs) and adherent junctions (AJs). Both junctions consist of several different types of proteins including cadherins, claudins,

occludins, and zonula occludens. Shen *et al*. have reviewed the molecular components of tight junctions (Shen *et al*., 2011). Alterations in these junctions affect intestinal permeability and may play roles in precipitating several diseases.

2.3.2.2 Intestinal Permeability: Definitions and the Role of Tight Junctions

Intestinal permeability is an intrinsic, measurable aspect of gut barrier function. Permeability is essentially the ease in which molecules can travel across the intestinal epithelium through passive diffusion and is typically measured in cm/s (Ménard *et al*., 2010). The permeability of the intestinal epithelium is often described as leaky or tight. Molecules can move across the intestinal epithelium through two separate mechanisms: paracellular transport and transcellular transport. The paracellular pathway, although limited by tight junctions, is more permeable than the transcellular pathway. Thus tight junctions are the rate-limiting step in transport across the epithelium and are the primary determinant of intestinal permeability (Turner, 2009). Tight junctions demonstrate selective permeability in terms of the size and charge of molecules. This selective permeability creates an electrical and concentration gradient across the apical and basolateral membranes of IECs. As such, transepithelial voltage and transepithelial electrical resistance (TER) can define the tightness or leakiness of the intestinal mucosa (Bischoff *et al*., 2014).

2.3.2.3 Influences on Permeability

Permeability varies throughout the GIT, with an increased permeability in the small intestine compared to the colon. Further, there are also differences in permeability between cells located at the intestinal villus tip and cells at the base of the intestinal crypts (Turner, 2009). Several environmental factors exist that influence intestinal permeability. For example, diet can influence permeability (Shimizu, 2010). Martinez-Augustin *et al*. have reviewed the influence of food derived bioactive peptides on intestinal barrier function (Martínez-Augustin *et al*., 2014). The influences of food on permeability are partly mediated through altering the gut microbiome (Tilg and Moschen, 2015). Forsyth *et al*. demonstrated that alcohol and circadian disruption also increase intestinal permeability

(Forsyth *et al*., 2014). Several medications including mesalamines, corticosteroids, and TNF inhibitors influence permeability (Scaldaferri *et al*., 2012).

Increased permeability of the intestinal barrier is associated with the pathogenesis of several diseases including IBS, celiac disease, and IBD. Additionally, altered intestinal permeability may play roles in the pathogenesis of autoimmune diseases and neurologic disorders such as multiple sclerosis and Parkinson's disease (Fasano and Shea-Donohue, 2005; Fasano, 2011). Accordingly, altering intestinal permeability is a potential mechanism for the health benefits of functional foods.

2.3.2.4 Absorption and Secretion

Barrier function, absorption, and secretion are co-dependent because they each depend on and contribute to an electrical gradient across the intestinal epithelium. In addition to passive diffusion, epithelial transport proteins mediate absorption and secretion via active or passive transcellular transport. These proteins include apical and basolateral channels, carrier proteins, and pumps. Absorption and secretion involve the coordinated transport of Na^+, Cl^-, K^+, and HCO_3. Secretion is primarily a product of the active transport of chloride and bicarbonate ions. The basolateral $Na^+/K^+/2Cl^-$ cotransporter (NKCC1) and apical chloride channels such as the cystic fibrosis transmembrane conductance regulator (CFTR) or ClC family channels are principally responsible for intestinal secretion. CAMP, cGMP, and Ca^{2+} primarily regulate these proteins involved in secretion (Banks and Farthing, 2002). Absorption is mostly due to the active transport of Na^+ via various apical transport proteins coupled with the basolateral sodium pump, Na^+/K^+ ATPase. Na^+/H^+ exchangers (NHEs) are notable apical transporters (Rao *et al*., 2016). A series of reviews by Banks *et al*., Venkatasubramanian *et al*. and Ghishan *et al*., highlight the mechanisms underlying fluid and electrolyte transport (Banks and Farthing, 2002; Venkatasubramanian *et al*., 2010; Ghishan and Kiela, 2012). Nutrient absorption is often coupled with Na^+ absorption. A notable example is the SGLT-1 cotransporter for Na^+ and glucose. Goodman *et al*. reviewed the transporters involved in the absorption of major nutrients (Goodman, 2010).

2.3.2.5 *In vitro* Models of Barrier Function, Absorption, and Secretion

The intestinal epithelium is central to gastrointestinal barrier function, absorption, and secretion. As such, *in vitro* modeling of these functions primarily involves recapitulating the characteristics of the intestinal epithelium and bidirectional transport across the epithelium. Several models of barrier function, absorption, and secretion will be highlighted.

2.3.2.5.1 Intact Tissue Methods

There are several models that attempt to emulate the functions of the intestinal epithelium by using intact tissue. The use of intact tissue provides some physiologic advantages over cell models due to the presence of multiple cell types, transporters, and enzymes. These methods include isolated intestinal perfusions, Ussing chambers, everted gut sacs, and precision cut slices (van de Kerkhof *et al*., 2007).

Isolated intestinal perfusions involves removing a segment of intestine from an animal (typically a rat), placing it in a water bath in order to maintain its viability, and then perfusing the segment's lumen with a study drug or bioactive compound. Since the segment's lumen remains isolated from the surrounding water bath, the presence of the compound of interest on the serosal side is indicative of intestinal absorption. A major limitation of this technique is that absorption, in this model, is defined by a substance's ability to traverse all of the GIT layers, not just the epithelium(van de Kerkhof *et al*., 2007).

Ussing chambers were developed by the Danish physiologist, Hans Henriksen Ussing over 60 years ago (Hamilton, 2011). The Ussing chamber technique utilizes excised intestinal segments as a barrier between two separate compartments or chambers continuously filled with an oxygenated Krebs–Ringer bicarbonate buffer (Deferme *et al*., 2008). The tissue is mounted in such a way that there are separate apical and basolateral sides or chambers. Due to the thickness of the human intestinal wall, the serosa must be stripped away prior to using human tissue in Ussing chambers. The Ussing chamber technique can assess the bidirectional transport of a compound of interest applied basolaterally or apically. Ussing chamber preparations are also used to determine electro-physiologic differences between the apical

and basolateral side which reflects permeability and ion transport (van de Kerkhof *et al*., 2007).

The everted gut sac technique is a method commonly used to assess absorption. The model was introduced in 1954 by Wilson and Wiseman. The technique involves everting intestinal tissue and clamping the ends to form a sac. The sac is filled with Krebs solution and then placed into an incubation flask filled with oxygenated media and a compound of interest. Alam *et al*. reviewed the preparation of everted gut sacs (Alam *et al*., 2011). The method is primarily used to assess the transport of compound across the serosa or the effect of a compound on the serosal transport of other substances. Although it is primarily used in pharmacologic studies, it is also relevant to the *in vitro* assessment of bioactive components seen in functional foods. A notable limitation of everted gut sacs is an underestimation of mucosal transport due to the presence of the serosa and muscularis propia (Le Ferrec *et al*., 2001).

Precision cut intestinal slices is an *in vitro* model that offers the advantage of retaining relevant structural and functional intestinal features seen *in vivo* including the three dimensional architecture, extracellular matrix, enzymes, co-transporters and multiple cell types of the intestine (Niu *et al*., 2013). The model begins with using a uniform tissue slicer such as Krumdieck or Vitron slicers to produce 200–250 μm thick "precision cut slices" of agarose-embedded intestinal tissue (Kanter *et al*., 2002). The processes of preserving and incubating the slices were reviewed by Niu *et al*. 2013 and Kanter *et al*., 2002. Precision cut intestinal slices, although still their infancy, have primarily been used in drug toxicity studies since the metabolic activity of the intestinal cytochrome P450 system (CYP) is maintained in this model. Likewise, the model also has potential as a high throughput model for assessing the metabolism and histologic effects of bioactive compounds found in functional foods.

2.3.2.5.2 Monolayer Cell Models

The primary culture of human IECs is an ideal model of the intestinal epithelium. However, the popularity of culturing IECs has been attenuated by the limited viability and slow growth rate of IECs (Le Ferrec *et al*., 2001; Shimizu, 2010; Pageot *et al*., 2000). Subsequently, the use of cell lines derived from human

colon adenocarcinoma, such as Caco-2, HT-29, and T84, has gained favor over the primary culture of IECs. These cell lines are commonly cultured on permeable transwell culture inserts. In this format, the cells form two-dimensional semipermeable monolayers with apical and basolateral polarity, emulating the intestinal epithelium. The transwell format has apical and basolateral chambers that allow for the evaluation of bidirectional transport of molecules across the monolayer and barrier function. Bidirectional transport of a substance can be determined by applying a substance of interest on one side of the monolayer then measuring its concentration on the other side after an incubation period. Similarly, measuring the flux of large molecules such as mannitol, dextran, or lactulose across the monolayer can approximate permeability or barrier function. Permeability can be determined by using electrodes or epithelial volt ohm meters. However, it is worth noting that the material used for supporting the monolayer can influence permeability studies (Foulke-Abel *et al.*, 2014). Additionally, immunohistochemistry, mRNA expression, or protein expression as determined by western blots or flow cytometry of proteins within tight junctions and adherent junctions within cultured cell lines can also provide insight into barrier function and permeability. One limitation of Caco-2 cells in approximating the intestinal epithelium is their lack of mucus production. Co-culturing Caco-2 cells with cell lines that secrete mucus such as HT-29-MTX compensates for this limitation (Le Ferrec *et al.*, 2001; Pontier *et al.*, 2001). In order to more closely approximate the complexity of *in vivo* physiology, co-culture and 3-dimensional culture or organoid models are used.

2.3.2.5.3 3-Dimensional Cell Cultures

Despite the ubiquitous use of monolayer cell models, these two-dimensional (2-D) models are limited in depicting the three-dimensional (3-D) *in vivo* architecture of the GIT. The 2-dimensionality of monolayer-based models generates altered chemical and molecular gradients (subsequently permeability) compared to *in vivo* studies since these gradients in *in vivo* studies occur in three dimensions in an *in vivo* setting (Nickerson *et al.*, 2007). The transwell monolayer setup does offer basal and apical polarity; but it still lacks a 3-D architecture, and thus it

provides a different cellular microenvironment than an *in vivo* background (Baker and Chen, 2012). Advances in culturing techniques have led to the development of 3-D models that more closely approximate the *in vivo* 3-D architecture of the GIT. One example is culturing cell lines a 3-D gel matrix or scaffold to create a 3-D culture system. These techniques often use MatrigelTM, a 3-D extracellular matrix gel containing collagen, laminin, and entactin (Hughes *et al*., 2010). Alternatively, Jabaji *et al*. used a collagen gel for culturing intestinal epithelial cells (Jabaji *et al*., 2013). Further, synthetic and semisynthetic gels have also been used (Ifkovits and Burdick, 2007; Burdick and Vunjak-Novakovic, 2009). Besides gel or scaffold based systems, suspension culture is another methodology for achieving cultures with a 3-D configuration. One notable suspension culture is the rotating wall vessel bioreactor technique. This techniques use implanting small porous beads coated with extracellular matrix with cultured cells and rotational forces to create 3-D cell culture models (Barrila *et al*., 2010).

2.3.2.5.4 Organoids

Beyond the aforementioned advances in 3-D culture techniques, recent advances in identifying and culturing stem cells by Hans Clevers and coworkers have led to organotypic 3-D culture systems known as organoids or enteroids (Foulke-Abel *et al*., 2014). These techniques utilize human-induced pluripotent stem cells (iPSCs) or adult intestinal stem cells (isolated from biopsies or surgical specimens). Sato *et al*. reviewed the mechanism and applications of growing "mini-guts" from intestinal stem cells (Sato and Clevers, 2013). These 3-D models are promising because they more closely emulate the GIT than traditional cell line cultures since they rely on cells grown from primary cell culture and yield multiple epithelial cell populations and express ion transport proteins involved in *in vivo* absorption and secretion (Foulke-Abel *et al*., 2014).

2.3.2.5.5 Gut-on-a-chip

As previously described in this chapter, the "gut-on-a-chip" is a micro-device that uses peristalsis-like microfluidics to induce Caco-2 cells to form intestinal villi and apical-basolateral polarity with tight junctions. This technique is still its infancy, but it

has potential for modeling absorption and secretion (Kim and Ingber, 2013).

2.3.3 Regulation of Immune Response

2.3.3.1 The Mucosal Immune Response Depends on IECs and GALT

The intestinal epithelium acting as a physical barrier is not the GIT's sole source of protection against the external environment. Interactions between IECs and GALT provide additional protection through both innate and adaptive immune responses. GALT consists of lymphoid tissues organized as mesenteric lymph nodes, Peyer's patches, isolated lymphoid follicles (ILFs), and cryptopatches (Goto and Ivanov, 2013). The GALT also includes scattered lymphocytes within the lamina propria and epithelium (Fasano and Shea-Donohue, 2005). These structures, along with IECs, generate immune responses targeting pathogenic, external antigens. Since non-pathogenic antigens including commensal bacteria and food components are also constantly present in the GIT, distinguishing and tolerating innocuous antigens while maintaining vigilance against pathogenic antigens is a key characteristic of mucosal immunity in the GIT. Disruption in the homeostasis between immune reactivity and tolerance contributes to the pathogenesis of several diseases, including celiac disease, inflammatory bowel disease, and food allergies. This balance is too complex to review here. Peterson *et al*. provides a thorough review of the role of IECs and GALT in achieving immune homeostasis (Peterson and Artis, 2014). We will briefly highlight key concepts involved in mucosal immunity relevant to *in vitro* models of mucosal immune responses.

2.3.3.2 Antigen Exclusion: The Importance of Secretory IgA

Excluding antigen from mucosal immune cells is critical in maintaining immune homeostasis. In addition to the mechanisms of barrier function we described earlier in this chapter, secretory IgA plays a prominent role in antigen exclusion. IgA is produced by plasma cells and GALT in both T-cell dependent and independent mechanisms (Nagler-Anderson, 2001; Goto and Ivanov, 2013). In the gut, IgA is secreted as a dimer. On the

basolateral side of IECs, IgA binds to the polymeric immunoglobulin receptor (pIgR) and is transported to the apical side. Secretory IgA is produced when part of the pIgR is cleaved and remains bound to dimeric IgA as the secretory component (SC). The SC maintains stability of secretory IgA (SIgA) within the harsh environment of the GI lumen (Nagler-Anderson, 2001). Secretory IgA participates in immune exclusion through agglutination of microbial and/or food antigens. Mantis *et al.* have reviewed the role of SIgA in immune exclusion (Mantis *et al.*, 2011).

2.3.3.3 Antigen Sampling is Necessary for Immune Homeostasis

Despite the GIT's multi-tiered barrier function, the gut is not perfect in excluding luminal antigens. Inevitably, nutrient absorption and the presence of gut microbiota expose the mucosal immune system to a plethora of potentially inflammatory exogenous antigens. The mucosal immune system tolerates these antigens by not generating an immune response. However, the gut simultaneously targets and reacts against pathogenic antigens. Balancing these two seemingly opposing tasks depends on the mucosal immune system's ability to selectively sample luminal antigens and deliver them to antigen presenting cells (APCs) within the lamina propria.

The mucosal immune system samples luminal antigens in several ways. The neonatal Fc receptor mediates the uptake of luminal antigens across IECs via vesicular transcytosis (Baker *et al.*, 2009). Antigen sampling and subsequent transport to lamina propria (DCs) also occurs via goblet cell-associated antigen passages (GAPs) (McDole *et al.*, 2013). Rescigno *et al.* demonstrated that lamina propia DCs can open TJs between IECs and extend dendritic processes to directly sample antigen from the gut lumen (Rescigno *et al.*, 2001). Luminal antigens also travel across the intestinal epithelium via paracellular leakage in conditions of increased intestinal permeability (Ménard *et al.*, 2010). Microfold cells (M cells) also mediate antigen sampling. These specialized IECs within the follicle-associated epithelium (FAE) facilitate epithelial exposure to luminal antigens through having microfolds instead of microvilli. M cells are specialized for transcytosis and deliver luminal antigens to Peyer patches

and isolated lymphoid follicles (Mabbott *et al*., 2013). Schulz provides a detailed review antigen sampling in the GIT (Schulz and Pabst, 2013).

2.3.3.4 Antigen Presenting Cells and IECs Modulate T-cell Adaptive Immune Responses

Dendritic cells and macrophages are the major APCs within the lamina propria. There is significant heterogeneity amongst lamina propria DCs and macrophages. Their function is often determined by expression patterns of CD103, CD11b, and/or CX3CR-1 (Chang *et al*., 2015). Depending on the expression pattern of these markers, DCs and macrophages can direct regulatory or inflammatory immune responses from naïve T-Cells. For example, CD11b + DCs can induce pro-inflammatory Th1 and Th17 responses, whereas CD103+ DCs promote differentiation of inflammation suppressing forkhead box P3 (FoxP3) expressing T-reg cells (MacDonald *et al*., 2011). IECs can also influence the function of DCs. For instance, IEC produced transforming growth factor (TGFβ) and thymic stromal lymphopoietin (TLSP) promote the development of CD103+ DCs. IECs also modulate T- cell immune responses via signaling with pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs) (Peterson and Artis, 2014).

2.3.3.5 *In vitro* Models of Mucosal Immunity

The *in vitro* modeling of gastrointestinal immune responses is an attempt to recreate the complex interactions between luminal antigens, the intestinal epithelium, and the immune cells of the GALT. *In vitro* models of the mucosal immune response typically consist of three components: an immune stimulus, modeling of the intestinal epithelium and/or immune cells, and the measurement and/or modulation of an immune response. These models often use bacteria, microbial associated molecular patterns (i.e. LPS), or antigenic components in food, cytokines, or antibodies (i.e. anti-CD3, anti-CD28) to evoke an immune response in various cell culture models including epithelial cell line cultures (Caco-2, T84, HT29), isolated intestinal biopsies typically co-cultured with peripheral blood mononuclear cells (PBMC), isolated peripheral blood mononuclear cells

(PBMCs) or isolated mucosal lymphocytes from either endoscopic biopsy samples or most commonly from surgically resected intestinal samples. Several studies use a transwell co-culture system containing IECs and PBMCs to better approximate the *in vivo* crosstalk between IECs and immune cells (Parlesak *et al*., 2004; de Kivit *et al*., 2014; Leonard *et al*., 2010). After stimulation, changes in cytokine production and changes in permeability reflect the mucosal immune response.

Recently, there has been increased interest in using newer models such as 3D cultures and organoids to study the mucosal immunity. Barrila *et al*. reviewed the use of the rotating wall vessel derived 3D cultures to assess host–pathogen interactions (Barrila *et al*., 2010). Similarly, Wilson *et al*. described using a small intestinal organoid model to assess the innate immune response of the epithelium to *Salmonella enterica* serovar *Typhimurium* (STM) (Wilson *et al*., 2014).

2.3.4 Storage, Fermentation, and Removal of Fecal Matter

2.3.4.1 Storage and Removal of Fecal Matter

The GIT's processing of ingested materials produces 100–200 g of fecal waste consisting of water, undigested fiber, soluble substances, and bacteria (Stephen and Cummings, 1980). The processing of fecal waste is a vital function of the GIT and the primary responsibility of the large intestine (colon). The large intestine anatomically consists of the cecum; ascending, transverse, descending, and sigmoid colon; and the rectum. Digested materials that are not absorbed in the small bowel and undigested materials traverse the ileal–cecal valve into the cecum mostly has fluid. The fluid waste becomes increasingly solid as the colon absorbs water and electrolytes during colonic transit. In order to allow for adequate absorption of water and electrolytes, feces transits slowly through the colon via the net propulsion of propagating and non-propagating contractions. Solid stool is eventually stored in the sigmoid colon and rectum prior to defecation. Although the absorptive and motility functions of the GIT were previously addressed, a review by Sarna provides a more detailed discussion of these concepts in regards to the colon specifically (Sarna, 2011).

As a whole, the transit, storage, and removal of fecal matter is a potential target for functional foods since colonic dysmotility is associated with chronic constipation, irritable bowel syndrome, and diverticular disease (Cook *et al*., 2010; Gras *et al*., 2013). However, modeling these conditions *in vitro* is complicated because their pathophysiology not only includes altered motility, but also visceral hypersensitivity and colonic immune regulation (Barbara *et al*., 2011; Humes *et al*., 2012). The *in vitro* models of absorption and motility previously described in this chapter are applicable to the colon. However, lacking the presence and activity of the colonic microbiome is a notable limitation in many physiologic models of the large intestine since the microbiome and its products of fermentation influence motility, immune regulation, and the ENS activity (Scheppach, 1994; Cherbut *et al*., 1997; Robinette and Colonna, 2014; Hurst *et al*., 2014). Models of *in vitro* fermentation take into account this limitation.

2.3.4.2 Colonic Fermentation

Fermentation is the anaerobic metabolism of carbohydrates to gas, alcohol, or acid and volatile organic compounds (VOC). The human intestinal microbiome contains large numbers of fermentive bacteria. Moreover, the colon has the highest population of microbes due to a slow transit time, favorable pH, and the presence of nutrients within feces facilitating a hospitable environment for microbial colonization in comparison to the rest of the GIT (Payne *et al*., 2012). Thus, most bacterial fermentation occurs within the colon. However, rates of fermentation vary within each region of the colon due to regional differences in pH and microbial colonization (Payne *et al*., 2012). Most fermentation occurs within the cecum and the proximal or ascending colon (Cook and Sellin, 1998). Colonic microbiota convert indigestible carbohydrates to short chain fatty acids (SCFAs), lactic acid, and mixed gases (e.g. H_2, CH_4) (Payne *et al*., 2012; Cook and Sellin 1998). The carbohydrate substrates for bacterial fermentation not only include indigestible soluble fiber, insoluble fiber, and resistant starches, but also malabsorbed fermentable oligo-, di-, and monosaccharides and polyols (FODMAPs) (Cook and Sellin, 1998; Payne *et al*., 2012; Khan *et al*., 2014). Moreover, many of these indigestible substrates are also considered prebiotics, a type of functional food.

2.3.4.3 Short-Chain Fatty Acids

The SCFAs resulting from bacterial fermentation include 2-carbon to 5-carbon weak fatty acids such as acetate (C2), propionate (C3), butyrate (C4), and valerate (C5) (Hurst *et al.*, 2014; Tazoe *et al.*, 2008). The total concentration of SCFAs in the colon is approximately 100 mM with acetate as the most abundant SCFA (Cook and Sellin, 1998). The location of fermentation within the colon, types of carbohydrates fermented, and bacterial composition of the microbiome influence the concentration and ratios of each type of SCFAs (Hurst *et al.*, 2014). Short-chain fatty acids primarily serve as nutrients to the colonic epithelial cells. Other functions of SCFAs include influencing colonic blood flow, fluid/electrolyte transport, colonic motility, and mucosal immune regulation (Tazoe *et al.*, 2008; Hurst *et al.*, 2014; Macfarlane and Macfarlane, 2011; Soret *et al.*, 2010)). SCFAs can also affect gut permeability (Camilleri *et al.*, 2012). The mechanisms through which SFCAs mediate these functions are not well understood. However, SCFAs activate three G protein coupled receptors, GPR43 (FFA2), GPR41 (FFA3), and GPR109a (only binds butyrate), which may mediate these effects (Milligan *et al.*, 2009; Natarajan and Pluznick, 2014). Further, each type of SCFA binds these receptors with differing affinities, indicating that each SCFA may have its own specific functions (Natarajan and Pluznick, 2014). *In vitro* models of fermentation can assess factors influencing SCFA production (e.g. dietary substrates) and determine the types of SCFAs produced.

2.3.4.4 *In vitro* Models of Fermentation

16S rRNA based methods and whole metagenomic sequencing (shotgun metagenomic sequencing) in the stool and intestinal mucosal samples provide insight into the taxonomic and phylogenetic composition and function of the gut microbiome; however, these methods do not assess fermentation and the overall functionality of the microbiome (Grice and Segre, 2012). There are several *in vitro* gut fermentation models that describe the function of the gut microbiome. Although variation exists between models, the models essentially entail inoculating chemostats with fecal microbiota while controlling environmental variables including pH, temperature, and oxygen saturation (Payne *et al.*, 2012). The models allow the cultivation of

intestinal microbiota and the assessment of fermentation of dietary components of interest by the cultivated microbiota. The models vary in complexity. Batch culture models are essentially closed suspensions of fecal material in a bottle or a reactor. They are used primarily for short-term fermentation since the cultivated microbiota will deplete substrate and nutrients over time. Continuous models address the challenge substrate and nutrient depletion by incorporating the continuous addition of nutrients and substrates. These models are thus better suited for long-term fermentation studies. Continuous models are performed in a single bottle or reactor (single-stage continuous fermentation) or in a series of connected bottles or reactors (multi-staged continuous fermentation). The single stage system is typically used to depict fermentation in a single region of the colon, whereas the multi-staged design allows for the replication of the physiologic characteristics of different regions of the colon in a series. Artificial digestive systems such as SHIME, TIM1, and TIM2 are multi-staged continuous fermentation models that seek to replicate the interdependent physiologic conditions of the stomach, intestine, and colon by including pancreatic enzymes, bile acids, and motility. These models are limited by a lack of host responses. Combining fermentation systems with cell culture lines can address this limitation. Payne *et al*. have reviewed *in vitro* fermentation systems (Payne *et al*., 2012).

2.4 Limitations of *In vitro* Modeling of the Gastrointestinal Tract

> *All models are wrong, some models are useful* – George E. Box (Tiao, 1984)

Despite the utility of *in vitro* models in understanding the physiology of the GIT and the effects of functional foods, these models have several limitations. The aforementioned *in vitro* models throughout this chapter represent isolated functions of the GIT. However, the functions of the GIT do not occur in isolation *in vivo*. In the fasting and fed states, the functions of the GIT are integrated and interdependent. Farré has reviewed

the complex interplay of the functions of the GIT in response to food (Farré and Tack, 2013). Isolating the functions of the GIT is useful for identifying mechanisms underlying physiologic processes, but it does not fully reflect the complex physiologic and chemical processes that occur *in vivo* (Hur *et al*., 2011). This limitation is known as methodological reductionism, the concept that analyzing components can lead to an overall understanding of complex systems (Glass and Hall 2008; Fang and Casadevall, 2011; Genot *et al*., 2013). Obviously, the overall functioning of the GIT is dependent on the interconnectedness of its individual components and their respective functions. *In vitro* models are also limited by the inclusion of artificial nonphysiologic conditions such as temperatures and electrolyte concentrations not reflective of *in vivo* physiology (Hartung and Daston, 2009). Cell culture models best exemplify these nonphysiologic conditions. First, cell culture models frequently depend on cell lines originating from cancer cells such as Caco-2. These cell lines have thousands of mutations that limit their correlation with noncancerous cells found *in vivo* (Frank and Nowak, 2004). Further, cell culture models do not reflect the physiologic impact of mucous, bile salts, and lipid emulsification on absorption (Le Ferrec *et al*., 2001). Other models also have specific limitations. Ussing chambers and everted gut sac models model absorption as occurring across the entire intestinal wall, which differs from *in vivo* absorption occurring across the epithelial layer alone (Le Ferrec *et al*., 2001). Aside from the artificiality of *in vitro* models, these models are also limited by variability in technique and a lack of standardization (Hartung *et al*., 2002). All in all, due to these limitations, *in vitro* techniques inadequately estimate bioaccessibility, the fraction of an ingested substance released into digestive fluids, and bioavailability, the fraction of an ingested substance absorbed through the GIT into the systemic circulation (Foltz *et al*., 2010; Versantvoort and Rompelberg, 2004). Nonetheless, its ease of use, its high throughput characteristics and its relative low expense made these *in vitro* systems a "work horse" of functional food industry and academic research laboratories involved in assessing GIT function and its role in healthy and disease states and provided valuable and clinically and scientifically relevant information.

2.5 Dynamic *In vitro* Models of Digestion

Dynamic *in vitro* models of digestion attempt to address the aforementioned limitations of *in vitro* models by closely modeling the *in vivo* complexities of human digestion. These dynamic models are well-suited for studying the bioaccessibility and bioavailability of ingested substances due to their 'global' *in vitro* representation of the functions of the GIT (Hur *et al*., 2011; Wickham *et al*., 2009). Dynamic *in vitro* digestion models use two to three compartments to simulate the physiology of the digestive tract. A two-compartment system represents the stomach and small intestine, whereas a three-compartment system represents the mouth, stomach, and small intestine or the stomach, small intestine, and colon. The conditions within each compartment such as temperature, pH, enzyme composition, and fluid dynamics are based on physiologic data obtained from *in vivo* studies. Examples of dynamic *in vitro* models of digestion include the Simulator of the Human Intestinal Microbial Ecosystem (SHIME), TNO gastro-Intestinal tract Model (TIM), and the Institute for Food Research (IFR) Dynamic Gastric Model (Molly *et al*., 1994; Krul *et al*., 2000; Chessa *et al*., 2014). Researchers have used these models to study the bioaccessibility, bioavailability, and digestion of food, soil contaminants, allergens, and mycotoxins without the ethical and experimental limitations of *in vivo* studies (Versantvoort and Rompelberg, 2004; Hur *et al*., 2011; Verstraete *et al*., 2002; Moreno *et al*., 2004). These models can also create conditions to assess the impact of digested substances on the microbiome (Van den Abbeele *et al*., 2010). Despite the advantages offered by these models, they do not duplicate the physiologic complexity of *in vivo* models (Coles *et al*., 2005).

2.6 Conclusions

The anatomy and physiology of the gastrointestinal system is both integrated and exceedingly complex. The GIT and its functions are essential to the mechanisms underlying functional foods since the GIT is the primary site of food processing. Compared to *in vivo* studies, *in vitro* techniques provide inexpensive, quick, high throughput models for exploring the

interactions between functional foods and the GIT. Throughout the text, we highlighted *in vitro* techniques relevant to assessing the effect of functional foods on gastrointestinal functions including motility, barrier function, absorption, secretion, immune response, and the storage, removal, and fermentation of fecal matter. Despite the advantages of *in vitro* testing, the models do not fully recapitulate the complexities of *in vivo* models. Therefore, the use of *in vitro* techniques should be coupled with appropriate *in vivo* models to fully elucidate the mechanisms by which functional foods may exert their benefit.

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3

In vivo Foundations of Sensory *In vitro* Testing Systems

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3.1 Introduction

While humans essentially eat food to meet nutritional needs they also eat for pleasure. The pleasure derived from eating a food often comes from its flavor and textural properties. While flavor appears to be localized in the mouth the perception of food flavor is multimodal and involves the senses of vision, hearing, taste, smell and touch. The senses of taste, smell and chemesthesis (chemical irritation) are known collectively as the chemosenses due to their ability to detect chemical stimuli in the environment and encode them to form a neural perception. The sense of smell provides information regarding stimuli in the local environment whereas taste and chemesthesis require stimuli to be in direct contact with receptor cells in the oral cavity. The chemosenses provide critical information regarding our chemical environment and have an important role in reducing the risk of ingesting toxic substances while also influencing eating behavior by providing information about the nutritional quality of a food (e.g. energy density).

Texture has been defined as the "sensory manifestation of the structure of food and the manner in which this structure reacts to applied forces, the specific senses involved being vision, kinesthesis and hearing" (Szczesniak, 1963). Texture is vital for

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the appreciation of foods and it has been shown that when foods are blended to remove their textural cues only 40% of foods can be identified by their taste alone (Schiffman, 1977). Moreover, it has also been proposed that texture provides influences the consumer's perception regarding the calorie content of foods (Biswas, Szocs *et al*., 2014).

A better understanding of the oro-sensory aspects of food perception is essential for food development. First, food choices are strongly influenced by food palatability and it is unlikely that any new or reformulated product will be chosen if its sensory characteristics do not meet expectations. Second, the taste, smell and flavor of a food contributes to the amount eaten through the process of sensory specific satiety (Rolls *et al*., 1981). Consequently, a better understanding of how oro-sensory perception may enable the development of new foods that maximize sensory specific satiety and reduce food intake. Third, the textural perception of foods may influence satiety through learned expectations (e.g., individual expectations that more viscous foods are more energy dense resulting in increased satiety).

The relationship between food stimuli and perception is usually assessed using human psychophysical studies. However, these studies can be relatively expensive, time consuming, require the use of trained sensory panelists and may have ethical approval constraints. Consequently, there is considerable interest in developing surrogate *in vitro* models that provide data that correlates with human psychophysical studies and can be used to obtain data quickly and inexpensively. While a growing body of research has been produced to understand how food is perceived there remain substantial gaps in knowledge which may prove to be substantial barriers to the development of *in vitro* models of the oro-sensory perception of food. This chapter will describe the physiological basis for the oro-sensory perception of food that would underpin the development of *in vitro* models.

3.2 Taste

3.2.1 Overview

Taste is the sensory perception produced when a food or other substance interacts with taste receptor cells on the tongue or other areas of the oral cavity. While humans can taste a wide

array of chemical entities it is generally, although not universally, accepted that there are only five fundamental taste modalities: sweet, sour, salty, bitter and umami (glutamate). Among these taste qualities, bitter and sour tastes are innately aversive whereas sweet and umami are generally attractive (Cowart, 1981). Salt is unique in that the concentration determines if it is attractive or aversive (Oka, *et al*., 2013). It has been argued that in addition to the five traditional taste modalities several others exist including calcium (Tordoff *et al*., 2012) or fatty acids (Mattes, 2009). However, despite a growing body of evidence to support these arguments they are not widely accepted as canonical taste modalities at this time. It has also been proposed that other "tastes", such as alkaline, metallic, astringent or pungent, exist although they are generally thought to be chemesthetic or olfactory in origin. However, these "tastes" may contribute significantly to the perception of foods and would need to be recognized in any *in vitro* model. For instance, if fat is a primary taste, the replacement of fat with fat mimetics in a food may not elicit the same sensory perception despite similar textural profiles. An *in vitro* model that did not include fat as a basic taste may ignore crucial information regarding the perception of that food. A more controversial viewpoint is that there are no primary tastes. For instance, Schiffmann *et al*. (1980) demonstrated that the taste range of sodium salts was beyond that defined by the primary taste system while Erickson (1982) proposed a synthetic system where the primary tastes combine to provide taste qualities that are distinct from the individual parts. However, a later study using various sugars (Breslin, Beauchamp *et al*., 1996) has challenged these findings and the primary taste model is still generally accepted.

3.2.2 Taste Anatomy

Taste buds are located throughout the oral cavity and contain the receptors for taste and are located throughout the oral cavity. Taste buds are located within three different types of structures called papillae. The fungiform papillae are mushroom shaped projections found on the front part of the tongue; circumvallate papillae are dome-shaped structures found at the back of the tongue and the foliate papillae which are found at the sides of

the tongue. In humans, the number of taste buds within the papillae varies greatly between individuals (Miller, 1988). However, the number of taste buds is not strongly influenced by factors such as age or health status (Miller, 1988; Miller and Reedy, 1990; Mavi and Ceyhan, 1999). While there are subtle differences in the sensitivity to the different taste stimuli across the tongue, the commonly-held concept of a "taste map" is now thought to be wrong (Lindemann, 1999).

Taste stimuli are detected by taste receptor cells (TRC) that are located within the taste buds (Miller, 1995). In humans, each taste bud contains between 50 and 100 TRCs (Chaudhari and Roper, 2010). TRCs are modified epithelial cells that project microvillae to the apical surface of the taste bud where they form the "taste pore". This is the site of interaction with tastants. As TRCs may be exposed to potentially harmful chemicals they are short-lived and have an average life-span of ten days (Hamamichi, Asano-Miyoshi *et al*., 2006). They are replenished from proliferative basal keratinocytes (Kapsimali and Barlow, 2013).

Ultrastructural studies reveal that there at least three morphologically distinct cell types contained in taste buds (types I, II, III). Type I cells are the most abundant cells in taste buds and are termed "glial-like" because they appear to restrict the spread of neurotransmitters such as ATP (which serves as a neurotransmitter in taste buds) or glutamate which is a candidate neurotransmitter in taste buds (Lawton *et al*., 2000; Bartel *et al*., 2006). Type I cells also engulf other taste buds with extended cytoplasmic lamellae (Pumplin *et al*., 1997). Intense taste stimulation would elicit prolonged trains of action potentials that would lead to the accumulation of K^+ in the limited interstitial space leading to diminished excitability of type II and III cells. However, type I cells express ROMK, a K channel that may be involved in K^+ homeostasis within the taste bud and which may serve to eliminate K^+ from the taste bud (Dvoryanchikov *et al*., 2009). Also, type I cells have been implicated in salt taste transduction (Vandenbeuch *et al*., 2008).

Type II (receptor) cells express G-protein-coupled receptors that are embedded in the membrane of these cells that are selective for sweet, bitter or umami taste stimuli (Tomchik *et al*., 2007). However, type II cells do not appear to be directly stimulated by salty or sour tastants. Type II cells also express

voltage-gated Na and K channels that are essential for the production of action potentials. Type II cells are tuned to bitter, sweet or umami (Nelson *et al*., 2001) and each cell only expresses a GPCR for one taste quality (Tomchik *et al*., 2007). Due to their role as the primary detectors of sweet, bitter and umami, type II cells have been renamed receptor cells (Defazio *et al*., 2006).

Type III cells are the most neuron-like cells and it is generally believed that they express proteins associated with synapses and that they form synaptic junctions with nerve terminals (Yang *et al*., 2000; Yee *et al*., 2001). Like type II cells, type III cells are excitable and express voltage-gated Na and K channels to support action potentials (Medler *et al*., 2003; Gao *et al*., 2009). A key feature of type III cells is that receptor transmits their signals to these presynaptic cells where the information is integrated (Tomchik *et al*., 2007). Hence, these presynaptic cells are not tuned to specific tastes but respond to sweet, salty, sour, bitter and umami compounds (Tomchik *et al*., 2007). This communication between different taste cells represents a convergence of taste information in the taste bud and results in taste cells that respond to multiple taste stimuli.

Each TRC is connected through a synapse to a sensory nerve ending through which the taste-coding information is sent to the brain. However, a single sensory neuron can be connected to several taste cells in each of the taste buds. Four different pairs of nerves innervate the tongue and make contact with the taste bud structures. The fungiform papillae are innervated by the chorda tympani branches of the facial nerves (cranial nerve VII). The glossopharyngeal nerves (cranial nerve IX) sends branches to the rear of the tongue and the vagus nerve (cranial X) to the far posterior areas on the tongue root. The greater superficial petrosal nerve sends branches to the palatal taste area. The taste information relating chemical stimuli to taste perception is encoded by the nervous system. In studies of primates it has been shown the primary taste cortex in the anterior insula and frontal operculum contains taste neurons tuned to sour, sweet, bitter, salty and umami stimuli (Scott *et al*., 1986; Yaxley *et al*., 1990; Baylis and Rolls, 1991). In addition, a secondary cortical taste area has been discovered in the orbitofrontal cortex (Rolls *et al*., 1990) which also responds to the five canonical taste stimuli (Baylis and Rolls, 1991; Rolls, 1997). Neuroimaging studies

using human participants indicate that taste activates an area of the anterior insula/frontal operculum (likely the primary taste cortex) and part of the orbital-frontal cortex (likely the secondary taste cortex) (Francis *et al*., 1999; O'Doherty *et al*., 2001).

3.2.3 Taste Coding

Taste coding refers to the process in which taste is identified, its concentration recognized and a hedonic value assigned through action potentials relayed to the brain. It has been demonstrated that each neuron responds to several different taste stimuli but it is not clear how the brain distinguishes among the various taste qualities. Two opposing theories to this problem have been proposed (Chandrashekar *et al*., 2006). One theory suggests that taste conforms to a labeled line model, in which each cell represents a distinct taste quality and communicates essentially without interruption to the CNS. The opposing theory is that taste conforms to a distributive model, in which cells respond in varying amounts to each taste quality, and the CNS makes sense of the chorus of activity. A third theory that does not garner widespread support is that the taste quality is denoted by a timing pattern of action potentials similar to those produced by auditory fibers. At this time, it is unclear how information gathered by TRCs in taste buds is coded for the eventual perception of distinct taste qualities.

The incomplete understanding of how taste is coded may be problematic for the development of *in vitro* models. For instance, while *in vitro* models can be developed to understand how tastants bind to taste receptors this provides only a partial explanation for the perception of foods. Several post-receptor events may modulate the perception of the food and at this present time it is not possible to model these processes accurately which may limit the usefulness of *in vitro* models at the present time.

3.2.4 Transduction Mechanisms

3.2.4.1 Overview

Biological and electrophysiological studies have shown that taste cells use a variety of mechanisms to transduce chemical information into cellular signals. A number of studies have

suggested that salt and sour stimuli modulate taste function by direct entry of Na^+ and H^+ through specialized membrane channels on the apical surface of the cell. By contrast, sweet and umami are mediated by a small family of G-protein-coupled receptors (GPCR): T1R1, T1R2 and T1R3. By contrast, bitter taste is mediated by a family of around 30 highly divergent GPCRs: the T2Rs.

3.2.4.2 Sour

Substances that taste sour are acidic and include a range of fermented foods or unripe fruits. It has been proposed that sour taste evolved to protect against the ingestion of excessive acid which would disturb the body's acid–base balance or the ingestion of spoiled foods. Consequently, sourness is generally aversive unless combined with another taste quality such as sweet. The sourness of acids is not tightly correlated with the concentration of protons in the acidic stimulus (Harvey 1920).It is interesting to note that acetic acid at pH 3.9 evokes a sour taste in humans whereas hydrochloric acid (HCl) at the same pH does not.

Lyall *et al.* (2001) demonstrated that it is a reduction in the intracellular pH of TRC that is the proximate stimulus for sour taste. This observation provides an explanation for the low correlation between the concentration of protons in the stimulus and the sourness of the acid. The protonated molecular forms of organic acids readily permeate the cell membrane, enter the cytosol, and disassociate to release protons inside the TRC. If present in high enough concentration, extracellular protons can also cross the cell membrane and lower the pH of the TRC thereby explaining the sour taste of relatively concentrated acidic solutions.

Considerable effort has been expended to identify sour taste transduction mechanisms. Over the years a number of candidates for sour receptors including ASICs, HCNs, K^+ channels and the TRP channels PKD2L1 and PKD1L3 have been proposed (Roper, 2007). However, there is insufficient evidence to directly link any of these candidate receptors to sour taste. Although an initial study found that PKD2L1 and PKD1L3 was activated by exposure to solutions with a $pH < 3$ (Ishimaru *et al.*, 2006) subsequent studies using PKD2L1 or PKD1L3 mouse knockout models report that chorda tympani nerve responses

to sour tastants were only reduced by 25–45% compared with those in wild type mice indicating that other mechanisms are involved in sour taste reception (Horio *et al.*, 2011).

3.2.4.3 Salt

The principle stimulus for salt taste is Na^+ although other cations such as Li^+ or K^+ may also be perceived as salty. Sodium is an essential nutrient and is required to maintain physiological functions such as muscle contraction or action potentials whereas excessive salt intake is deleterious and may contribute to chronic disease (Feldstein, 2002). Due to the beneficial or deleterious effects it is possibly not surprising that salt can elicit two opposing behavioral responses with low concentrations (<100 mM NaCl) of salt being appetitive but high concentrations (>300 mM) being aversive and provokes a rejection response (Oka *et al.*, 2013). In humans, the detection threshold for NaCl is 1–7 mM (McMahon *et al.*, 2001) although in complex foods the recognition threshold may be blurred due to the effect of other tastes on salt taste (Omahony *et al.*, 1976).

Studies have shown that in rodents salt tastants modulate taste cell function by direct entry of Na^+ through amiloride sensitive Na^+ channels on the apical surface of the cell (Heck *et al.*, 1984). However, the role of amiloride are less clear for salt taste in humans and it is likely that an amiloride insensitive pathway also plays a role in the transduction of sodium salts (Ossebaard and Smith, 1995). It has also been postulated that a vanilloid receptor, a VR1 variant, also transduces salt taste (Lyall *et al.*, 2004) although a subsequent behavioral study using a mouse V1R knockout model found that the knockout mice preferred NaCl over water at concentrations avoided by the wild-type mice and consumed higher amounts of salt (Ruiz *et al.*, 2006). This suggests that that the VR1 variant receptor is involved in salt aversion. In addition, paracellular pathways through taste buds have been identified for Na^+ and these may contribute to salt taste (Simon, 1992).

3.2.4.4 Bitter

It is thought that bitter taste has evolved to detect toxins in the environment that are primarily produced by plants. A large number of structurally diverse chemicals taste bitter to humans

including caffeine or quinine. However, many bitter compounds are harmless and it has been proposed that the knowledge that a compound is bitter provides little information whether it is nutritious or toxic (Glendinning, 1994).

In humans, bitter chemicals are detected by a small family of receptors (T2R) with 25 different genes coding for 25 T2Rs (Behrens *et al.*, 2007). It is interesting to note that these relatively few receptors suffice to detect thousands of structurally different bitter compounds. It has been shown that bitter receptors fall into two classes: specialist cells that detect one to a few different bitter chemicals and generalists that detect many (Behrens *et al.*, 2009). Receptors that detect many ligands are less sensitive than specialist cells. Moreover, mutations in the receptor that increase affinity for one agonist will decrease the affinity for another (Born *et al.*, 2013). It is interesting to note that some bitter tasting compounds can function both as agonists for one set of bitter receptors and antagonists for others meaning that these competing actions may produce responses to complex mixtures of foods that are not the sum of their respective components (Brockhoff *et al.*, 2011).

3.2.4.5 Sweet

Sweet taste is elicited by sugars, artificial sweeteners and a small number of sweet tasting proteins (Jiang *et al.*, 2005). Up until recently, there was a tight link between sweetness and the caloric content of a food. However, the rise in the use of artificial sweeteners has weakened this link which may lead to a reduction in the effectiveness of learned associations between sweet tastes and post-ingestive caloric outcomes (Davidson *et al.*, 2011).

A functional sweet taste receptor is a dimer of T1r2 and T1R3 (Ohkuri *et al.*, 2009). Studies using knockout mice indicate the T1R2/T1R3 receptor is the predominant mechanism for detecting sweet taste they also indicate another mechanism may exist (Zhao *et al.*, 2003). The T1R1 receptor is expressed in fungiform papillae and the palate and the T1R2 receptor almost exclusively in the circumvallate and foliate papillae (Hoon *et al.*, 1999) while the T1R3 receptor is found in all three types of papillae on the tongue (Nelson *et al.*, 2001). Due to the pattern of the taste receptor expression, TRC are divided into three types: T1R1/T1R3 in the fungiform papillae and palate, T1R2 and T1R3 in the

circumvallate and foliate papillae, and T1R3 alone in the fungiform papillae and palate (Bachmanov and Beauchamp, 2007).

3.2.4.6 Umami

The taste of umami is primarily elicited by L-glutamate which is predominantly supplied in the diet in the form of monosodium glutamate (MSG). Umami is derived from the Japanese word umami which means "delicious". The umami taste is also elicited by other amino acids including L-aspartate, some dipeptides, tripeptides, oligopeptides and some organic acids (Roper, 2007). The taste of umami is amplified by the presence of inosine 5'-monophosphate (IMP) and guansine-5'-monophosphate (GMP) (Zhang *et al*., 2008). For instance, although IMP does not elicit an umami taste response itself, in humans it can increase sensitivity to umami by 15-fold (Li, Staszewski *et al*., 2002). Umami taste is best exemplified in western cuisine by the taste of meaty broths.

Cell studies have demonstrated that T1R1 and T1R3 GPCRs combine to form a broadly tuned L-amino acid receptor (Nelson *et al*., 2002). Further evidence that T1R1 and T1R3 combine to provide the glutamate receptor comes from mice knock-out studies. Homozygous mutants lacking either the T1R1 or T1R3 subunit showed an overwhelming loss of umami taste and the behavioral attraction to MSG (Damak *et al*., 2003; Zhao *et al*., 2003). These data provide an example of how heteromeric GPCRs alter their selectivity according to a combinatorial arrangement of subunits (T1r2 + T1R3 = sweet taste and T1R1 + T1R3 = umami). They also reveal that these two taste modalities share a common receptor and evolutionary origin.

3.2.4.7 Downstream Signaling of T1R and T2R

Binding of sweet, umami or bitter ligands to T1R and T2R located in the apical membrane of type II TRC cells leads to the dissociation of heterotrimeric G proteins and the activation of signal transduction pathways. It is thought that the a subunit of G proteins cause an increase in the secondary messenger cyclic adenosine monophosphate (cAMP) due to the activation of adenylyl cyclase. Elevated cAMP activates Protein kinase A which in turn phosphorylates potassium channels, causing them to close and depolarize the TRC. Stimulation of calcium channels and the influx of calcium ions results in the release of

neurotransmitters (Roper, 2007). The binding of the stimuli to its receptor may also lead to a change in cyclic guanosine monophosphate (cGMP) (Sugita, 2006).

The second signaling pathway involves the activation of PLC-b2 by the b/g subunits of G proteins. PLC-b2 hydrolyzes PIP2 to DAG and IP3. Binding of IP3 to the IP3R3 receptor leads to the release of calcium ions from intracellular compartments (Ishimaru and Matsunami, 2009). The involvement of PLC-b2 and IP3R3 in the signal transduction of sweet, bitter and umami tastes is confirmed by a study that found that mice lacking PLC-b2 mice show a diminished response to sweet, bitter and umami tastes (Zhang *et al*., 2003).

Another key participant in chemosensory transduction is the nonselective monovalent cation channel TRPM5 which is expressed in taste bud cells (Hofmann *et al*., 2003). Significantly, TRPM5 knockout mice are less responsive to sweet, bitter and umami tastes (Damak *et al*., 2006). It appears that TRPM5 channels are transiently and directly opened by increases in intracellular Ca^{2+} (Prawitt *et al*., 2003). It is believed that the result of TRPM5 activation is cation influx and membrane depolarization leading to neurotransmitter release.

3.2.5 Non-Canonical Taste Modalities

3.2.5.1 Fat Taste

The oro-sensory detection of fat has traditionally been attributed to its texture (Grabenhorst *et al*., 2010), or nasal or oral irritancy (Chale-Rush *et al*., 2007). Although not currently accepted as a canonical taste accumulating evidence suggests that non esterified fatty acids (NEFA) may also be a primary taste. Psychophysical studies repeatedly report the presence of a detection threshold for various NEFA stimuli (Chale-Rush *et al*., 2007). However, a widely accepted mechanistic explanation for these results has not been fully elucidated although several potential mechanisms have been proposed.

One proposed mechanism is that fatty acids are detected through an effect on delayed-rectifying K^+ channels. When receptor cells that have been isolated from rat fungiform papillae are exposed to *cis*, long-chain polyunsaturated fatty acids (PUFA) there is an inhibition of delayed-rectifying

K^+ channels (Gilbertson *et al.*, 1997). However, saturated, monounsaturated and trans-saturated fats have little effect on K^+ channels. From this data it was proposed that the net effect of fatty acid stimuli was to prolong stimulus induced depolarization of taste receptor cells and this was the mechanism though which fats was detected in the oral cavity. However, subsequent studies suggest that DRK channels are more important for enhancing the response to other tastants and are not the primary mechanism responsible for fat taste (Gilbertson and Khan, 2014).

It has been proposed that CD36 contributes to fat taste (Fukuwatari *et al.*, 1997) CD36 (also known as fatty acid translocase (FAT)) is an integral membrane ditopic glycoprotein (Abumrad *et al.*, 1993) that is present in the circumvallate papillae and, to a lesser extent, the foliate papillae of mice (Laugerette *et al.*, 2005). In humans, CD36 has been detected in human foliate and circumvallate papillae (Simons *et al.*, 2011). CD36 has a reversible binding affinity for long-chain fatty acids (Baillie *et al.*, 1996). CD36 knockout mice show behavioral changes and in contrast to wild-type mice do not express a preference for fatty acids (Laugerette *et al.*, 2005; Sclafani *et al.*, 2007); however, they continue to prefer sucrose and avoid quinine indicating that the effects are specific to lipids (Laugerette *et al.*, 2005). It is interesting to note that while CD36 knockout mice do not show a preference for fatty acids they exhibit an enhanced ability to compensate for energy from dietary fat (Sclafani *et al.*, 2007).

3.2.5.2 Calcium

Calcium is essential for life and is involved in many fundamental physiological processes. While calcium homeostasis is largely regulated by physiological processes it has also been proposed there is a behavioral component that is regulated behaviorally through a calcium appetite (Tordoff 2001). Data from several studies suggest that animals can detect calcium although it is not clear if this is due to taste (Inoue and Tordoff, 1998; McCaughey *et al.*, 2005). The data supporting a primary calcium taste remains limited although there is evidence that calcium is tasted through its action on T1R3 (Tordoff *et al.*, 2012).

3.3 Factors that Influence Taste Acuity

3.3.1 Saliva

Saliva plays an important part in taste function, both as a carrier of sapid molecules to the receptors and because it contains substances capable of modulating taste response. Saliva is produced by three pairs of major (the parotid, submandibular and sublingual) and minor glands (labial, lingual, buccal and palatal). Taste pores are exposed to fluids which are derived from the salivary glands. It has been speculated that changes in the quality and quantity of saliva influence taste sensitivity during the initial phase of taste stimulation. This is due to tastants having to dissolve in saliva before they can interact with taste pores. Moreover, saliva contains components which also stimulate taste receptors so continuous stimulation decreases taste sensitivity to the salivary components. However, since taste receptors are usually adapted to the salivary environment we do not normally recognize the taste of saliva.

Taste substances are present in foods of various physical properties and the rate at which they are dissolved can differ. For instance, taste stimulants in aqueous solution are more rapidly dissolved than those in dry solid. This will influence the rate in which taste receptors are stimulated. For instance, in one study the taste responses of rats were recorded from the chorda tympani nerve (Matsuo *et al.*, 1994). When rats lick taste solutions (NaCl or sucrose dissolved in distilled water) the taste responses of the chorda tympani were characterized by an initial transient phase followed by a prolonged steady-state phase. When the rat chewed a dry food pellet the summated chorda tympani discharges gradually increased and then reached a relatively stable level. In addition, it has been shown that a slower flow rate of taste solution causes a smaller magnitude of the phasic response and the longer it takes to reach the peak of the phasic response (Marowitz and Halpern, 1977).

The saliva may also influence the intensity of taste perception. For instance, the intensity of sour taste is reduced by saliva. This is because HCl acid becomes highly dissociated in aqueous solutions and H or H_3O determines taste stimulation by HCl and a reduction of the taste intensity of HCL results from the buffering action of bicarbonate in saliva (Christensen *et al.*, 1987).

3.3.2 Genetic Differences

There are large within- and between-species variations in taste perception. For instance, studies have shown large species differences in sweet taste between species. For instance, studies have shown that cats are behaviorally insensitive to sweet taste (Jiang *et al*., 2012) while sea lions and dolphins have atrophied taste systems with a low number of taste buds in their lingual epithelium (Jiang *et al*., 2012). In mice, a mutation in the *Tas1R3* gene influences the intake and preference for sweet substances (Inoue *et al*., 2004). An examination of *TAS1R3* in humans found that a mutation in this gene influenced the pleasantness, but not the sweetness intensity ratings, of aqueous sucrose solutions (Reed *et al*., 2001). It is not clear what effect this has on human food choices. In addition, several studies of humans have shown that sensitivity to the bitter tasting compound propylthiouracil (PROP) is an inherited trait (Hansen *et al*., 2006). It has been hypothesized that PROP supertasters may consumer fewer vegetables or have other suboptimal dietary habits due to a hypothesized increased sensitivity to bitter taste (Feeney *et al*., 2011). While some studies support this hypothesis (Bell and Tepper, 2006) others do not (O'Brien *et al*., 2013) and report that socio-economic status and gender (Feeney *et al*., 2014) or cultural factors (Catanzaro *et al*., 2013) have a greater influence on vegetable intake than PROP status. Moreover, a clear relationship between PROP status and disease has yet to be established (Schembre *et al*., 2013). While a link between taste sensitivity, food choices and health is intuitively appealing and theoretically plausible further research is required to conclusively demonstrate this link.

3.4 Chemesthesis

The burn of hot peppers, the tingling of carbonation and the coolness of peppermint are all qualities of chemesthesis. In the oral cavity, chemesthesis is mediated by three different nerves: the trigeminal nerve (cranial nerve V), the glossopharyngeal (cranial nerve IX) and the vagus (cranial nerve X). The trigeminal nerve has an extensive innervation field

that includes the anterior oral cavity and tongue, the nasal cavity and the face. While chemesthetic sensitivity has frequently been described as trigeminal sensitivity this is limiting as inhaled irritants that pass into the nasal and oral pharynx stimulate the vagus nerve. Chemesthetic stimuli that are ingested reach the posterior tongue and oral pharynx during mastication and swallowing where they stimulate the glossopharyngeal and vagus nerves.

Much of the understanding of the sensory receptors for chemesthesis has come from the study of capsaicin which is the principle pungent component of chilli peppers. Capsaicin was first identified as a stimulus for pain receptors over 50 years ago (Jancso *et al.*, 1961). The molecular receptor responsible is the vanilloid receptor 1 which is sensitive to capsaicin, other vanilloids, heat and low pH (Caterina *et al.*, 1999; Jordt, 2003). VR1 is a member of a family of transient receptor potential (TRP) ion channels (Gunthorpe *et al.*, 2002). Psychophysical studies have shown that neurons possessing VR1 mediate the chemesthetic qualities of numerous other compounds. Other members of the TRP family are also important for oral chemesthesis. For instance TRPm8 was recently shown to responds to menthol (McKemy *et al.*, 2002) and is thought to mediate sensitivity to cooling temperature from 10°C to 25°C. Capsaicin is of interest not only due to its presence in chilli peppers but also from its ability to desensitize afferent fibers (Carpenter and Lynn, 1981; Wall and Fitzgerald, 1981; Kenins, 1982).

3.5 The Olfactory System

The human olfactory system can sense a vast number of chemical stimuli, and any airborne molecule that as a sufficiently low molecular mass and a sufficiently high hydrophobicity to allow it volatility, is a potential odorant. The olfactory system helps to identify foods and provides information about its quality (e.g., the presence of spoilage microorganisms) while also protecting against potential toxic environments (e.g., gas).

Odorants reach the olfactory epithelium through two routes: through the nose (orthonasal olfaction) or through the nasopharynx (retronasal olfaction). Only a limited subset of the

orthonasal odorants are likely to be retronasal odorants as the odorant sources must be in the oral cavity to be detected restricting potential retronasal natural odorant sources to substances that are ingested. In all cases, and certainly for solid foods, lingual manipulation and interaction with saliva can result in substantial changes in the characteristics of the food that was initially ingested.

3.5.1 Olfactory Anatomy

In humans, the sensory receptors of the main olfactory system are located in the upper recesses of the nasal chambers within a neuroepithelium that lines the cribriform plate and areas of the middle and superior turbinate and septum. Most of the olfactory epithelium is found in the olfactory cleft, which is linked to the olfactory bulb of the brain by the cribriform plate. Olfactory neurons are bipolar with a single dendrite that extends to the central nervous system and a dendrite that terminates on the olfactory epithelium. A thin axon projects from the proximal pole of the cell directly to the higher brain regions. From the apical end of the dendritic terminal, narrow appendages extend towards the outside world from which project 20–30 cilia that contain olfactory receptors and the transduction mechanisms. These cilia lie in a thin layer of mucus which covers the tissue which offers protection against foreign agents and drying. Secretion of mucus originates from supporting cells called Bowman's glands. Some six million neurons project through the cribriform plate towards approximately 800 glomeruli in each olfactory bulb.

3.5.2 Olfactory Binding Proteins

Airborne odorants are commonly hydrophobic molecules; however, to reach their membrane receptors they have to be transported through the aqueous nasal mucus. The odorant-binding proteins (OBPs), which are abundant low-molecular-weight soluble proteins (around 20 kDa) secreted by the olfactory epithelium in the nasal mucus, are candidates for playing a carrier role (Steinbrecht, 1998). However, the essential role of OBPs in odor has only been demonstrated in insects (Kim and Smith,

2001) and their role in human olfaction remains to be established. Odorant-binding proteins may also have a role in the deactivation of odorants which may prevent the saturation of olfactory receptors when odorant are highly concentrated (Pelosi, 1996). They have also been shown to bind to cytotoxic and genotoxic compounds and may have a role in protecting the nasal mucosa and olfactory neurons (Pevsner *et al.*, 1990; Eckl *et al.*, 1993).

Odorant binding proteins are a member of the lipocalin superfamily that display low sequence similarity (usually lower than 20% amino acid identity) but share a conserved folding pattern, an 8-stranded β-barrel flanked by an R-helix at the C-terminal end of the polypeptide chain. The β-barrel defines a central apolar cavity, called calix, whose role is to bind and transport hydrophobic odorant molecules (Blanchet *et al.*, 1996; Tegoni *et al.*, 1996; Spinelli *et al.*, 1998). Odorant-binding proteins bind with high efficiency to a large number of odorants belonging to different chemical classes. For instance, rat OBP-1 preferentially binds heterocyclic compounds, OBP-2 is more specific for long-chain aliphatic aldehydes and OBP-3 binds strongly with odorants composed of a saturated or unsaturated ring structure (Lobel *et al.*, 1998; Briand *et al.*, 2000; Lobel *et al.*, 2002). Consequently, OBP could contribute to odor discrimination although any discriminatory power would be limited.

3.5.3 Olfactory Receptors

If OBPs do not have great discriminatory power, how does the olfactory system mange to discriminate between thousands of different odors? Olfactory receptors were discovered in the early 1990s by Buck and Axel (Buck and Axel, 1991). It is believed that approximately 1000 odorant receptors exist making them the largest known gene family and account for ~1% of all expressed genes (Buck and Axel, 1991). Olfactory receptors are members of the G-protein coupled receptor (GPCR) family. It is striking to note that the number of putative functional olfactory receptors in humans is approximately 350, which is far smaller than the myriad odors we can perceive, which strongly suggests a combinatorial system for odor coding.

3.5.4 Transduction Mechanisms

The G protein coupled receptor is a large family of membrane proteins that share many features in common. They are the first element of a three step process involving a receptor for external signal reception, a transducer and an effector enzyme. They all have 7TM domains, with an external domain outside the cell, corresponding to the N-terminal end of the protein, and an internal cytoplasmic C-terminal domain. The seven membrane-spanning sequences are characterized by highly hydrophobic amino acids, and are linked by six loops, three outside the cell and three in the cytoplasm. The 7TM domains form a seven-α-helix bundle, which composes a central core whose global structure is common to all GPCRs. In many cases, a pocket formed by the membrane spanning segments is involved in ligand binding.

This diversity allows GPCRs to bind to an amazing number of ligands of very different types. Following odorant binding to an olfactory receptor, the enzyme adenylate cyclase is activated to produce the secondary messenger adenosine monophosphate (cAMP) (Lowe *et al*., 1989). G_{olf} -induced cAMP diffuses through the cell and activates cellular depolarization via the opening of cyclic-nucleotide-gated ionic channels and Ca^{2+} dependent Cl^- or K^+ channels (Restrepo *et al*., 1993). In a study using a frog model, the amount of adenylate cyclase activity produced by various odorants in a ciliary preparation is positively correlated with the magnitude of the electro-olfactogram (a surface potential associated with the number of receptors activated) (Sklar *et al*., 1986). Some odorants also activate cyclic guanosine monophosphate (cGMP) which is believed to play a role in the modulation of the sensitivity of olfactory neurons, such as during adaptation (Leinderszufall *et al*., 1996). Although G proteins other than G_{olf} have been identified in olfactory receptor cells they do not appear to be involved in early transduction events but likely assist in processes such as axonal signal propagation, axon sorting and target innervation (Wekesa and Anholt, 1999).

3.6 Texture

It is well documented that the texture perception of a food product is crucial to its acceptability (Guinard and Mazzucchelli, 1996) Therefore understanding texture perception will help in

the development of acceptable products for consumers. The oral cavity has several means to obtain information regarding the textural properties of a food. This information is transmitted to areas of the brain linked to texture perception.

3.6.1 Mechanoreceptors

The mouth contains sensitive tactile receptors called mechanoreceptors. Mechanoreceptors are responsible for relaying information about pressure, vibration, viscosity and movement in the mouth and have a major role in the perception of foods. Mechanoreceptors can be classified as fast adapting or phasic receptors or slow adapting or tonic receptors. Fast adapting receptors signal changes in the environment while slow adapting receptors produce a constant rate of firing when a stimulus is maintained. The oral cavity is richly innervated with nerve fibers and receptors and is exceedingly sensitive to tactile stimulation (Vanboven and Johnson, 1994).

Four types of mechanoreceptors have been identified in the oral cavity. These are slowly adapting type 1 receptors (SA1) that terminate in Merkel cells, fast adapting (FA) afferents that terminate in Meissner corpuscles, slow adapting type 2 receptors that terminate in Ruffini corpuscles and Pacinian receptors (PC) that terminate in Pacinian corpuscles. Each of these mechanoreceptors responds to deformation or motion of the cutaneous surfaces in a different way.

SA1 afferents are sensitive to curvature, edges and points. They are especially sensitive to dynamic rather than static stimuli. It has been suggested that SA1 receptors are well suited for the perception of food form and texture and would be useful to sense the changes a food texture during mastication (Vanboven and Johnson, 1994). SA2 afferents are likely responsible for the perception of larger food particles in the mouth and may be important for the manipulation and swallowing of food. It is not clear what role FA1 mechanoreceptors have in the perception of food.

3.6.2 Proprioreceptors

Proprioception is the sense of the relative position of neighboring parts of the body and strength of effort being employed in movement. It is provided by proprioceptors in skeletal striated

muscles and in joints. During eating, it is of great importance to know the position of the tongue, cheeks, and lips while eating so the food can be manipulated for swallowing. Three types of receptors in the muscles and joints transmit proprioceptive information: muscle spindles that signal changes in the length of muscles, Golgi tendon organs that signal changes in muscle contraction and receptors that are located in joint capsules which sense extension of the joint. In the oral cavity proprioreceptors are found in the tendons and muscles of the masseter, temporalis and pterygoid muscles as well as the tempomandibular joint.

3.6.3 Periodontal Receptors

Teeth are attached to the alveolar bone by the periodontal ligament. The periodontal ligament contains periodontal mechanoreceptors which respond to the forces on the teeth and provide information about the direction in which the forces are applied. The presence of these receptors make human teeth sensitive to very small forces applied to them (Jacobs and Vansteenberghe, 1994). Periodontal receptors are mostly slowly adapting afferents that encode information on the orientation, magnitude, rate and position of loads applied to the teeth. When chewing a food, a force is applied to the tooth which moves in its socket which induces stresses and strains on the periodontal ligament. It has been shown that chewing movements change during the mastication sequence (Wang and Stohler, 1991). It is interesting to note that individuals with dentures often chew with the same masticatory pattern throughout the masticatory sequence (Haraldson, 1983). A study using a rodent model suggests that stimulation of periodontal ligaments is involved in the satiation response and may contribute to body weight regulation (Sakata *et al*., 2003).

3.6.4 Central Processing of Texture

Accumulating evidence indicates that the orbitofrontal cortex (OFC) contains neurons that are responsive to food texture (Rolls, 2011; Grabenhorst and Rolls, 2014). In one study of the macaque OFC it was found that oral viscosity stimuli in the range of 1–10,000 centipoise elicits a response in a subset of

neurons (Rolls *et al.*, 2003). Some of the neurons had increasing responses to increasing viscosity, some had decreasing responses, and some neurons were tuned to a range of viscosities.

3.7 Convergence of Taste, Smell and Texture to Produce Flavor

Flavor is the sensory impression of a food and is determined mainly by the chemical senses of taste and smell with further contributions from chemesthesis, temperature and texture. At some stage, it would appear likely that information from these different modalities converge to produce flavor. Rolls and Bayless (Baylis and Rolls, 1991) found that in the orbitofrontal cortex taste areas there were 112 single neurons which responded to taste, olfaction or visual inputs. Many of these neurons were unimodal (taste 34%, olfactory 13%, visual 21% although some single neurons showed convergence, responding for example to taste and visual inputs (13%), taste and olfactory inputs (13%) and olfactory and visual inputs (5%). Consequently, the authors suggested it is the OFC where gustatory and olfactory inputs converge to produce flavor. It is interesting to note that the response of these neurons decline due to eating a food to satiety (Critchley and Rolls, 1996) and this may contribute to the development of sensory specific satiety (Rolls *et al.*, 1981).

3.8 Concluding Remarks

The oro-sensory perception of foods involves a complex interaction between several different sensory modalities. While many advances have been made in understanding the oro-sensory perception of foods there are still many significant unknowns that may hinder the development of good *in vitro* models of the oro-sensory perception of foods. Moreover, it must be acknowledged that the breakdown of foods in the oral cavity is an extremely complex process that will have a marked influence on food perception. For instance, masticatory

efficiency may alter the rate at which odorants and tastants are released from the food matrix altering the flavor profile of the food. The amount of saliva produced and its composition may alter the taste and textural perception of the food. In addition, the breakdown of foods is a dynamic process meaning the oro-sensory perception of a food will change through a meal. This means that while instrumental measures may correlate with aspects of oro-sensory perception of foods they may not capture the full experience. For instance, as a food particle size is reduced by mastication the textural perception will change due to a reduction in particle size and also due to mixing with saliva. If a food does not behave as expected through the total oral phase of ingestion it is likely that the food will have poor acceptability. Moreover, as a meal progresses, the breakdown of the food is likely to change due to changes in masticatory parameters while the hedonic value of the food will change due to the development of sensory-specific satiety.

This chapter described the physiological basis for the oro-sensory perception of foods. It is clear that these processes remain poorly understood and are enormously complex. Developing useful *in-vitro* models that adequately represent these processes will be an extremely challenging endeavor.

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4

In vitro Models of Host–Microbial Interactions Within the Gastrointestinal Tract

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4.1 Introduction: The Human Gastrointestinal Tract

The human gastrointestinal tract (GIT) refers to the anatomical features and organs that are linked sequentially and participate in the digestion and absorption of nutrients and water. The adult GIT is approximately 1.5 m in length and supports a volume of 500 mL, and often contains 200 g of solid content (Macfarlane and McBain, 1999). Beginning with the oral cavity and esophagus, the GIT progresses through the stomach and into the small and large intestine, ending at the rectum. The term "gut" is often used colloquially to describe the entire GIT, and particularly the colon proper. Food moves through the colon more slowly compared to the upper GIT, which is aided by peristaltic movements (see Chapter 2). Colonic digesta consists of exogenous dietary molecules in addition to microbial cells which are estimated to contribute to 50% of the total mass (Macfarlane and McBain, 1999). Therefore, microbial metabolic processes that occur within the gut contribute to host homeostasis in a significant fashion. On the

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average, the nutrient content that reaches the colon from the small intestine is about 20–60 g/day carbohydrates (i.e. polysaccharides) and 5–20 g protein (Power *et al.*, 2013).

The human colon is segregated into three primary anatomical sites, termed the proximal, transverse, and distal colon. In the proximal region, saccharolytic fermentation proceeds due to high concentrations of non-digestible carbohydrates that colonizing microbiota utilize. As a result, short chain fatty acids (SCFA) are secreted by microbes which decrease the local pH to 5–6 and promote rapid bacterial growth (Payne *et al.*, 2012). As the digesta transits to the transverse colon, fermentable carbohydrates decrease to coincide with less microbial acid production to promote an environment that approaches neutral pH (Olano-Martin *et al.*, 2000). In the distal colon, microbial proteolysis rapidly results in the production of ammonia, amines, phenols, thiols, and indoles in addition to other metabolites including SCFAs (Macfarlane *et al.*, 1992; Macfarlane and Macfarlane, 1995). As available substrates change across the length of the colon, the microbial communities and their inherent metabolic function are somewhat distinct in these environments (Macfarlane *et al.*, 1992; Macfarlane *et al.*, 1998).

The human gut is colonized by bacteria and archaea in quantities of up to 10^{12} cells per gram (Payne *et al.*, 2012; Power *et al.*, 2013; Ventura *et al.*, 2009). The development or microbial succession within the mammalian gut shares an evolutionary relationship with milk and soluble factors delivered within (Sela, 2011). In newborn infants that are breastfed, bifidobacterial populations often dominate the microbiome due their ability to utilize human milk oligosaccharides (Marcobal, 2013; Sela *et al.*, 2008; Turroni *et al.*, 2012). Post-weaning, gut microbial community diversity is influenced by their host's nutrition and genotype (Fan *et al.*, 2013; Guaraldi and Salvatori, 2012; Li *et al.*, 2012; Ventura *et al.*, 2009). This is in addition to environmental factors such as antibiotic and other exposures. The impact of diet on guiding the microbial community has been studied in comparing children from Europe and Burkina Faso (De Filippo *et al.*, 2010) as well as other human and animal studies (Claesson *et al.*, 2012; Kabeerdoss *et al.*, 2012; Kang *et al.*, 2014; Wu *et al.*, 2011, 2015; Zimmer *et al.*, 2012).

The human gut may host up to 1200 microbial species within the microbiome (Claesson *et al.*, 2009; Rajilic-Stojanovic *et al.*, 2007), with several bacterial phyla that are consistently represented including Actinobacteria, Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Verrucomicrobia. Of these, Bacteroidetes and Firmicutes are typically dominant and comprise up to 90% of the GIT microbiota (Dethlefsen *et al.*, 2006, 2007; Eckburg *et al.*, 2005; Tap *et al.*, 2009). The diversity within microbial communities is influenced by host diet and genotype among other host–microbial interactions and microbe–microbe interactions (Dethlefsen *et al.*, 2006; Turnbaugh *et al.*, 2009; Wu *et al.*, 2011). Microbial species that colonize the mature human gut are facultative anaerobes or strict anaerobes (Finegold *et al.*, 1974). In addition to maintaining an anaerobic environment, studying GIT microbial ecosystems often requires culture-dependent approaches.

Accordingly, some bacteria require complex cultivation requirements that are not well understood or are impossible to recapitulate in the laboratory. For example, some bacteria require the metabolic activities of heterologous species or hosts to provide essential molecules (Macfarlane and Macfarlane, 2004). Since culturing-based approaches require optimal viability within the growth media of choice, it often underestimates bacterial diversity or biases the representation. This is often referred to as the "great plate count anomaly" (Staley and Konopka, 1985). In response, culture-independent approaches are often deployed to determine the phylogenetic diversity present in the gut from fecal community extracts (Eckburg *et al.*, 2005; Franks *et al.*, 1998; Rajilić-Stojanović *et al.*, 2009; Wang *et al.*, 2003). This includes massively parallel sequencing of the small subunit ribosomal RNA (16s) gene to enable the identification of uncultivable species. Accordingly, the community composition could be characterized in previously unprecedented resolution (Blottière *et al.*, 2013; Kurokawa *et al.*, 2007). Shotgun metagenomics of total community DNA allows the classification of the genetic functional capacity of the microbiome. This is coupled with other system-level approaches such as metatranscriptomics to infer the gene expression profile of the community, as well as metabolomics to characterize metabolite production within the gut (Jansson *et al.*, 2009; Lozupone *et al.*,

2012; Van Baarlen *et al*., 2013; Verberkmoes *et al*., 2009). These methods have been used in large consortium-level collaborations such as the European Metagenomics of Human Intestinal Tract (MetaHIT) and the US Human Microbiome Project (HMP) (Qin *et al*., 2010; The Human Microbiome Project Consortium, 2012; Turnbaugh *et al*., 2007). Regardless of taxonomic diversity within one's microbiome, the core functions of their community remains similar in healthy subjects (Lozupone *et al*., 2012; Tap *et al*., 2009). This functional redundancy is a consistent theme that has emerged in the study of the human microbiome.

The gut microbiota extract energy and nutritive molecules from dietary components that are otherwise impervious to host digestion. This leads to the production of metabolites such as SCFAs, vitamins and amino acids to be utilized in host metabolism. Microbial metabolic activity is influenced by both endogenous factors (e.g. secreted mucins, bile, enzymes, immune cells) and exogenous factors (e.g. diet, xenobiotics, environmental exposures). Specific carbohydrates that are encountered by the gut microbiota include resistant starch, non-starch polysaccharides, milk oligosaccharides, plant cell wall oligosaccharides, non-digestible oligosaccharides (fructo-oligosaccharides (FOS), galactooligosaccharides (GOS) and inulin) (Adamberg *et al*., 2014; Duncan *et al*., 2003; Garrido *et al*., 2015; Gonzalez *et al*., 2008; Han *et al*., 2014; Martens *et al*., 2011; Rossi *et al*., 2005; Shen *et al*., 2011; Stiverson *et al*., 2014; Ze *et al*., 2012). In addition to carbohydrates, proteins and peptides released during digestion could also interact with microbial communities established along the length of the GIT (Davila *et al*., 2013; Richardson *et al*., 2013; Walker *et al*., 2005). Phenolic-based compounds participate in reciprocal interactions with the microbiota, as they are transformed by the community metabolism (Ozdal *et al*., 2016). The interactions of phenolics with the gut microbiota may dictate the function of these dietary molecules as well as their bioavailability. Often these phenolic compounds are transformed *in vivo* and may modulate the gut microbiota to promote health benefits for the host (Crozier *et al*., 2009; D'Archivio *et al*., 2007; Etxeberria *et al*., 2013; Hervert-Hernández and Goñi, 2011; Kemperman *et al*., 2010; Landete, 2012). Several studies have been performed to elucidate the

complex interactions between dietary polyphenolics and gut microbiota (Manach *et al*., 2005; Stoupi *et al*., 2010; Tzounis *et al*., 2011; Van der Hooft *et al*., 2012; Van Dorsten *et al*., 2012). In order to utilize these dietary molecules, microbes have evolved an assortment of enzyme systems to degrade molecules both extracellularly and within intracellular compartments following transport (Andreasen *et al*., 2001; Flint *et al*., 2008, 2012; Turroni *et al*., 2012).

Microbial metabolism in the gut is not restricted to linear pathways, or bound by the cellular envelope. Microbes compete for substrates, with metabolites secreted by one member often utilized by a secondary taxon (Belenguer *et al*., 2006). Syntrophic metabolism is extended towards interactions with their host. Among other activities, microbial SCFAs (i.e. acetate, propionate, butyrate, formate) provide an energy source for specific host tissues including butyrate utilization by gut enterocytes (Canani *et al*., 2011; den Besten *et al*., 2013). In addition, SCFAs interact with the immune cells that further regulate the microbiome (Huda-Faujan *et al*., 2010; Puertollano *et al*., 2014). Along these lines, dietary phenolic derivatives may have local or systemic anti-inflammatory activity in the host (Crouvezier *et al*., 2001; Russell *et al*., 2007; Sang *et al*., 2004). Moreover, proteolytic catabolism may lead to the formation of toxic compounds such as ammonia and amines, both of which have been linked to colon cancer (Hughes *et al*., 2000).

Since the gut microbiome is composed of both commensal and potentially opportunistic pathogens, analysis of microbial ecosystems in aggregate is required to test scientific hypotheses to ultimately be applied in maintaining a healthy gut state. Thus *in vitro* systems to model complex community interactions have been innovated to study associated scientific questions.

4.2 The Current State of *In vitro* Model Systems to Model Gut Ecosystems

In vitro systems enable the investigation of microbial communities and their respective functions by modeling microbial ecology and the interface with host physiology. This could include single or multiple bioreactors that are seeded by fecal

inocula or defined communities (e.g. 8 bacterial species) (Payne *et al.*, 2012). Parameters are manipulated to reflect GIT physiology and include temperature, pH, oxygen tension, and substrates introduced to the system at defined intervals and flow rates. These systems vary by experimental approach and differ by inoculation, transit/resident time, and the specific bioreactors.

In vitro models are preferred in many instances, as *in vivo* studies are expensive and require special facilities to maintain animals or interact with human subjects in an ethical manner. For the latter, access to the internal anatomy of human subjects is prohibitively restricted, thus most studies focus on analyzing fecal excreta (Payne *et al.*, 2012). This is a limitation for investigations of other GIT sites beyond the distal colon. Moreover, nutritional interventions require a controlled diet over extended time intervals, and sufficient participants who must comply with inconvenient study parameters. In contrast, *in vitro* studies are not restricted by similar ethical constraints, are often cost effective, and enable mechanistic studies. A strong argument for their use is to allow pre-clinical hypothesis testing prior to *in vivo* trials with human and animal subjects.

Other advantages for *in vitro* models include facile sampling across time with strict control of variables. Some models allow parallel experiments to account for inter-community variation while measuring several outcomes simultaneously. The accumulation of microbial metabolites allows for cross-sectional and time course studies and access to molecular markers that would otherwise be rapidly sequestered *in vivo*.

There are, however, limitations to *in vitro* experimental systems in comparison to *in vivo* approaches. Fecal inocula does not fully model the spatial complexity of microbial ecosystems along the GIT (Auchtung *et al.*, 2015). Moreover, reproducibility and stability is a challenge to establishing an informative *in vitro* model. The priority lies in managing the inoculation and colonization efficiency of fecal microbiota, both qualitatively and quantitatively.

The reproducibility and stability of modeled microbiomes has been evaluated using various approaches (Auchtung *et al.*, 2015; Child *et al.*, 2006; Macfarlane *et al.*, 2005; Minekus *et al.*, 1999; Possemiers *et al.*, 2004; Probert and Gibson, 2004; Rajilić-Stojanović *et al.*, 2010; Van de Wiele *et al.*, 2004; Venema *et al.*,

2000, 2003). The HITChip microarray analysis and next-generation sequencing (NGS) have been employed to test the stability of *in vitro* microbiomes (Auchtung *et al*., 2015; Rajilić-Stojanović *et al*., 2010). These approaches enable characterization of microbial succession with great precision. As sequencing technologies improve, taxonomic discrimination is enhanced with NGS approaches as NGS approaches rapidly become the tool of choice (Auchtung *et al*., 2015).

Experimental reproducibility is a concern often cited with these approaches. There is evidence that the initial fecal inocula must be fresh to reduce errors in composition due to freeze-induced bias (Bonten *et al*., 1997). Another approach is to pool fecal specimens from multiple donors to approximate a stable microbial community, albeit theoretical or otherwise ideal (Auchtung *et al*., 2015). This approach may inadvertently lead to artifacts resulting from inter-microbial interactions that do not occur *in vivo* (Payne *et al*., 2012). Inoculation with recently acquired fecal samples is generally preferred for batch culture systems used in short-term studies (Macfarlane and Macfarlane, 2007).

4.3 Batch Culture Systems to Model the Gut Microbial Consortium

Batch culture typically involves small reactor vessels (i.e. 70–1000 ml) inoculated with either axenic cultures or a mixed community that is allowed to grow in a medium for a short period of time (e.g. 72 hours) with regular sampling (Macfarlane and Macfarlane, 2007; Payne *et al*., 2012). These are either closed or fed-batch bioreactor systems that are maintained under anaerobic conditions by sparging with oxygen-free gas (usually nitrogen). Closed systems are used to model a single component of the human gut, such as the distal colon, but other sites have been developed as well (Macfarlane and Macfarlane, 2007; Payne *et al*., 2012). A common use of batch models is to measure the response to a given treatment or perturbation to dominant populations maintained within the simple system (Macfarlane and Macfarlane, 2007; Payne *et al*., 2012; Williams *et al*., 2014). Although this may not represent the full functional potential of the community, it permits investigation of simplified

mechanistic relationships. In general, batch systems seeded with fecal inocula perform well for initial characterizations of inter-individual variation in community metabolism (Gross *et al*., 2010). These systems are tractable for many research groups, cost-effective, and operate well when large amounts of substrates and seed microorganisms are available (Macfarlane and Macfarlane, 2007; Payne *et al*., 2012). Batch models are typically stirred continuously to promote homogeneity, sparged with nitrogen to reduce oxygen tension, with pH and temperature controlled throughout the experimental cycle.

Batch culture systems to represent the GIT are made up small volume of reactor vessels in order to represent the typical volumetric space of GIT. It also points out a scientific shift from commonly used fermentation experiments to *in vitro* gut model systems. Additionally, it is important to validate the usage of model systems.

Batch culture systems have been developed to investigate carbohydrate catabolism and the interactions of modeled GIT communities with other exogenous factors (Oufir *et al*., 2000; Pompei *et al*., 2008). This includes both prebiotics and probiotics that are believed to influence host physiology by influencing endogenous microbiota (Barry *et al*., 1995; Gietl *et al*., 2012; Likotrafiti *et al*., 2014; Macfarlane *et al*., 1988; Wang and G.R. Gibson, 1993). In 1992, Macfarlane and colleagues investigated the fermentation within different regions of the GIT using contents harvested from sudden death victims (Macfarlane *et al*., 1992). *In vitro* fermentations were conducted in 70 ml capacity serum bottles gassed with argon and incubated at 37°C anaerobically in an orbital shaker using 10 % (w/v) fecal slurries. Interestingly, the secretion of fermentative end products correlated with variations in the donors. In general, SCFAs, lactate, and ethanol concentrations were higher in the ascending colon whereas protein fermentation products such as ammonia and phenolic compounds increased in the descending colon. In 1993, Wang and Gibson (Wang and Gibson, 1993) investigated oligofructose and inulin utilization in an *in vitro* fermentation by *Bifidobacterium* and *Clostridium* spp. in a 280 ml volume fermentation vessels which were run for 48 h at 37°C and pH of 7.0. They were sparged with oxygen-free nitrogen and continuously stirred using 5% fecal slurries collected from 6 individuals.

These were prepared freshly by homogenizing in anaerobic phosphate buffer. Thus it was determined that oligofructose and inulin are utilized by intestinal commensals as SCFA coincides with the increase in specific beneficial populations and reduces *Escherichia coli* and *Clostridium perfringens*. This Gibson model has been replicated in several additional studies to be discussed in subsequent sections.

Phenolic biotransformation within the human gut has been modeled in batch culture systems. Microbial metabolism of anthocyanins and quercetin were conducted either in head-space bottles or large-scale fermenters under controlled temperature, pH, and oxygen concentration (Aura *et al*., 2002, 2005). In 2008, Tzounis and colleagues (Tzounis *et al*., 2008) developed a batch culture model similar to the Gibson system with 300 mL vessels under expected conditions within the gut. Afterward, this model was used in several studies of flavanol and anthocyanin metabolism transformed by colonic microbiota and assessed by metabolite and microbial profiling using liquid chromatography (HPLC) and fluorescent in situ hybridization (FISH), respectively (Cueva *et al*., 2013; Hidalgo *et al*., 2012; Sánchez-Patán *et al*., 2012). Flavanol and anthocyanin phenolics are degraded by gut microbiota, with products increasing the populations of *Bifidobacterium* and *Eubacterium* reflecting the potential enriching activity of precursor phenolics. Several static batch colonic fermentation models were developed to investigate flavonol and phenolic acid degradative pathways (Appeldoorn *et al*., 2009; Gross *et al*., 2010; Serra *et al*., 2011, 2012).

Two batch systems were prepared with phosphate-buffered saline (PBS, oligotrophic) and basal culture medium (BCM, eutrophic) and seeded with fecal inoculum to characterize the response of the modeled community to a prebiotic formulation of GOS and guar gum (Long *et al*., 2015). As a result, significant shifts within specific populations receiving treatment were observed in the pyrosequencing community analysis.

Despite frequent use of batch culture fermentation systems, models have limitations related to over-simplification of the ecosystem being modeled in the absence of the host physiology. Moreover, accumulation of toxic compounds and substrate diminishment may restrict microbial activity as the system will not achieve steady-state equilibrium (Macfarlane *et al*., 1992). A simple *in vitro* batch culture fermentation study conducted in

multiple labs illustrates limitations of the batch culture systems (Barry *et al.*, 1995). Accordingly, dietary fiber was fermented within several laboratories with outcomes varying in terms of the rate of substrate degradation as well as the amount of SCFAs produced. It was concluded that the most significant potential source of variation is the fecal inoculum in *in vitro* models. Thus batch systems are typically appropriate for short-term resident times with continuous, semi-continuous, and dynamic model systems used in more complex models.

4.4 Continuous Systems to Model the Human GIT

Continuous model systems contain multiple chemostats used in long-term studies with constant provision of substrates and removal of toxic compounds under defined parameters. In general, optimal conditions for microbial growth may be achieved at steady-state conditions unless experimental goals dictate otherwise. Chemostat conditions reflect distinct parts of the human GIT and often consist of the proximal, transverse, and distal colon (Payne *et al.*, 2012). Environmental conditions are simulated including substrate retention time and may involve modeling peristaltic movements. Continuous model systems are appropriate for studies that require longer response measurements to a given treatment or perturbation. Important considerations in designing these experimental schemes include maintaining physiological concentrations of metabolites and relative concentrations of microbial populations to reflect fermentation rates of substrates and interactions between microbes (Minekus *et al.*, 1999). Often *in vitro* models are constrained by maintaining stable communities of microbes to model *in vivo* states. Stabilization requires a period of adaptation to the simulated environment that involves microbial interactions within this unique environment. This is often not considered in batch culture models due to shorter resident times. However, in continuous models the adaptation period must be addressed. The validation of the stability of these systems was tested using culture-independent techniques (Possemiers *et al.*, 2004; Rajilić-Stojanović *et al.*, 2010).

An early semi-continuous system was developed by Miller and Wolin in a glass vessel to maintain 500 ml of stirred anaerobic culture over 81 days (Miller and Wolin, 1981). Fermentation was initiated with 156 g of freshly collected feces diluted in 200 ml of solution containing sodium bicarbonate, urea, hemin, casein and a vitamin mixture. The microbial community was left to stabilize for 18 days before a suspension of dietary material was added derived from various fruits and vegetables. Sodium deoxycholate represented bile salts and mixed with nutrient suspensions to be pumped into the fermentation vessels once or twice daily. In the same year, Veillux and Rowland developed a 2-stage continuous reactor to simulate the distal part of the rat intestine (Veilleux and Rowland, 1981). Two-stage cultures were connected in series with sterile media added to the first stage. The continuous system was run in a volume of 600 ml in an agitated, temperature, and pH controlled fermenter and continuously flushed at a rate of 0.5 l/min. The medium reservoir was held under anaerobic conditions and agitated to ensure a homogenous distribution of the components and pumped continuously. Fecal suspensions were homogenized shortly after acquisition and allowed to stabilize for 12 h before culture medium addition. These model systems were developed based on a number of assumptions. The first is that most microbial growth and metabolism occurs immediately after ileal fluid entry into the colon and prior to water removal that concentrates substrates. Another assumption is the use of fecal communities is representative of the colonic microbial community. Furthermore, the high concentration of insoluble carbohydrates in dietary substrates in the study is predicted to evade digestion in the stomach and small intestine. These assumptions are necessary in order to simplify the system in these *in vitro* systems. Regardless, the metabolite and microbiological profile was found to be consistent between the model and *in vivo* systems.

A three stage continuous system was developed by the Gibson group to investigate the influence of mucin on sulfate reduction and methanogenesis by mixed populations of human gut microbiota (Gibson *et al*., 1988). This three-stage continuous culture system was inoculated with 20% (w/v) of feces in an anaerobic sodium buffer and maintained under controlled conditions for 120 days. A mixture of polysaccharides and proteins were added

as carbon and nitrogen sources respectively. The vessels' operating volumes (0.3, 0.5, and 0.8 liter) and pH (6.0, 6.5 and 7.0) reflected the physiological characteristics of the colon. Porcine gastric mucin was added to the first vessel after 48 days, with microbial degradation allowed to proceed for 22 days until the mucin was replaced by distilled water. As a result, the mucin was extensively hydrolyzed which stimulated volatile fatty acid production. Mucin participated in sulfate-reduction, however methanogenesis was the major route for the disposal of electrons in the absence of mucin. Of primary interest, this study modeled competition between sulfate-reducers bacteria and methanogens over a limited nutrient source.

In another approach, a three-stage continuous system was developed to test the effects of retention time of various organic carbon and nitrogen sources on the community ecology and metabolism of gut microbes (Macfarlane *et al.*, 1998). This system consisted of three vessels simulating the ascending, transverse, and distal colon by judiciously dictating pH, temperature, operating volumes, and retention time. Since fecal extracts do not reflect the community structure in the proximal colon, the researchers used content harvested from four sudden death victims. Operating volumes of the three vessels were set as 0.22, 0.32 and 0.32 L and pH was maintained at 5.5, 6.2, and 6.8 under automated control. The environmental conditions sought to recapitulate the spatial, temporal, nutritional, and physicochemical characteristics encountered by the gut microbiota. The first vessel (V1) was fed with culture media containing polysaccharides, proteins, vitamins, and salts under anaerobic conditions. The three stages were continuously linked and the vessels were inoculated with 100 ml of 20% (w/v) fecal slurry. The system was equilibrated overnight and allowed to achieve steady state after at least 336 h assessed by monitoring SCFA production. The retention time for each vessel was determined as the reciprocal of dilution rate and evaluated by comparing the chemical and biological characteristics of the fecal suspensions from donors.

Further advances to the development of the 3-stage *in vitro* model were made to investigate fermentation of dextran, oligodextran, and maltodextrin by human gut microbiota (Olano-Martin *et al.*, 2000). In this approach, a cascade of three

fermenters inoculated with 10% (w/v) freshly collected and homogenized fecal slurry were linked in series, with operating volumes of 250, 250 and 300 ml, and pH values of 5.5, 6.2 and 7.1 under continuous stirring and anaerobic conditions. Vessel 1 simulated the acidic conditions and rapid bacterial growth in response to high substrate availability that are experienced in the proximal colon. In contrast, vessel 3 was maintained at a neutral pH and with limited substrates to model conditions in the distal colon. In total the retention time was 60 hours which was selected according to previous three stage continuous models validated for retention time (Macfarlane *et al*., 1998). Oligodextran IV was shown to enrich for bifidobacteria and lactobacilli with steady-state populations achieving higher densities in all three vessels. However, substrate utilization varied among the three stages of the colonic model. Whereas dextran is a suitable growth substrate for bifidobacteria and lactobacilli in the proximal colon (Vessel 1), maltodextrin was used as the carbohydrate source for both bacterial groups in Vessel 3. Recently, a similar system was used to model the effects of the antibiotic rifaximin on the colonic microbiota of patients with Crohn's disease (MacCaferri *et al*., 2010). Interestingly, rifaximin promoted a relative abundance of *Bifidobacterium*, *Atopobium*, and *Faecalibacterium prausnitzi* that was confirmed using fluorescent *in situ* hybridization (FISH), quantitative PCR, and other culture-independent methods. The coinciding shift in microbial metabolism was observed with ^{1}H-NMR and solid phase microextraction coupled with gas chromatography/mass spectrometry. Accordingly, SCFAs, propanol, decanol, nonanone, and aromatic organic compounds were increased, and ethanol, methanol and glutamate were decreased. In addition, wheat dextrin soluble fiber influenced the composition and metabolic activity of the gut microbiota in this model as measured by 16S rRNA-based FISH and gas chromatography (Hobden *et al*., 2013). Wheat dextrin fermentation significantly increased production of acetate (Vessels 2 and 3) and propionate (Vessel 3), coinciding with a significant increase in key butyrate-producing bacteria assigned to clostridial cluster XIVa and the genus *Roseburia*.

Another three stage continuous model was developed and termed GIS2 (gastrointestinal simulation) to study the effects of

a probiotic product (i.e. PROEXO) on the modeled infant gut microbiota (Vamanu *et al*., 2014). This three-stage system consists of three Duran bottles (1,000 ml capacity) and maintained at pH values of 5.5, 6.2 and 6.3. Culture media and the substrate which functions as both a probiotic and prebiotic were introduced to the first vessel to be pumped to Vessels 2 and 3. The model was seeded by fecal samples obtained from healthy infants of 6 months, and stabilized after an initial period of 10 days assessed by culture-dependent quantification. In this system, polysaccharides present in the tested product enriched the lactic acid bacteria in the modeled ascending colon.

A four stage, semi-continuous colonic model system (i.e. EnteroMix human colon simulator) was developed to model the effects of lactose on the growth and fermentation dynamics of colonic microbiota (Mäkivuokko *et al*., 2006). This system contains four glass vessels connected in sequence, with conditions adjusted to represent each compartment of the human colon under anaerobic conditions. pH values were maintained at 5.5, 6.0, 6.5 and 7.0 from the ascending colon towards the distal colon. The system was run in parallel with six treatment and three baseline simulations using the same fecal inoculum preconditioned at 37°C for 24 h, anaerobically. The microbial density was adjusted from vessel 1 to vessel 4. Lactose was initially dissolved in sterile simulator medium, which mimics the contents of the human ileum and was semi continuously fed to the first vessel. Microbial populations and metabolite (SCFA) profiling were monitored by flow cytometry and HPLC. In a subsequent study, the effects of polydextrose and xylitol were investigated in the EnteroMix four-stage colon model (Mäkeläinen *et al*., 2007). While polydextrose degradation leads to higher concentrations of all SCFA, especially acetate and propionate, xylitol degradation increased the concentration of butyrate in the medium. This was attributed to population shifts of *Bifidobacterium* and *Lactobacillus*. In addition, this model was used to study the effects of xylo-oligosaccharides on bifidobacteria in the human colon (Mäkeläinen *et al*., 2010). Interestingly, xylo-oligosaccharides were preferred by *Bifidobacterium lactis* strains and lactitol by lactobacilli in this four stage *in vitro* model, whereas galacto-oligosaccharides, fructo-oligosaccharides and gentiobiose were preferred by a larger group of microbes.

Molly and colleagues developed a novel model that was termed the simulated human intestinal microbial system (SHIME) (Molly *et al*., 1993). This is a five-stage bioreactor system consisting of a large intestine phase that encompasses three bioreactors, and is fed by a small intestine reactor 1 and 2 (duodenum/jejunum and ileum) simulated by a two-step fill and draw system. This system was based on the presence of indicator bacterial groups, volatile fatty acids, enzymatic activities (e.g. α- galactosidase β-galactosidase, α-glucosidase, β-glucosidase, β-xylosidase), and headspace gases. The first substrates tested in the SHIME system included dietary carbohydrates such as arabinogalactan, xylan, pectin, dextrin and starch. In addition, SHIME has been deployed to model microbial activity in the GIT in separate operations (Molly *et al*., 1994). Each reactor vessel was designed with eight ports for medium transfer, sampling of the liquid phase and headspace gases, pH-electrode, pH-control (acid and base) and for flushing of the headspace. Pumps were used to transfer the reactor contents between the successive vessels. Media containing polysaccharides were introduced to the first vessel semi-continuously (3 times a day) and two hours after first feeding, pancreatic juice supplemented with bile (pancreas acetone powder dissolved in NaHCO) was added to reactor vessel 1 to simulate the conditions in dueodenum/jejunum. In order to model the GIT, stool specimens (10 % w/v) from eight healthy individuals were fed into reactor vessels 3-4-5 (cecum and ascending colon, transverse colon, descending colon) (Molly *et al*., 1994). Validation of the SHIME system was performed by analyzing a number of microbial activities, degradation of four polysaccharides and direct enumeration of bacteria by plate count (Molly *et al*., 1993, 1994). A validation of a model system is generally performed on the available data in literature by comparing the outcomes with *in vivo* clinical trials. Additionally, validation also requires the reproducibility, stability and consistency within the same system. Not much sufficient information is available in the literature for every model system validation that is used *in vitro* experiments. From a scientific perspective, it was assumed that the validation of a system had done before the related outcomes for the treatment were established.

SHIME has been employed in several applications since its initial development. In 1999, Alander and colleagues tested six

stages of SHIME for screening and selection of five probiotic strains (*Lactobacillus plantarum*, two strains of *Lactobacillus paracasei* subsp. *paracasei*, *Lactobacillus rhamnosus* and *Bifidobacterium* sp.) (Alander *et al*., 1999). In this study, they developed a SHIME reactor consisting of five closed vessels in series to which a sixth reactor was added (simulating the stomach). Stabilization of probiotic strains required a 7-day pretreatment period, followed by a 7-day inoculation period during which the supplement was added daily. A 2-week post-treatment washout period was included. The impact of the strains on the composition of modeled gut microbiota and its metabolic activities (production of lactic acid and SCFA) were measured by plate count enumeration and gas chromatography coupled with flame ionization detector. Accordingly, lactic acid increased in the SHIME probiotics were added as well as the relative increase in lactobacilli and bifidobacteria. Interestingly, the SCFA profile remained somewhat static. Members of Enterobacteriaceae and Clostridia decreased markedly during the intervention, while Enterococci tended to increase post-treatment. This suggests that the positive effects attributed to probiotic treatment are reversible.

The effects of a synbiotic mixture of *Lactobacillus acidophilus* and fructo-oligosaccharides (FOS) were also tested in a six stage version of SHIME (Gmeiner *et al*., 2000). Alterations to the microbial community structure and their metabolic activity were monitored by culture-dependent methods and assayed for β-galactosidase and β-glucuronidase activity. These experimental conditions were similar to those performed by Alander and colleagues including pretreatment (10 days), treatment (addition of probiotic strains and FOS; 17 days) and post-treatment (10 days) (Alander *et al*., 1999). To avoid the deleterious effects of stomach acid on the integrity of the synbiotics, the treatment feeding started from the second set of vessels. The addition of the synbiotic product increased the bifidobacterial population and production of butyric and propionic acid.

A synbiotic mixture of *Lactobacillus* GG and fermented oat bran was introduced to a modeled gut microbiome in a six-stage SHIME system (Kontula *et al*., 1998). This system diverges from the canonical SHIME model (Gmeiner *et al*., 2000) as the model stomach and small intestine vessels were fed semi-continuously.

Accordingly, pre-feeding occurred for 8–10 days, feeding totaled 12 days, and post-feeding lasted for 19 days. Lactic acid bacteria, bifidobacteria, enterobacteria and clostridia were enumerated by culture-dependent methods. It was concluded that *Lactobacillus* GG may colonize in the human gut for several weeks with the fermented oat bran enriching bifidobacteria and the production of several desirable metabolites. However, one limitation of the SHIME model is that the lack of absorption led to an overestimation of digesta constituents that reached the colon (Gmeiner *et al*., 2000; Kontula *et al*., 1998).

Possemiers and colleagues (Possemiers *et al*., 2004) employed 5-stage SHIME (stomach, small intestine, and three colonic phases) to produce a stable bacterial community for 27 days. The microbiome members were quantified by enumeration of total aerobes and anaerobes, total coliforms, and other targeted groups by culture-dependent methods and PCR-DGGE. SCFAs and ammonium in the reaction vessels were monitored by gas chromatography coupled with flame ionization and as a released of ammonia to determine the metabolic activity of the microbial community. Microbiome stability was achieved after two weeks, while metabolic activity required three weeks to achieve steady-state.

In another application of the five-stage SHIME system, the prebiotic potential of inulin that varied by degrees of polymerization was evaluated (Van de Wiele *et al*., 2004; Van De Wiele *et al*., 2007). Moreover, the SHIME system was used to screen for prebiotic sources from soy germ and to model potential alterations in the microbial ecology of the human colon (De Boever *et al*., 2000; Decroos *et al*., 2006). In 2009, the prebiotic potential of arabinoxylan oligosaccharides and inulin was evaluated in a TWIN-SHIME system which is two systems run in parallel to compare treatments simultaneously (Grootaert *et al*., 2009). This enables real time inline monitoring of oligosaccharide hydrolysis and the production of marker metabolites in parallel. Microbial colonization was developed in TWIN-SHIME that was simultaneously inoculated with the same human fecal seed. The microbial diversity was assessed with a high-resolution phylogenetic microarray: the human intestinal tract chip (HITChip) (Van den Abbeele *et al*., 2010). This model was found to be reproducible in SHIME units and resulted in highly diverse

microbial communities that were colon region specific, with the proximal regions harboring saccharolytic microbes (e.g., *Bacteroides* spp. and *Eubacterium* spp.) and the distal regions harboring mucin-degrading microbes (e.g., *Akkermansia* spp.). Subsequently, the TWIN-SHIME system was used to evaluate the microbial metabolism of red wine and black tea extracts (Van Dorsten *et al*., 2012).

In another effort to model an interaction between food constituents and gut microbiota, the TWIN-SHIME system was used in conjunction with key dietary polyphenols (Kemperman *et al*., 2013). Ingestion of polyphenols might influence health through absorption, as well as interactions with resident gut microbiota. Black tea and a red wine grape extract (RWGE) both contain complex dietary polyphenols and influence the microbiome structure and function. This was measured by both culture-independent and culture-dependent methods and gas chromatography. Pyrosequencing of community phylogenetic markers indicated that the Firmicutes:Bacteroidetes ratio was altered in the presence of polyphenolic extracts. Moreover, black tea stimulated *Klebsiella*, enterococci and *Akkermansia* and reduced bifidobacteria, *B. coccoides*, *Anaeroglobus* and *Victivallis*. Likewise, RWGE promoted growth of *Klebsiella, Alistipes, Cloacibacillus, Victivallis* and *Akkermansia* while bifidobacteria, *B. coccoides, Anaeroglobus, Subdoligranulum* and *Bacteroides* were decreased. In addition, the effects of cranberry and grape seed polyphenols were tested on a similarly modeled microbial community in SHIME (Sánchez-Patán *et al*., 2015). Finally, a single strain of *Lactobacillus plantarum* IFPL935 was evaluated for their ability to metabolize a polyphenolic red wine extract by monitoring phenolic derivatives using UPLC-ESI-MS/MS and community changes using qRT-PCR (Barroso *et al*., 2013).

Currently, next-generation sequencing (NGS) beyond pyrosequencing is commonly used to determine to measure phylogenetic diversity of modeled gut microbiota. These techniques have been particularly valuable in prebiotic studies (Finegold *et al*., 2014). As an example, fructo-oligosaccharides and inulin were introduced to a model community in SHIME and the microbial communities assessed by Illumina sequencing (Marzorati *et al*., 2015).

In addition to simulations of the GIT, a multi-compartmental dynamic computer-controlled system for the stomach and small intestine (TIM-1, TNO intestinal model) was developed (Minekus *et al.*, 1995). A similar system to simulate the large intestine with peristaltic mixing, and the dynamic absorption of fermentation products and water (TIM-2) was developed as well (Minekus *et al.*, 1999). The TIM-2 was engineered to host the relatively high concentrations of microbes to model *in vivo* conditions. Moreover, hollow-fiber membranes were included inside the reactor compartments to simulate absorption of water and dialysis of metabolites. In this system, a dense chyme model is mixed and transported to the tubular loop-shaped segment with movement to mimic peristalsis where the colonic fermentation occurs. Water and SCFAs were removed from the system to obtain the desired cell density, which provides an advantage over batch culture systems. The TIM-2 system contains four glass units, all connected and each with flexible inner walls to permit peristaltic movements by controlling the hydraulic pressure to circulate the chyme through the loop-shaped system. The tubular shape of the model lumen prevents blockage or obstipation. The water activity of the luminal content is maintained via absorption controlled by a level sensor, and the system is kept anaerobic reflecting conditions in the proximal part of the large intestine. In addition to metabolite removal, the TIM-2 system also maintains metabolite concentrations through controlling the dilution rate and providing a limited amount of substrates. In addition, TIM-2 models the proximal colon, as most carbohydrate fermentation occurs in this region allowing for the mechanistic study of the transformation and bioactivity of exogenous molecules (Venema *et al.*, 2003). Accordingly, the TIM-2 system was used to model the effects of lactulose on the composition of anaerobic bacterial groups within an intestinal microbial community by plate counting and SCFA production by gas chromatography (Venema *et al.*, 2003). The TIM-2 model has been used in a variety of experiments to model host–microbial interactions and diet–microbial interactions This includes investigating bacterial metabolism of black tea polyphenols (Gao *et al.*, 2006), the diversity of glucose fermenting microbiota (Egert *et al.*, 2007), phylogenetic analysis of starch fermenting bacteria (Kovatcheva-Datchary *et al.*, 2009), and the

effects of inulin (degree of polymerization = 3–25) on the metabolic activity of modeled gut microbiota in the presence of *Clostridium difficile* (van Nuenen *et al*., 2003).

In an effort to benchmark the performance of the SHIME and TIM-2 systems, the models were compared in parallel characterizations of the fermentation patterns of arabinoxylan and inulin (Van Den Abbeele *et al*., 2013). This revealed similar effects for arabinoxylan and inulin on structure and function of the modeled microbiome in both models. In the TIM-2 model, ten fecal samples were pooled and stabilized for 16 h prior to the addition of either inulin or arabinoxylan in three days. In contrast, the microbial ecosystem in the SHIME model was derived from a single fecal suspension that was stabilized for 14 days with inulin or arabinoxylan introduced for 21 days. Both *in vitro* systems exhibited an increase in propionate and butyrate in response to a given substrate. Furthermore, arabinoxylan enriched *Bifidobacterium longum* in the modeled community, whereas inulin stimulated populations of *Bifidobacterium adolescentis*.

In addition to previously described models, the dynamic SIMulator of the GastroIntestinal tract (SIMGI) was innovated to simulate operations of digestion and fermentation (Barroso *et al*., 2015). This system incorporates three-stage culture reactors to model the ascending, transverse, and descending colon. In addition, the SIMGI model includes a gastric compartment that simulates peristaltic mixing and a dedicated reactor to simulate the small intestine. Most *in vitro* gut model systems were engineered to simulate either the upper GIT or the colon (Minekus *et al*., 1995, 1999; Molly *et al*., 1993; Van Den Abbeele *et al*., 2013). Thus the SIMGI model represents an advance compared to previous models as this fully computer-controlled, multi-compartmental system allows joint or independent simulation of gastric and colonic processes. The stomach compartment is comprised of two transparent, rigid, methacrylate plastic modules covering a reservoir of flexible silicone walls. The gastric content is mixed by peristaltic movements created by pumping thermostated water into a jacket between the plastic modules and the flexible reservoir. Beyond mimicking peristalsis, water movement is used to maintain temperature of the gastric compartment. The initial food intake is mixed with gastric

electrolytes and enzymes in the stomach compartment. The other four vessels, representing the small intestine and the three distinct colonic regions, are magnetically stirred, continuous flushed by nitrogen and pH and temperature controlled, and connected sequentially through pipes and peristaltic pumps. The succession and stability of the microbial community was evaluated by PCR-DGGE and quantitative PCR in this particular study.

Recently, a high-throughput continuous-flow system was developed to allow microbial community dynamics to be examined in minibioreactor arrays (MBRA) (Auchtung *et al*., 2015; Robinson *et al*., 2014). The MBRA was designed in continuously stirred six reactors, with a working volume of 15 ml to operate under an atmosphere of 5% CO_2 –5% H_2 –90% N_2 at 37°C in a heated anaerobic chamber. To simulate microbial community, pooled fresh fecal samples were mixed in anaerobic phosphate buffer, inoculated in 5 % (w/v) in each bioreactor and stabilized for 72 h. This MBRA system was used to describe community differences between epidemic and non-epidemic *Clostridium difficile* infections which is a common cause of severe cases of antibiotic-associated diarrhea (AAD) (Robinson *et al*., 2014). In this study, the *tcdA* gene was used to quantify *C. difficile* invasion and 16S rRNA amplicon sequencing was used to profile microbiome diversity in the presence of *C. difficile* and antibiotic treatment. Interestingly, clinical strains outcompeted the other strains in the presence of complex microbiota.

4.5 Mucus-Immobilized Models of the Gut

Mucosal microbiota are difficult to study *in vivo* as human intervention studies are typically restricted to observations on fecal samples. Microbes do not typically interact with the host epithelia and are instead found within the mucin layer secreted by goblet cells (Macfarlane and Dillon, 2007; Moal and Servin, 2006; Van den Abbeele *et al*., 2011; Zoetendal *et al*., 2002). Bacteria that reside in the mucosal gel layer contribute to inhibiting pathogenic epithelial invasion, either by excreting antimicrobial molecules or competing for adhesion sites (Moal and Servin, 2006).

Model systems that incorporate mucosal immobilization are used to study interactions between luminal and mucosal microbiota. Models that simulate dynamic interactions that occur with long-term residence times typically do not address the mucosal environment which may restrict their predictive power.

Immobilized mucus systems utilizing cell immobilization in anaerobic continuous-flow cultures for modeling the GIT were first established in the Lacroix model (Lacroix *et al*., 2004). Thereafter, the model was used for the immobilization of infant fecal microbiota in *in vitro* gut fermentation model system by Cinquin *et al*. (Cinquin *et al*., 2004). The system is predicated on a three-stage bioreactor that models immobilization of mucosal microbiota with xanthan-gellan gum beads to simulate bacterial cells entrapped in fibrous particles or forming biofilms on the intestine epithelium (Lacroix *et al*., 2004). Bacteria isolated from infant feces were immobilized in xanthan-gellan gum beads using a two-phase dispersion process previously described for the immobilization of lactic acid bacteria in kappa-carrageenan/locust bean gum gel beads (Lamboley *et al*., 1997). This is done in order to study the compositional changes in the microbial ecosystem over a long time period of fermentation. The medium was formulated to approximate the carbohydrate:nitrogen ratio present in infant chyme, with the bile salt concentrations reduced to the levels of secretion observed in infants. This model has several improvements including growing bacteria within biofilm structures, achieving high cell density in gel beads, maintaining community stability, protection of sensitive bacterial taxa from shear stress, prevention of washout and loss of less competitive bacteria. Based on these characteristics, a model with immobilized fecal bacteria was developed to simulate intestinal *Salmonella* infections and long-term shedding in children was developed (Le Blay *et al*., 2009).

In order to model sessile gut bacteria populations, a single stage chemostat could house mucin beads to encourage microbial adhesion encased within a dialysis membrane (Probert and Gibson, 2004). Water and metabolites are removed by osmosis and diffusion respectively using polyethylene glycol (PEG) and the media is maintained under anaerobic conditions. This model system supports the growth of bacteria associated with mucin beads and planktonic bacteria in the culture medium. Distinct differences were observed between the biofilm fermenter system

and traditional chemostats including microbial populations and their secreted metabolites.

Recently, a chemostat gut model was developed and validated to model planktonic and sessile states of colonic bacteria in biofilm formation associated with *Clostridium difficile* infections (CDI), (Crowther *et al*., 2014). In this model, macroscopic biofilms were facilitated with 18 glass rods inserted into the biofilm vessel, positioned to ensure the sampling portion of the rod extended across the liquid/gas interface to simulate CDI. Rods were affixed to the lid under an anaerobic environment with the vessel modified to encourage an even distribution of gas flow across the rods. The continuous chemostat model was inoculated with a pooled human fecal emulsion. Planktonic and sessile bacterial populations were enumerated during culturing methods and *C. difficile* toxin production was monitored using a Vera cell cytotoxicity assay. Interestingly, *C. difficile* spores preferentially persisted within biofilm structures.

The SHIME model has been adapted to simulate surface attached mucosal microbes (M-SHIME) (Van den Abbeele *et al*., 2012). Two SHIME units used in parallel (i.e. Twin-SHIME) were inoculated with luminal microbes (i.e. luminal-SHIME or L-SHIME), along with mucin-enveloped microcosms to the ascending colon model vessel for a mucosal environment (i.e. mucosal-SHIME or M-SHIME). In order to achieve a representative mucosal surface in the M-SHIME, microcosms were created with mucin-encapsulated suspensions. Mucin-adhesion assays were performed to track the succession of the community in addition to typical microbiological assays. The study reported *Lactobacillus mucosae* and *Pediococcus acidilactici* were both present in the modeled lumen, though *L. mucosa* was strongly enriched in the mucosal phase due to a specific mucus binding (mub) protein. In addition, *Lactobacillus rhamnosus* GG specifically colonized mucus upon inoculation.

The Polyfermentor Intestinal Model (PolyFermS) was developed to study the ecological distribution of intestinal microbiota (Berner *et al*., 2013). In this model, the proximal colon is simulated in the primary reactor containing fecal microbiota from healthy children immobilized in gel beads. The reactor continuously inoculates a set of parallel second stage reactors referred to as an inoculum reactor (IR). This is to enhance reproducibility by

allowing testing of the same complex microbiota in parallel reactions. A single reactor serves as intra-model control for assessing system stability. The PolyFermS system favors stable microbial compositions and metabolite production consistent with bacterial activity reported *in vivo*. The intestinal communities were validated as stable communities using HITChip microarray analysis and metabolic activity over a 38-day culture were observed at ratios similar feces of healthy donors. Enhanced butyrate production at the expense of acetate in inoculum reactors were detected, which was accompanied by a donor-specific reorganization of the reactor community. This suggests a metabolic adaptation and induction of community-specific lactate or acetate cross-feeding pathways in response to varying pH conditions. In addition, the PolyFermS was used to model fermentation that occurs in the gut of elderly individuals (Fehlbaum *et al*., 2015). Three platforms were deployed to conduct this study. The first vessel system was in three-stages to emulate the proximal, transverse, and distal colon similar to adult gut microbiota previously validated in infant and child microbiota fermentation in the Lacroix model. Vessel systems two and three were based on the PolyFermS composed of an IR that was seeded with immobilized fecal microbiota, and used to continuously inoculate second-stage reactors in parallel. The modeled gut microbial diversity and metabolite production was monitored using qRT-PCR, pyrosequencing, and HPLC (Fehlbaum *et al*., 2015).

In addition to human GIT models, PolyFermS was utilized to simulate the conditions of the porcine proximal colon (Tanner *et al*., 2014). This was composed of a first-stage IR seeded with immobilized fecal porcine microbiota and used to inoculate (10% v/v) five second-stage reactors, with all reactors fed fresh media with carbohydrate and protein concentrations to closely mimic the ileal chyme found in pigs. First, the IR was operated in batch mode to colonize the fecal beads and the nutritive medium was replaced by fresh medium every 12 hours. After colonization, continuous operation in IR was initiated, followed by a stabilization period of 5 days before connection to CR (control reactor) and TR1-4 (treatment reactors). The entire two-stage system was then stabilized for another 5 days. Thus, the model provides a tool for efficient, reproducible and cost-effective screening of environmental factors, such as dietary additives, on colonic fermentation.

4.6 Models to Simulate Complex Host–Microbial Interactions

Canonical bioreactor systems such as those described previously are ideal for studying single microbial species and inter-microbial interactions. However, they do not fully account for all features of the human GIT that add complexity to host–microbial interactions. While iterations of these models have been developed in order to achieve accurate simulations of the human GIT, *in vitro* bioreactor modeling systems typically do not account for the three-dimensional architecture of the colon. The colonic walls present intricate patterns of epithelial invaginations. Whereas conventional bioreactors mimic luminal conditions, including pH, gas content, and temperature, direct microbial–epithelial interactions cannot be simulated in such systems. Furthermore, it is difficult to model the planktonic and sessile phases as they exist *in vivo*, despite development of mucosal systems. This is not representative of the complex interplay between the host epithelia and commensal microbes that reciprocally interact.

As *in vitro* model systems have evolved to include peristaltic movement and fluid flow under constant parameters, there have been efforts to develop systems to bridge the three-dimensional gap. One solution to this concern is the recent development of an approach referred to as "gut-on-a-chip" (Kim *et al*., 2012). The general setup of the gut-on-a-chip is a small chamber made of a flexible polymer with several microchannels traversing its length. Upper and lower channels of the same dimensions, 150 μm high by 1000 μm wide, are separated by a porous layer of the same flexible polymer, and this membrane is then coated with an extracellular matrix solution, typically containing collagen of mammalian origin. The channels are connected to both a vacuum source and a source of fluid medium, both of which can be manipulated to achieve the desired flow rate and peristaltic simulation. A common epithelial cell line such as Caco-2 is deposited on the top surface of the polymer membrane using a syringe and maintained in culture. The cells are deposited at a density of 1.5×10^5 cells/cm^2, and have been observed to adhere to the membrane in 30 minutes, and form inter-cellular adhesions in one hour. Once the Caco-2 cells have formed attachments, culture medium is perfused through the channels with enough

shear stress to ensure that any superimposed cells or cell aggregates are washed away, and only a monolayer remains.

Prior to the development of gut-on-a-chip, complex host–microbial interactions were best performed in animal studies. Not only are these studies costly, they may also require dedicated resources, such as gnotobiotic centers, and come with ethical concerns inherent to working with live animals. Gut-on-a-chip does not present any ethical concerns, as human cell lines are a widely distributed and available. Also, interactions between human gut microbiota and the cells may better reflect *in vivo* interactions over time. Microbial viability under these experimental conditions was determined through assays of enzymatic activity, and the level of activity remained comparable to those observed *in vivo* (Kim *et al*., 2012).

Most significantly, however, is how accurately the gut-on-a-chip model emulates intestinal physiology, including response to laminar flow, peristalsis, and cell morphology. While other models of planar epithelium have been previously utilized, the gut-on-a-chip promotes the three-dimensional arrangement of epithelium observed *in vivo*. Multiple factors of the model's design work in conjunction to shape the cells in villus-like structures. These influences include the shear stress of laminar fluid flow, mechanical strain, and cyclic strain.

In the static system, the Caco-2 monolayer lies flat, and does not undergo any dynamic changes in cell structure or arrangement regardless of shear stress or mechanical strain. On the contrary, when cells on the gut-on-a-chip membrane were exposed to a shear stress of 0.02 dyne/cm^2 an obvious directionality was observed. The cells grew to a columnar shape and height of 30–40 μm, the expected characteristics of epithelium in a healthy human large intestine (Kim *et al*., 2012). Forces of laminar flow that epithelial cells experience *in vivo* are effectively represented in this system, and results indicate their important role in cellular morphology. In addition to the spontaneous formation of a columnar cell shape, when the same levels of laminar flow are maintained for a longer period of time, in the presence of cyclic strain, the monolayer also began to spontaneously fold. Through histology, the folds were confirmed to mimic the natural villi comprised of columnar epithelium that line the colon, thus achieving a model of the barrier environment that much of the gut microbiome inhabits.

The effects of mechanical stress and cyclic stress were also experimentally determined, and while the phenomena did not directly affect the formation of villi from the cellular monolayer, both of these stressors are critical to simulate peristaltic motion that occurs *in vivo*. Peristalsis is also involved in determining whether a model system could withstand strain similar to the host organism, and how this may interact with microbial commensals. Classic static epithelial monolayers do not mimic the degree of permeability observed in the human gut. However, the introduction of peristaltic, cyclic strain to the gut-on-a-chip system led to a four-fold increase of monolayer permeability, without compromising the integrity of the tight junctions (Kim *et al*., 2012). Barrier integrity continued to be measured in the presence of bacteria, and was determined to increase over time as the epithelial and microbial cells interacted. This supports previously reported findings that "probiotic" strains promote the health of intestinal epithelium and enhance its barrier function (Kim *et al*., 2012; Tottey *et al*., 2013).

Gut-on-a-chip performs well in studies of complex microbial communities, such as those derived from human fecal samples. Specific analytical platforms have been created in conjunction with microarray technologies to assess mass quantities of phylogenetic information (Tottey *et al*., 2013). In addition, massively parallel sequencing could now be applied towards analyzing data from 454-pyrosequening of a variable region of the 16srRNA gene (Tottey *et al*., 2013).

Overall, the gut-on-a-chip *in vitro* model holds a great deal of promise for studying host–microbial interactions that take place in the human colon. Due to their representation of colonic forces, including strain and flow, epithelial cell morphology, and barrier strength and permeability, these models provide a degree of accurate mimicking of colonic physiology and microbial interactions at the epithelial barrier.

4.7 Gastric–Small Intestine Model Systems

Although the majority of microbes colonize the large intestine, other GIT segments are relevant to health outcomes. Accordingly, modeling the colon in the absence of the upper

GIT does not represent the harsh barriers experienced by food and microbes once consumed or delivered.

Modeling the stomach and small intestine requires maintenance of proper pH in both simulated organs (2 in the stomach, 6–7.4 in the small intestine) (Chen *et al.*, 2011; Minekus *et al.*, 1995). Besides pH, modeling the proper composition of gastric juice is imperative in the correct sequence and concentration (Chen *et al.*, 2011; Minekus *et al.*, 1995).Pepsin and hydrochloric acid are encountered in the stomach. In contrast, bile, bicarbonate, and pancreatic enzymes are found in the small intestine, particularly in the duodenum. Modeling upper GIT enzyme secretions pose another challenge in these systems. In the stomach, acid and enzymes are secreted evenly throughout directly from the stomach lining (Vardakou *et al.*, 2011). Thus the direct addition of physiological concentrations of enzymes and hydrochloric acid to a model system may not emulate digestion *in vivo*.

In addition to modeling biochemical aspects of the upper GIT, there are many mechanical forces to be considered to achieve a representative simulation. The secretions themselves, whether from the stomach walls or duodenum, exert forces of flow onto the digesta present (Chen *et al.*, 2011; Vardakou *et al.*, 2011). These hydrodynamic forces and waves cause shear stress. As food progresses through the GIT, its own viscosity contributes to a decreased rate of diffusion of nutritive food components to their site of absorption in the small intestine (Mackie *et al.*, 2015). The stomach possesses active churning, and an accurate model must mimic the peristaltic contractions that cause churning (Chen *et al.*, 2011; Minekus *et al.*, 1995; Vardakou *et al.*, 2011). Churning is necessary for the appropriate mixing and production of chyme. The small intestine experience peristalsis as well, as it pushes chyme along its length, the duodenum, jejunum, and ileum, so nutrient absorption can occur.

At the junction of the stomach and small intestine lies the pyloric sphincter, which selectively allows digested material through, to control the rate of stomach emptying (Maathuis *et al.*, 2010). Liquids pass through the pyloric sphincter with relative ease, while larger particulates will be forced back into the stomach for further breakdown (Kong and Singh, 2010; Minekus *et al.*, 1995). Without a model sphincter with the proper pressure in place, this critical function cannot be

simulated (Chen *et al*., 2011). Indirectly, this affects the modeling of transit time through the GI tract as well (Minekus *et al*., 1995). Each of these parameters has been included based the results of on studies done on human subjects and animal *in vivo* studies (Minekus *et al*., 1995).

The role of the nervous and endocrine systems in digestion, such as stimulation by the vagus nerve, has yet to be emulated in an *in vitro* GIT model (Guerra *et al*., 2012). Pancreatic enzymes and bile can be accurately added to a duodenal approximation, but the hormones that act upon the pancreas and gallbladder are not typically considered. Feedback mechanisms that occur in the stomach and small intestine digestion and absorption have not been factored in either.

With these challenges in mind, a successful gastric and upper intestinal simulator can encompass the dynamic nature of these organs' physiology. One of the earliest dynamic models, and one that is widely used and highly regarded today, is the TNO *in vitro* Gastrointestinal Model (TIM), by Minekus *et al*. (Minekus *et al*., 1995). The first model, TIM-1 was created in 1995, followed by TIM-2 in 1999 (Minekus *et al*., 1999). This model is significant because it was the first of its kind to be both dynamic and multi-compartmental (Guerra *et al*., 2012; Minekus *et al*., 1995). At its core, the system consists of four compartments, representing the stomach, duodenum, jejunum, and ileum. Comprising each region is a glass vessel with an internal compartment, with flexible walls. The space between the glass container and the inner compartment is filled with water controlled by a rotary pump. A separate three part valve pumps connects each discrete region and emulates the forces of peristalsis, contracting 3 times per minute in the stomach compartment, and 9 times per minute in the models of the small intestine. Dialysis was performed after digestion was completed with an added glucose load to simulate absorption by the small intestine, and it was reported that 96% of the glucose was found in the dialysis, demonstrating successful absorption. The pressure exerted on the walls inside also allow the system to simulate churning and mixing of the meal (Minekus *et al*., 1995).

Although the mechanical forces that are exerted in the stomach and small intestine are accounted for, some of the more sophisticated aspects of contraction and peristalsis may be

improved. The regular contractions that TIM-1 exerts through the pumping of water are not entirely representative, as within a single peristaltic contraction, there is gradient in the force felt by the stomach, increasing as the contraction approaches the antrum, the lower, fuller portion of the stomach (Kong and Singh, 2010). Antral grinding is a critical component of the mechanical digestion that occurs in the stomach, as particulate matter is turned into chyme. Sufficient decrease in size of digested food is necessary for entry into the duodenum. If larger pieces of digesta within the stomach are not broken down, the human pylorus is responsible for the phenomenon of retropulsion. Liquids and sufficiently small particles pass through the pyloric sphincter with ease, all the while preventing the entry of large constituents, forcing them back into the stomach for further mechanical breakdown with almost elastic properties (Kong and Singh, 2010).

One model created to meet these needs is the Human Gastric Simulator (HGS) from University of California Davis (Kong and Singh, 2010). In practice, this model is similar to TIM, but contains only a stomach chamber, and addressed the concern specific to gastric simulation that the former system lacked. The stomach itself is modeled with a latex chamber, held up with clamps and supports at a height of 13 inches. Rather than enclosing the "stomach" in a solid vessel and using water to simulate pressure and contraction, this system uses a series of 12 rollers on belts that apply force directly to the latex walls. Three rollers are connected via pulley system on each of the four sides of the vessel such that peristalsis is experience evenly throughout the model. A motor drives each of the four belts to operate simultaneously. This model also uses a rate of 3 contractions per minute to mimic *in vivo* observations (Kong and Singh, 2010). In contrast to the previous models, the roller and pulley system is more sophisticated because they can be adjusted to simulate the pressure differential that occurs in one single peristaltic contraction. The stomach experiences stronger contractions at the antrum, and as such food used in an *in vitro* gastric modeling system should feel the greatest contraction force in the bottom portion of the chamber simulating the stomach. The HGS accomplished this by tapering the latex vessel, indirectly decreasing the area over which the four sets of rollers exert their force, thus

increasing the force of the contraction (Kong and Singh, 2010). The latex of the vessel combined with the intricate application of mechanical force make the HGS better for modeling specific parameters of peristalsis, such as frequency, amplitude, and intensity (Guerra *et al*., 2012).

Gastric secretions are delivered to the chamber via variable flow mini peristaltic pump. The pump flows into a piece of tubing that splits into 5 equivalent tubes, ensuring that the simulated juice is distributed evenly throughout the vessel, as occurs in the human stomach (Kong and Singh, 2010). The pyloric function of selective filtration and retropulsion that is observed *in vivo* is achieved with the addition of a polythene mesh bag inside of the latex vessel, with an average pore size of 1.5 mm. Particles less than 2 mm in diameter are able to continuously pass through the pore for emptying, as is true of liquids and small particles through the pyloric sphincter. However, large particles are retained in the chamber, exposing them to further digestive action. As such, the "sieving" function of the pyloric sphincter is retained in the HGS (Kong and Singh, 2010).

The success of this model was determined based on properties of the experimentally digested food, in this case apples and rice. The criteria they hoped to fill were particle size distribution, pH profile, and desired content remaining in the stomach compared to what was emptied (Kong and Singh, 2010). Experiments performed comparing the HGS to older shaking bath techniques of food disintegration showed that particle size was significantly reduced in the HGS, meaning that it better approximates *in vivo* gastric digestion due to its additional mechanical forces. Smaller particle size confers a greater surface area to the digested food. As a result, it is implied that this *in vitro* system is able to simulate the kinetics of nutrient release for absorption *in vivo*, as surface area plays an outsized role in increasing absorption.

An *in vitro* upper GI tract simulator that has been commonly used in pharmacological studies is the Dynamic Gastric Model (DGM) (Vardakou *et al*., 2011). While similar in design to other models of its kind, there are differences in how mechanical forces are applied. The DGM consists of inner and outer compartments, like HGS and TIM, but food is exposed to mechanical digestion is found in between the two chambers, as the inner

compartment is mobile and acts to crush the food against the walls of the outer chamber (Kong and Singh, 2010). Contractions, at 3 times per minute, are achieved through water pressure, as in the TIM system (Vardakou *et al*., 2011). Gastric juice, including enzymes, is also carefully distributed such that it is delivered evenly, but rather than through a system of tubes, the mixture is released from a dispenser floating atop the contents of the vessel. Overall, the DGM was designed to account for many of the considerations mentioned previously, including dynamic pH change, shear stress, churning, and transit time. As this particular study was concerned with the absorption of pharmacological agents in the presence of different meals, the success of the model was determined by the measure of mean breaking time of agar beads, used to represent ingested tablets or capsules (Vardakou *et al*., 2011).

This model places particular emphasis on simulating antral grinding forces, as discussed with the HGS (Kong and Singh, 2010; Vardakou *et al*., 2011). A piston is used to achieve shearing in the antrum of the model, and the sieving of the pyloric sphincter is mimicked using an elastic ring (Vardakou *et al*., 2011). However, it has been claimed that the mechanical forces applied in the DGM do not accurately reflect the forces of peristalsis (Guerra *et al*., 2012; Kong and Singh, 2010).

All three models – TIM, HGS, and DGM – are dynamic representations of gastric digestion, but each comes with its own strengths and attributes. Only TIM is multi-compartmental, which is useful for whole-system studies, but its approximations of gastric mechanical forces are weakest. Both the HGS and DGM attempt to recreate forces in the stomach, considering both peristalsis and the gradient of force exerted along the stomach. The HGS arguably accomplishes this task with more accuracy, using the system of rollers at different applied pressures, while the DGM uses a water based pressure simulation like TIM. However, TIM is also a better model with which to observe absorption after digestion is complete. HGS allows for the approximation of nutrient absorption, but this phenomenon cannot be directly measured in this model.

In vitro models of the stomach and small intestine are most representative of *in vivo* conditions when a meal or food matrix is incorporated along with the bacteria of interest. Since some

strains have been demonstrated to be resistant to harsh conditions *in vivo* it is important to be able to distinguish between the success of the model and a particular microbe simple exhibiting resilience. For example, sporulating bacteria are known to withstand the conditions of the upper GI tract (Maathuis *et al*., 2010).

In conventional testing of microbes in highly acidic conditions, 100% of strains exposed to hydrochloric acid did not survive. However, food itself has significant buffering capabilities that can protect microbes from pH profiles that would otherwise render them inviable (Mainville *et al*., 2005). It is not enough to consider the effects of acid or bile, which is thought to be antibacterial, on their own (Marteau *et al*., 1997). Rather, the combination of acid exposure with a buffering meal and subsequent exposure to bile and enzymatic action must be considered as is *in vivo*. Studies using TIM but with these additional considerations, namely a kefir based meal, demonstrated that more bacterial strains survive when in a dynamic model rather than static, and the survival is significantly greater than when on media incorporating acid or bile alone (Mainville *et al*., 2005).

Another difference from the conventional TIM set-up is that this study only used the stomach and duodenal compartments. The duodenum is the site of most of the digestion in the small intestine, and is considered to be the threshold between the upper and lower gastrointestinal tract. If viability is determined upon emptying into the duodenum, it is likely that the microorganisms will successfully pass through to the colon. Survival rates of microbes in the duodenum were shown to be more uniform than those of the stomach, likely due to the higher pH (Mainville *et al*., 2005). Five strains of lactic acid bacteria were found to have survival rates of over 72%, implying likelihood of a significant number of them remaining viable and reaching the colon (Mainville *et al*., 2005). Known genera of beneficial microbes are lactic acid bacteria, including *Lactobacillus* and *Bifidobacteria*, so this is a promising result.

Though each model discussed is best fit for certain types of studies, at large these models represent a comprehensive arsenal of methods for approximating the human gastrointestinal tract, especially in relation to commensal microbiota. Although the majority of the human gut microbiome resides in the large

intestine, it is important to consider the effects of the upper GIT on digested food, and how this affects the nutrients that become available to the microbiome. Future directions for improvement of these studies is to emulate mucosal interactions more accurately, such as gut-on-a-chip strives to do, as well as continue to improve the dynamics of multi-compartmental models. Current studies have also begun to look at the effects of non-nutritive bioactive components in *in vitro* models, which can also have some bearing on the interactions between microorganisms, their human hosts, and the food we digest.

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5

Macronutrient Nutritional Functionality of Carbohydrates, Proteins and Lipids: Digestibility, Absorption and Interactions

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5.1 Introduction

Simulated digestion protocols in laboratory equipment, known as *in vitro* testing, are used for a wide variety of investigative purposes. While *in vivo* animal and, ideally, human testing is the gold standard for determining digestibility of foods and beverages (FAO, 2013), it is not practical or required in many situations. Investigations with humans and animals are expensive, time consuming and there are ethical considerations which must be closely monitored (Minekus *et al*., 2014).

The use of *in vitro* models has several advantages. It gives researchers the ability to test a large number of samples under identical conditions, avoiding the between animal variability of *in vivo* tests (Ménard *et al*., 2014). Simulated digestion methods are frequently used for screening large numbers of samples to guide decisions as to which should proceed to *in vivo* trials (McClements and Li, 2010). They also have advantages related to understanding mechanistic phenomena. Sampling can be easily performed throughout the process making it possible to analyze breakdown products and rates of reactions. Interactions between food components during digestion can also be more

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easily observed. For example, the effects of enzyme inhibitors can be measured (Gilani *et al.*, 2005). The influence of food structure, including the effects of cellular structure in plant materials and emulsification, can be observed by microscopy (Brummer *et al.*, 2015). Partitioning of nutrients between lipophilic and hydrophilic phases can also be studied as digestion progresses (McClements and Li, 2010). Furthermore, where differences in digestibility have been observed *in vivo*, *in vitro* methods can be useful to examine underlying reasons for the differences (Fanbin *et al.*, 2011), offering the potential to further optimize *in vitro* systems for desired performance.

There is a wide variety of *in vitro* digestion models in use. These vary greatly in terms of complexity and design (Butts *et al.*, 2012; McClements and Li, 2010; Woolnough *et al.*, 2008). The choice of protocol and equipment depends on the application and available resources. The two basic categories of *in vitro* methods are dynamic and static systems. Dynamic systems are more complex and therefore more closely simulate *in vivo* conditions. They generally consist of multiple chambers and pumps so that enzymes and simulated digestive fluids can be added over time, the pH can be adjusted at a predetermined rate, and the meal bolus can be mixed by peristaltic motions (Guerra *et al.*, 2012). Static procedures are often conducted in one vessel (Butts *et al.*, 2012), with enzyme additions and pH adjustments made at one time. Mixing is accomplished by shaking, orbital motions or rotation. While dynamic systems are complex and require specialized equipment and additional user inputs, static systems can be set up with common and inexpensive laboratory equipment. More information on the differences between apparatuses can be found in Chapter 4.

Digestion protocols used to investigate the different macronutrients (carbohydrates, proteins and fats) follow the same general approach (Minekus *et al.*, 2014). Minced food is subjected to conditions which mimic the oral, gastric and upper intestinal phases of digestion at human body temperature, 37 °C (Figure 5.1). The degree to which starch, protein and lipids are broken down is an indicator of their digestibility and availability for absorption and therefore the potential nutritional value of the food. *In vitro* digestion methods are frequently adapted and optimized, depending on the food matrix being investigated and the

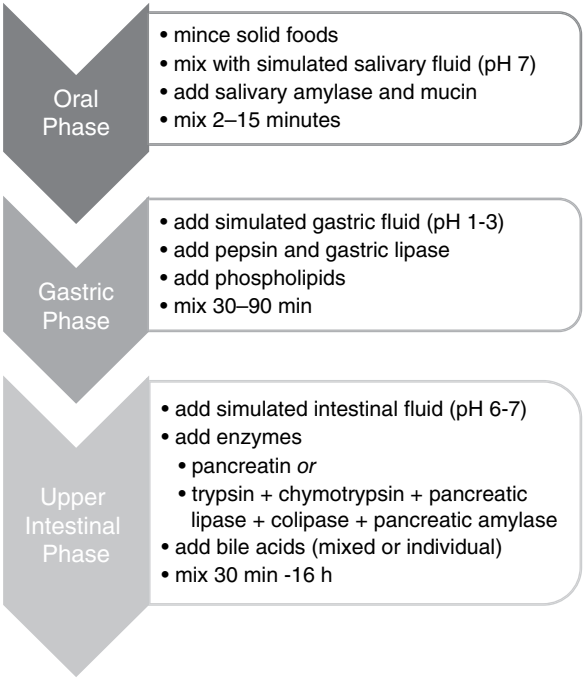

Figure 5.1 General protocol of three-stage *in vitro* digestion for solid foods, including essential enzymes, biochemical environment, pH and duration of digestive phases.

nutrients of interest. As such, the literature reflects the use of a wide range of equipment and experimental protocols, all of which complicates the comparison of results between studies and groups.

5.2 Applications and Considerations

5.2.1 Carbohydrates

Available carbohydrates are the mono- di- oligo- and polysaccharides which provide energy for metabolism (Leturque and Brot-Laroche, 2013). For practical purposes, they are the sugars which can be absorbed directly from the upper intestinal tract as

well as soluble starches and dextrins which are broken down to simple sugars by digestive enzymes. Plant foods, including fruits, vegetables cereals and grains, may contain a wide variety of sugars. Glucose, fructose and sucrose are the most common sugar molecules, but xylose, mannose, galactose and arabinose can also be present. Milk contains 5–6% of the sugar lactose, while animal tissues contain only low levels of available carbohydrates, in the form of glycogen. Many plants store starch in seeds, roots or tubers.

Other carbohydrates present in foods, including sugar alcohols, most oligosaccharides and non-starch polysaccharides, pass through the upper digestive system unchanged (AACC, 2001). Humans do not produce the enzymes necessary to break down galacto-oligosaccharides, fructo-oligosaccharides, cellulose, pectin, arabinoxylan or other hemicelluloses. In some foods, a portion of the starch is not digested because of its physical or chemical characteristics. These portions are referred to as resistant starches (RS) and further divided into four types (Zhang and Hamaker, 2010): RS1 – physically inaccessible, RS2 – naturally resistant, RS3 – retrograded, and RS4 – chemically modified. Dietary fiber is made up of indigestible non-starch polysaccharides, oligosaccharides and resistant starch. Since dietary fiber is not absorbed in the upper digestive tract it passes into the colon where it can be fermented by the gut microbiota.

In vitro methods are frequently used to investigate the partitioning of carbohydrates into so-called available and unavailable fractions. In fact, the official methods for determining resistant starch and dietary fiber include *in vitro* digestion steps (AOAC, 2015). The application of *in vitro* models in the study of dietary fiber is described in Chapter 4. In addition to total amount of available carbohydrates, *in vitro* digestion protocols are frequently used to assess potential rate of digestion (Englyst *et al*., 2003). Sugars and rapidly digestible starch move quickly into the bloodstream increasing blood glucose concentrations and stimulating the secretion of insulin. A rapid influx of a large amount of glucose results in a high glycemic response. On the other hand slowly digestible starch (SDS) is absorbed over the length of the small intestine, eliciting lower but sustained blood sugar concentrations (Jenkins *et al*., 2004). An attenuated postprandial

blood glucose response is preferred, from a health perspective, because of the decreased risk of developing insulin resistance and associated complications.

5.2.2 Proteins

Protein digestibility is a very important issue since amino acids derived from dietary protein are essential for the growth and maintenance of muscle and other tissues (Moughan, 2009). While protein from meat, dairy, and eggs are considered high quality and generally bioavailable, plant proteins are less so. The amino acid balance in most plant proteins is not optimum for human growth and maintenance (Jahan-Mihan *et al*., 2011). Of the twenty-six amino acids used to synthesize proteins, the majority can be synthesized by humans but some, called the indispensable amino acids must be obtained from foods (FAO, 2013). Most plant proteins are low in at least one indispensable amino acid. For example cereal proteins are low in lysine, whereas legumes tend to be low in methionine (USDA, 2015). In addition to amino acid balance, the nutritive value of proteins depends on digestibility. The ability of digestive enzymes to hydrolyze proteins into short peptides and free amino acids which can be absorbed by the gut epithelium can be hindered by a number of factors. For example, plant proteins may be physically inaccessible because of intact cell walls that partition protein bodies away from enzymes (Brummer *et al*., 2015). The presence of protease inhibitors, like the trypsin inhibitor found in beans and tannins in rye, also reduces the efficacy of digestive enzymes (Butts *et al*., 2012).

In vitro digestion is frequently used to compare the digestibility of proteins (Butts *et al*., 2012). Traditionally, protein quality was compared by measuring the growth rate in rats against the growth rate with a standard protein (FAO, 2013). This protein efficiency ratio method was adapted to account for the proportion of essential amino acids in the protein digestibility corrected amino acid score method. Digestibility of protein cannot be accurately measured using fecal nitrogen content because the majority of nitrogen in feces originates from bacterial protein. Determining the amount of undigested protein entering the colon in healthy humans is also very difficult. Currently, the Food

and Agriculture Organization (FAO) of the United Nations Expert Committee on Dietary Protein Quality Evaluation in Human Nutrition recommend the use of cannulated pigs to determine true ileal amino acid digestibility (FAO, 2013). However, the committee noted that "ideally, a rapid *in vitro* protein digestibility assay in food would be available" and has stated there is an "urgent need to develop a standardized, independently validated *in vitro* amino acid digestibility assay".

Protein digestibility is also of interest because the rate of digestion can impact satiety. Specifically, slow digestion of proteins leads to the release of amino acids into the blood stream over a longer period of time (Jahan-Mihan *et al*., 2011). This may extend feelings of satiety and help people aiming to reduce energy intake by reducing insulin secretion, thereby contributing to glucose homeostatis (Van Loon-Bouwman *et al*., 2014) and have also been associated with satiety-related incretins (Nilsson *et al*., 2004).

5.2.3 Triglycerides

Triglycerides are the major class of storage lipids in both animal and plant tissues (Brannon *et al*., 2013). They can be present in foods as bulk solid fats (i.e. butter, lard, shortenings) and liquid oils or they can exist as dispersed lipids, as in emulsions or oil bodies. Triglycerides consist of a glycerol backbone with three fatty acid molecules attached through ester bonds. Triglycerides are hydrolyzed within the gastrointestinal tract, enabling the absorption of the liberated fatty acids and monoglycerides by the gut epithelium. The fatty acids present in foods are generally a mix of saturated and unsaturated molecules. Most are between 14 and 18 carbons long although, depending on the source, much shorter or longer chained fatty acids can be present. For example, dairy fat contains a very wide range of fatty acids, including the 4 carbon molecule butyric acid, whereas fish oil tends to contain high proportions of very long chain polyunsaturated fatty acids (Harwood and Weeselake, 2015). The unsaturated fatty acids linoleic acid (18:2) and linoleic acid (18:3), are considered essential for health and must be obtained from the diet (FAO, 2010). Health benefits of other fatty acids, in particular the omega-3 fatty acids (eicosapentanoic acid (20:5, EPA)

and docosahexanoic acid (22:6 DHA) have been established. Importantly, ensuring bioavailability of specific fatty acids from foods and supplements is necessary to ensure efficacy of fortified products.

Triglycerides have a higher energy density than other nutrients, providing 9 calories per gram compared with 4 calories per gram for starch, sugar or protein (USDA, 2015). Therefore, their digestibility and absorption can have a proportionally greater impact on energy intake. As is the case with proteins and starches, food structure can significantly affect lipid digestibility. For example, native oil bodies in undisrupted cells of plant tissues can be resistant to digestion because of decreased accessibility to digestive enzymes (Ellis *et al*., 2004). There is also interest in the ability of emulsions and microencapsulated oils to delay or minimize digestion and absorption of oils in the upper intestine (McClements and Li, 2010). Indigestible encapsulated oil systems could be useful to deliver omega-3 oils and oil soluble bioactive components to the microbiota in the colon. *In vitro* digestion protocols can be very useful for assessing the rate and extent of triglyceride digestion from different food systems. They also have wide application in the study of digestive behavior of other lipid molecules, including phospholipids, cholesterol, phytosterols, mono- and diglycerides, carotenoids, etc. (Hur *et al*., 2011) and the potential bioavailability of minor lipids.

More details about the application of *in vitro* models to study micronutrient bioaccessibility and bioavailability can be found in Chapter 6.

5.3 Simulating Digestive Processes

5.3.1 Oral Food Processing and Implications for Sample Preparation

During the oral phase of human digestion, food is mixed and broken into small pieces. This increases the surface area of the food particles, promoting enzymatic activity and facilitating swallowing. The tongue mixes the food particles with saliva which contains α-amylase to begin the process of hydrolyzing starch to α-limit dextrins (Leturque and Brot-Laroche, 2013).

When the food bolus is swallowed, the α-amylase is partially or completely denatured by stomach acid, depending on the buffering capacity of meal (Bornhorst *et al*., 2014). In the stomach, peristaltic contractions further reduce the particle size and gastric fluids dilute the meal bolus.

For carbohydrate digestibility analyses, sample preparation is extremely important. Foods should be prepared as they would be for consumption. Food processing and storage can significantly affect the digestibility of starch and the absorption rate of sugars (Lan-Pidhainy *et al*., 2007; Regand *et al*., 2009). In intact plant tissues, sugars and starch granules are enclosed in cell walls which are resistant to enzyme and acid disruption. Milling or grinding can disrupt the cell walls, making the sugars and starches more available (Edwards *et al*., 2014). Therefore experiments conducted on foods made from flours, pastes or juices will yield different results than from foods with intact tissues.

Because processing can appreciably affect the results obtained, it is recommended that, prior to *in vitro* digestion, foods be processed as they would be eaten (Englyst *et al*., 1999). The variability observed in starch digestibility highlights the potential impact of processing. Heating starch granules in the presence of water causes gelatinization, meaning that the crystalline structure is disrupted. Partial gelatinization results in disruption of crystal structure and opens the starch granule structure, allowing the infiltration of water and increased enzyme access. Complete gelatinization results in the solubilization of starch and the disintegration of the starch granules, allowing easy access by amylases and leading to rapid depolymerization. Cooling and storage of these foods can result in starch recrystallization, known as retrogradation. In such cases, the ordered structure again limits the access of amylases to starch molecules. Over time, as crystalline starch structures reorganize and grow, slowly digestible starch becomes resistant starch. Thus, starch digestibility is a continuum, with no sharp distinctions between rapidly, slowly and resistant starch. Because the accessibility is time and temperature dependent and the definitions somewhat arbitrary, it has been difficult to obtain consistent, repeatable results.

Oral processing is frequently combined with sample preparation. Simulated saliva is often added to food before it is minced. The simulated saliva can be a simple NaCl solution (Astwood

et al., 1996) or a complex mixture of electrolytes (Minekus, *et al*., 2014), closely mimicking human saliva. USP formulations have also been published for salivary and other digestive fluids (USP, 2015) and, in some jurisdictions, there are commercially available options. If salivary amylase is used, 0.75 mM calcium needs to be included because it is a cofactor required for the proper enzyme function. In early *in vitro* digestion protocols, food was sometimes chewed by an investigator and spit into the simulated gastric fluid (Woolnough *et al*., 2008). Now that human and porcine salivary amylases are available commercially, this is rarely seen in the literature. For foods with low levels of starch, the addition of salivary amylase is sometimes omitted and, in other instances, the composition is more complex with the addition of mucin, for example. Of note, although lipid digestion is initiated by the presence of lingual lipase, this enzyme is rarely included in *in vitro* digestion experiments, given the lack of commercial availability and the supposition that it exerts a relatively minor influence.

Chewing time varies greatly between individuals and for different food items. Not surprisingly then, the length of time that should be used for the oral phase can be controversial. It is important to have uniform mixing of salivary amylase with the sample but not allow excess starch degradation at this stage (Minekus *et al*., 2014). Generally salivary enzyme is left in contact with the food for 1–5 minutes. Because salivary amylase can be inactivated in the stomach, it is not appropriate to allow the amylase reaction to proceed to completion. For the analysis of protein and lipid digestion the length of time of oral processing is less critical. However, for starch-rich foods, the starch matrix is likely to impact the digestibility of protein and lipid, rationalizing the need for careful consideration.

5.3.2 Gastric Phase

Simulation of the gastric phase is initiated by a decrease in sample pH. In a dynamic *in vitro* apparatus, acid is added slowly to decrease the pH gradually and mixing is accomplished by peristaltic motion. This more closely resembles the situation *in vivo* than in static models where acid, electrolytes and enzymes are added in one aliquot at the beginning of the gastric phase

(Minekus *et al*., 1999). In the stomach, amylase is denatured by acid secreted (Leturque and Brot-Laroche, 2013). The length of time amylase remains active in the stomach depends on the buffering capacity of the food and the cohesiveness of the food bolus.

Pepsin and simulated gastric fluid are added to hydrolyze protein. The simulated gastric fluid, containing hydrochloric acid and electrolytes, is available commercially or may be made in house. Pepsin is stable at low pH and initiates the process of cleaving the proteins into smaller peptides (Moughan and Stevens, 2013). Pepsinolysis opens up the structure of the food matrix increasing the accessibility of other enzymes. For protein-based products, the gastric phase of digestion is very important. The low pH causes denaturation of many proteins, a change in the conformation of the protein structure (Moughan and Stevens, 2013). Below their isoelectric point, the protein chains unfold, opening up the structure of globular proteins and making them more accessible to protease activity. Protein denaturation can also result in intermolecular associations leading to aggregation and gelation. For example, the colloidal casein micelles in milk are destabilized in the acidic stomach environment. The casein coagulates which slows passage of the meal into the duodenum.

In static *in vitro* systems, hydrochloric acid and pepsin are added all at once and mixed at 37 °C for between 30 min to 2 h (Butts *et al*., 2012). The amount of acid added may be either a fixed amount or the mixture can be titrated to a predetermined pH, usually between 1.5 and 3. The length of the gastric phase varies widely: from 30 min to 2 h (Hur *et al*., 2011). The appropriate incubation time is dependent on the characteristics of the food. For instance, the gastric emptying rate is faster for liquid meals than for solid meals (Santangelo *et al*., 1998). MRI imaging of digestion *in vivo* have also shown that the viscosity of foods can affect the rate of mixing of digestive juices with the meal bolus (Marciani *et al*., 2000). Therefore, foods containing significant amounts of viscous soluble fiber, can delay gastric emptying. The presence of protease inhibitors in foods, for example trypsin inhibitor a small peptide found in legumes, can also slow the digestion rate in the gastric phase. Therefore, choosing a gastric incubation time should be carefully considered,

taking into account any relevant *in vivo* information available on gastric emptying time, as well as the nature of the test meal. Samples may be taken at the end of the gastric phase to determine degree of denaturation and depolymerization. In dynamic *in vitro* systems, the acid, enzymes and bile acids can be added incrementally while peristaltic mixing takes place (Guerra *et al*., 2012). When a closer approximation of *in vivo* conditions is desired, gastric lipase and phospholipids are also added. Phospholipids are emulsifying agents which migrate to the fat/water interface, aiding in the formation of a coarse emulsion in the stomach. It should be mentioned that gastric lipase plays an important role in infant digestion (Abrahamse *et al*., 2012), rationalizing its inclusion in studies seeking to simulate that gastrointestinal environment.

Of note, where gastric processing is deemed less relevant, some static model experiments do not include a gastric phase at all. Streamlined methods to determine the rate of digestion of starch in different food products (Edwards *et al*., 2014) or the rate of emulsion breakdown (McClements and Li, 2010) have not involved a pH change or pepsin addition. While this can isolate factors relevant to intestinal processing, the limitation exists that *a priori* processing may have impacted dynamics relevant to the experiments. For example, gastric lipase accounts for 10–30% of lipid hydrolysis, but is often omitted from *in vitro* digestion models. Based on the surface activity of the partial glycerides and free fatty acids generated, it is possible even a limited extent of digestion could impact subsequent interfacial dynamics. This highlights the importance of clearly rationalizing and reporting on the selection of experimental parameters utilized. A strength of *in vitro* modeling is that it allows protocols to be tailored for specific purposes, including mechanistic investigations of digestion dynamics, including rate.

5.3.3 Upper Intestinal Phase

As the gastric contents, called chyme, empty into the upper small intestine through the pylorus, they are further dispersed by shear forces and mixed with pancreatic enzymes, bile from the gall bladder and bicarbonate to neutralize the pH and electrolyte solution. The formation of a fine emulsion is important

since droplet size dictates the surface area available for lipolytic activity. A major difference between static and dynamic *in vitro* systems is seen in the upper intestinal phase of the digestion process. While static systems move material from the upper intestinal phase at a fixed time point and treat the entire suspension at once (Butts *et al.*, 2012), dynamic systems are capable of releasing small volumes of chyme into the duodenum phase, creating a flow of the suspension through the upper intestinal chamber (Guerra *et al.*, 2012). This approach more accurately reflects *in vivo* digestion and can be especially relevant when considering the effects of dynamics such as phase separation of stomach contents on subsequent GI processing.

Human pancreatic secretions contain a mixture of hydrolytic enzymes, including amylases, proteases and lipases (Thomson and Tso, 2013). Porcine pancreatin is typically used, as the enzymes are relatively similar to those in humans (Minekus, *et al.*, 2014). Alternatively, purified trypsin and chymotrypsin are available. As protein digestion continues in the upper intestine, the increase in pH allows proteases and peptidases, including trypsin, chymotrypsin, elastase and carboxypeptidases A and B, secreted by the pancreas to continue hydrolysis (Moughan and Stevens, 2013). Proteases are synthesized in an inactive form, known as zymogens, to prevent autolysis and damage to the surrounding tissues. Once they enter the lumen of the gut, they are activated by functioning proteases which were previously secreted. Peptides that prevent the substrate from entering the active site are removed and the enzymes begin to hydrolyze proteins. The bile acids, which are amphiphilic in nature, facilitate further denaturation of the proteins.

The majority of starch digestion and sugar absorption takes place in the upper intestine. The pancreas secretes α-amylase into the duodenum to continue starch hydrolysis (Leturque and Brot-Laroche, 2013). Glucose and other monosaccharides are absorbed through the wall of the intestine by a wide variety of transporters located within the epithelial cells of the small intestine. Some allow passive transport, whereas others require ATP or $NADH_2$ for absorption. Some transporters move cofactors, like sodium or calcium ions, in or out of the cell at the same time. Brush border enzymes, like sucrase and maltase, hydrolyze disaccharides and maltodextrins into monosaccharides for

absorption. Individuals who do not produce β-galactosidase, the enzyme which hydrolyzes lactose into galactose and glucose, lack the ability to metabolize lactose and are considered to be lactose intolerant. Because brush border enzymes are not present in pancreatin, they must be accounted for in other ways in an *in vitro* digestion protocol. Amyloglucosidase is often added to hydrolyse maltose and malto-oligosaccharides into glucose (Englyst *et al*., 1999). When other sugars are present, HPLC methods can be used to quantify various mono- and disaccharides. Of note, bile acids are often omitted in analyses of available carbohydrate analyses; they are not thought to influence starch digestion significantly and are unpleasant to work with.

To be absorbed and contribute to postprandial elevations in blood lipids, dietary triglycerides must be hydrolyzed. The majority of lipid digestion takes place in the upper small intestine. Biliary constituents, including bile salts and phospholipids, are particularly critical components of lipid digestion. Both contribute to lipid emulsification and the solubilization of lipid digestion products (i.e. free fatty acids and monoglycerides), as well as other lipophilic molecules such as vitamins. While bile acids play a lead role to emulsify the fat into small droplets (Turumin *et al*., 2013), proteins, peptides and other amphiphilic molecules can also act as emulsifiers, further increasing the surface area. The rate of triglyceride digestion increases with decreases in emulsion droplet size (McClements and Li, 2010), owing to the greater surface area for lipase activity. Colipase adsorbs to the interface and binds lipase anchoring it to the interface and activating it (Brannon *et al*., 2013). Pancreatic triglyceride lipase, with the help of colipase, removes fatty acids at either end (carbon positions 1 and 3) of the triglycerides. The activity of pancreatic triglyceride lipase and colipase are supported by other lipases, including cholesterase. As with many aspects of *in vitro* modeling, adequate characterization of the composition and enzymatic activities of the digesta should be considered. Porcine bile extract is often used (Minekus *et al*., 2014), although, depending on the application, single or combinations of individual bile acids may be used, as is fresh porcine bile, when a reliably consistent supply is available. *In vitro* models sometimes investigate the rate of lipid hydrolysis by direct or indirect quantification of

liberated fatty acids or the partitioning of lipid species into the aqueous phase of the digesta. In order to cross the unstirred water layer and reach the epithelial cells, lipids must be incorporated within the mixed bile salt micelles, vesicles and other aqueous phase colloidal structures which form (Wilde and Chu, 2011). When lipids are not hydrolyzed and absorbed, they are excreted, as they can be when insoluble complexes form, such as fatty acid calcium soaps.

5.4 Interactions and Structural Considerations

Interactions between macronutrients in foods can significantly affect digestion. *In vitro* digestion experiments have been applied to investigate these interactions and the nutritional implications. For example, the impacts of dietary fiber on macronutrient digestion are discussed in Chapter 4. The structure of food materials can also impede the access of digestive enzymes to the nutrients of interest. For example, the presence of intact cell walls in plant materials blocks the access of enzymes to protein bodies, oil bodies and starch granules within the cell. In a human study of almond digestion it was observed that only 8–12% of the oil was released during digestion of whole almonds cut into 2–3 mm sized particles (Berry *et al.*, 2008) This corresponded with the clinical observation that the postprandial serum triglyceride response was significantly higher for almond oil and defatted almond meal as compared with whole almond particles, all consumed in equivalent amounts.

Interactions between food ingredients and the gastrointestinal environment can also impact particle size. Particle size has a pronounced effect on digestibility because it increases the surface area for enzyme activity. This is particularly relevant for lipid digestion since pancreatic triglyceride lipase, with the cofactor colipase, acts at the oil–water interface. Proteins and other amphiphilic molecules located at the surface of oil droplets can act as emulsifiers facilitating reduction in particle size, impacting the absorption of digestive enzymes, and even form impenetrable coatings, depending on the characteristics of materials at the interface.

The amounts of protein and fat in a meal affect the gastric emptying rate *in vivo* (Jahan-Mihan *et al*., 2011). This means that a food bolus can effectively be mixed at low pH in contact with gastric enzymes for variable lengths of time. Fat, protein and, to a lesser extent, resistant starch, trigger the ileal brake mechanism, thereby increasing the upper intestinal phase digestion time (Van Citters and Lin, 2006). Thus the digestion time for different phases can vary depending on the composition of the meal. These factors should be taken into consideration when choosing an *in vitro* protocol.

Anti-nutritional factors found in certain foods can also impact protein and starch digestibility (Alonso *et al*., 2000; Dhingra and Jood, 2002; Rehman and Shah, 2005). Tannins, phytate, lectins, polyphenols, hemagglutinins as well as trypsin, chymotrypsin and α-amylase inhibitors are commonly found in plant foods including legumes and cereals. Starch and protein digestibility have been shown to be negatively correlated with phytic acid content, polyphenol content, trypsin inhibitor activity and α-amylase inhibitor activity in bread containing barley or soy flour (Rehman and Shah, 2005).

Lastly, regardless of the *in vitro* digestion apparatus, digestion parameters should be selected to best approximate the likely gastrointestinal environment. For example, the presence of lipids leads to the secretion of fluids that significantly increase the bile, phospholipid and pancreatic lipase activity in the fed versus fasted states (Brunner *et al*., 1974). Similarly, health status and underlying physiology may also impact the digestive environment such that the most relevant conditions to utilize need to be adjusted. For example, research is underway to develop protocols which simulate digestion in an elderly population (Levi and Lesmes, 2014).

5.5 Post-Digestion Analysis

The chemical analyses conducted during or after *in vitro* digestion depend on the research objectives. For simple digestibility studies, the digesta is analyzed for small molecules which can be absorbed into the gut epithelium. However the digesta can also be sampled and analyzed for other purposes.

For available carbohydrate analysis, samples are frequently withdrawn at several time points to investigate the rate of digestion. Sugar analysis can be carried out either spectrophotometrically or by high performance liquid chromatography (HPLC). In foods where all of the available carbohydrate is glucose and starch, glucose oxidase methods can be used to measured glucose concentration spectrophotometrically. If brush border enzymes are not included in the digestion protocol, it is important to include di- and oligosaccharides in the analysis. Sucrose, lactose and maltose are all simultaneously hydrolyzed and transported into the gut epithelium by brush border enzymes (Leturque and Brot-Laroche, 2013). This can either be done by breaking the disaccharides down to simple sugars using enzymes like amyloglucosidase and invertase or by quantifying them using HPLC (Englyst *et al*., 1999). The starch not broken down to glucose can also be of interest (Fuentes-Zaragoza *et al*., 2011). The amount of resistant starch, the size of remaining dextrins and the structure, especially the degree of crystallinity, of the undigested starch, in the final digesta can be characterized (Brummer *et al*., 2015). Samples of digesta can also be analyzed for viscosity, particularly in dietary fiber research where it is desired to relate fiber physicochemical properties with gastrointestinal dynamics and nutrient digestibility (Regand *et al*., 2009).

To determine protein digestibility, free amino acids in solution are measured by HPLC (FAO, 2013). The cationic resins used are able to separate individual amino acids to be quantified and the profiles of the digestible protein can be compared with the total initial protein to see whether indispensable amino acids remain in the undigested fraction. *In vitro* digestion assays are widely used to investigate peptides which lead to allergic reactions (Wickham *et al*., 2009) and immune diseases (Shan *et al*., 2002). Bioactive peptides produced during digestion are of increasing interest. These small peptides, which are resistant to digestion, can be transported into the bloodstream where they may exert additional functions (Jahan-Mihan, 2011). For example, cholecystokinin (CCK), peptide YY (PYY) and glucagon-like peptide 1 (GLP-1) are gut hormones which regulate digestion and feelings of satiety. Two tripeptides, i.e. valine-proline-proline (VPP) and isoleusine-proline-proline (IPP) produced during the digestion

of milk have been shown to lower blood pressure by inhibiting angiotensin-converting enzymes (ACE). *In vitro* digestion protocols have been very useful for isolating and studying these peptides. Larger peptides and undigested proteins can be characterized by HPLC or polyacrylamide gel electrophoresis (Butts *et al*., 2012).

Lipid digestion can be monitored by a variety of methods, some of which include a step to isolate the hydrolyzed from the non-hydrolyzed lipids. Centrifugation of digesta samples can be used to obtain the fraction of digested lipids solubilized within the aqueous phase (i.e. micelles, vesicles) since undigested lipid will rise and calcium soaps and other material will precipitate (Kean *et al*., 2008; Kean *et al*., 2011). The fatty acids released during hydrolysis of triglycerides can be quantified by using a non-esterified fatty acid enzymatic kit or by titration, as is commonly done using the pH-stat method. Lipid digestion can also be studied using thin layer chromatography (TLC) (Sek *et al*., 2001), gas chromatography (GC), and HPLC-MS. Samples of digesta can also be observed under the microscope to study inaccessible fat, emulsions or microcapsules, potentially designed to protect fat from digestion (McClements and Li, 2010) and digesta emulsion size distribution can be determined by light scattering techniques (McClements *et al*., 2008). The physical state of the undigested lipid and the ratio of liquid to solid fat can also be determined using nuclear magnetic resonance (NMR) or differential scanning calorimetry (DSC).

This chapter is intended to focus on nutrient digestibility. However, samples of digesta are commonly used in cell culture assays with gut epithelial cells where the intent is to determine the potential bioavailability of nutrients following the *in vitro* digestion assay (Coles *et al*., 2013). Upper gut *in vitro* digestion protocols can also be used to obtain "feedstock" fluids for use with lower gut *in vitro* digestion systems. In such cases, macronutrients need to be digested from foods before the resultant materials can be fermented by the gut microbiota. The digesta is then used to obtain information on the influence of foods and supplements on the, types, numbers and proportions of different bacteria likely to be present in the large intestine (Guerra *et al*., 2012).

5.6 *In vitro* Models

Investigators have been using *in vitro* digestion protocols for more than 100 years (Lea, 1890). More than 150 methods have been published (Guerra *et al*., 2012; Hur *et al*., 2011; McClements and Li, 2010; Woolnough *et al*., 2008). Although they share common elements, they vary in terms of the details. The number of digestion phases and incubation times vary. Similarly, the choice of enzymes and their concentrations range widely. Some protocols include bile acids, phospholipids and mucin, but many do not. The composition of simulated digestive fluids and pH also often differ. The equipment used ranges from a beaker on a hot plate to complex, computer-controlled machines with sophisticated engineering and which require significant skill and training to operate. Only the temperature, 37 °C, remains constant. Rather than attempting to summarize all of the different protocols below, a few have been chosen to describe in detail. A recent general approach proposed by a group of experts working in the field is explained (Minekus, *et al*., 2014). In addition, streamlined protocols specific to available carbohydrates (Englyst *et al*., 1999), proteins (Astwood *et al*., 1996) and lipids (McClements and Li, 2010) are described. The basic parameters are compared in Table 5.1.

5.6.1 Static Models

5.6.1.1 INFOGEST Method for General Nutrient Digestion

Recently, a collaborative effort by an international network of scientists resulted in the proposal of a standardized static *in vitro* digestion method for use with foods (Minekus *et al*., 2014). The group reviewed the available literature concerning the conditions in the gastrointestinal tract and current *in vitro* methods and proposed a general framework for the study of available carbohydrates, protein and lipids. Formulations for electrolyte solutions mimicking those found in salivary, gastric, and intestinal fluids were rationalized for diluting food samples and enzyme addition. These fluids contain physiological levels of potassium, sodium, chloride, phosphate, carbonate, magnesium and calcium. The paper recommended that the particle size of solid food samples be reduced using a mincer similar to a

Table 5.1 Description of basic *in vitro* digestion parameters: general procedure and specific foods (available carbohydrates, proteins and triglycerides in emulsion).

		General	Available carbohydrate	Protein	Triglyceride in emulsion
		Minekus *et al.*, 2014	Englyst *et al.*, 1999	Astwood *et al.*, 1996	McClements and Li, 2010
Oral phase	Particle size reduction	Food mincer (<2 mm)	Minced crushed or broken	Ground in a mill, defatted	Not required for liquids
	Diluent	Simulated salivary fluid	None		
	pH	6.8	As is		
	Amylase	75 U/mL	None		
	Time	2 min	Not defined		
Gastric phase	Mixing	Stirrer, shaker or impeller	Shaking water bath, tubes with glass balls	Shaking water bath	
	Diluent	Simulated gastric fluid	50 mM HCl + 5 g/L guar gum	30 mM NaCl	
	pH	3	2	1.2	
	Enzymes:				
	Pepsin	2000U/mL	5 g/L	0.16%	
	lipase	None	None	None	
	Time	2 h	30 min	60 min	

(*Continued*)

Table 5.1 (Continued)

		General	Available carbohydrate	Protein	Triglyceride in emulsion
Upper intestinal phase	Mixing	Stirrer, shaker or impeller	Shaking water bath, tubes with glass balls		pH-stat titrator with stirring at $4\,s^{-1}$
	Diluent	Simulated intestinal fluid	500 mM acetate buffer		Simulated small intestinal fluid
	pH	7	5.2		7
	Enzymes	Pancreatin (100 U/mL trypsin) or individual	3.4 mg/mL pancreatin + amyloglucosidase + invertase		2.4 mg/mL pancreatin
	Bile acids	10 mM	None		20 mg/mL
	Time	2 h	2 h		30 min

meat grinder and that the resulting mince should be mixed with simulated salivary fluid to form a thin paste. The particle size should be less than 2 mm and mixing with salivary amylase should proceed for 2 min at 37 °C. Although this is longer than typical chewing, the time was selected to ensure thoroughly mixing with the enzymes. *In vitro* samples are recommended to be "homogenized using a stirrer, shaker or impeller", with no particular mixing technique suggested. Both these points highlight the challenges that exist with respect to standardizing a method which is physiologically relevant and can be used across a variety of applications.

To simulate the gastric phase, an equal volume of the simulated gastric fluid is added. Porcine pepsin is added to obtain a final concentration of 2000 U/mL and mixing continues for 2 h at 37 °C. The pH is allowed to acclimatize based on the characteristics of the food bolus and is therefore variable across samples and can fluctuate with digestion time. But an average pH 3 is maintained throughout the gastric digestion. *In vivo*, the stomach pH drops gradually and chyme leaves the stomach in small increments over 1–4 h. However, average conditions were chosen for practical reasons. For some samples, such as purified proteins or fats which do not contain surfactants, phospholipids may be added to increase the rate of digestion.

The upper intestinal phase is the most complex stage described in Minekus *et al*. (2014). To transition to this step, the gastric chyme is diluted with an equal volume of simulated intestinal fluid (less other additions) and adjusted to pH 7.0. Porcine pancreatin containing all of the digestive enzymes and cofactors in appropriate proportions can be used. The amount added should be calculated to deliver 100 U/mL of trypsin activity based on the final volume. Alternatively, purified pancreatic trypsin (100 U/mL), chymotrypsin (25 U/mL), α-amylase (200 U/mL lipase (2000 U/mL), and colipase (half the weight of lipase) can be used. Bile extract (bovine or porcine) or fresh (frozen) porcine bile (10 mM) may be added to help emulsify lipids. The intestinal digesta is then mixed for 2 h at 37 °C.

One of the important strengths of the INFOGest consensus method is that it included consultation and validating experiments from many groups. The general method is advanced as a set of conditions that are practical to work with and which are

close enough to approximate physiological conditions that they can effectively answer many research questions. The approach can be adapted, depending on the investigational purpose, making it either more complex or simpler, as needed.

5.6.1.2 Englyst Method for Rate for Carbohydrate Digestion

The Englyst Method is probably the most widely known *in vitro* method for carbohydrate digestibility (Englyst *et al*., 1999). It is a simplified method designed to measure the rate of starch digestion. Foods should be prepared "as eaten" so that the starch is gelatinized to the appropriate degree. The particle size is reduced as though the food were chewed, for example by being passed through a mincer with 0.9 cm holes. There is no oral digestion phase in this model and no salivary amylase is added. A sample of food containing ~500 mg of carbohydrate (weighed accurately) is mixed with 10 mL of pepsin-guar gum solution which contains 5 mg/mL pepsin and 5 mg/mL guar gum in 50 mM HCl. The guar gum helps to standardize the viscosity and prevent sedimentation. The Englyst method is very specific about the apparatus to be used. The digests are prepared in 50 mL capped test tubes. The tubes are clamped into a rack in a shaking water bath (37 °C) so that they are horizontal and with the long axis in the direction of the movement. The samples are shaken for 30 min. Five mL of 500 mM sodium acetate buffer (pH 5.2) and 5 glass balls (1.5 mm diameter) which aid in mixing are added to each tube. One at a time, enzyme solutions containing pancreatin, amyloglycosidase and invertase are added to the tubes. The final pancreatin concentration in the digesta is 3.4 mg/mL. The amyloglucosidase and invertase are added to mimic the activity of brush border enzymes. Exactly 20 min after addition of the enzymes, 0.2 mL digesta is removed from each tube, in order, and mixed with ethanol to stop the enzyme reaction. The tubes are immediately returned to the water bath before the next tube is sampled. After 120 min, another 0.2 mL of digesta is collected and mixed with ethanol. Once all of the tubes have been sampled this second time, the remaining digesta is boiled.

The digestion pattern is assessed by measuring the sugar present in each of the three samples obtained. The free sugars

in the original food samples are measured separately. The proportion of starch hydrolysed to glucose in the first 20 minutes plus the free sugars is referred to as rapidly available glucose. The starch degraded between 20 and 120 min is referred to as slowly digestible starch (SDS) and the starch not digested after 2 h is called resistant starch. In many cases, there is good correlation between the amount of slowly digestible starch and glycemic index, a measure of glycemic response to starchy foods (Englyst *et al*., 2003). However, the results are extremely dependent on the sample storage and preparation; cooling, freezing, heating, drying and particle size all affect the susceptibility of starch to amylase activity.

Recently the above method was automated. Predicted glycemic index (pGI) can now be determined using a NutriScan GI20 Glycemic Index Analyser (Next Instruments, Condell Park, NSW, AU). The instrument mixes, adds enzymes and measures glucose concentration at predetermined intervals. The data are collected automatically and the pGI is calculated by the software.

5.6.1.3 Streamlined Protein Digestibility

Because of the importance of gastric processing for protein digestibility, protein-based foods are sometimes studied using *in vitro* methods which include only the gastric stage. This is true in studies of food allergenicity, including in the widely cited Astwood *et al*. (1996) publication.

A flask or tube containing simulated gastric fluid (SGF) with pepsin is preheated to 37 °C for 5 min. The SGF-pepsin consists of 0.32% pepsin in 30 mM NaCl and the pH is adjusted to 1.2 using HCl. After addition of an equal amount of dilute protein solution, the flask is shaken in a temperature-controlled water bath. Aliquots are withdrawn into vials containing 0.2 M sodium carbonate solution (ratio of 3 parts $NaCO_3$ to 10 parts SGF) to inactivate the enzyme at intervals up to 2 h of incubation. The degree of proteolysis at each time point can be determined by polyacrylamide gel electrophoresis.

This method is quick, easy, inexpensive and requires minimal equipment. It has been widely used as per Astwood *et al*. (1996), or with modifications, to identify allergenic peptides and to produce bioactive peptides. It has also been used to investigate

how methods of protein modification, such as glycation and crosslinking, affect gastric digestion.

5.6.1.4 pH Stat Method for Testing Emulsified Lipids

McClements and Li (2010) recommended a simple method for use in screening lipid digestibility from emulsion samples. This method simulates only the small intestinal phase of digestion and is conducted in a pH-stat titration unit. The sample is mixed with simulated intestinal fluid to give final concentrations of 150 mM sodium chloride, 10 mM calcium chloride, 2.4 mg/mL pancreatin (containing 100–400 U/mg protein) and 20 mg/mL bile extract in the digesta. As the digestion proceeds and fatty acids are released, 0.1 mM NaOH is used to automatically maintain the pH at 7.0, allowing the user to monitor and quantify increases in acidity with fatty acid hydrolysis over time. To correct for an underestimation of fatty acid lipolysis due to the formation of calcium soaps, a back titration at higher pH can be applied (Williams *et al*., 2012). Generally, lipid digestion in the pH stat reaches a plateau within 30 min, allowing multiple digestions to be completed in a day. The relatively simple preparation and use of a common apparatus also make the method attractive, although it omits the oral and gastric stages. The pH stat technique has been used to demonstrate that emulsions containing medium length fatty acids were digested faster than those containing long chain fatty acids and that small emulsion droplets are digested faster than large emulsion droplets. It has also been used to investigate how different emulsifiers, proteins and polysaccharides at the interface influence digestion rate.

5.6.2 Dynamic

Dynamic *in vitro* digestion methods have the advantage over static models of greater physiological relevance. Mechanized systems intended to closely approximate key and/or targeted aspects of human digestion and which can be modified based on the application have been developed. For example, TNO in the Netherlands manufactures a commercial *in vitro* digestion instrument with high predictive quality (Minekus *et al*., 1999). The TIM-1 instrument mimics the gastric and upper intestine

in chambers with peristaltic mixing. TIM-2 is the TNO platform specific to the lower intestine. Enzyme and bile salt addition and pH control in the TIM-1 are computer controlled. There are multiple sampling ports for easy access to the digesta and sampling to monitor digestion. All aspects of mixing, dilution, chamber emptying, pH, biosurfactants, enzyme addition, etc. can be programmed to occur as per a pre-defined set of conditions. This fact, combined with investment to optimize the system and parameters against conditions of the human gastrointestinal tract, have resulted in the TIM-1 being widely used for *in vitro* digestion modeling of food systems.

The Institute of Food Research (Norwich, UK) has also developed a dynamic gastric model (DGM) which is comprised of a fundus and antrum of the stomach integrated with a simulated upper intestine (Kong and Singh, 2008; Fanbin *et al.*, 2010). This system was designed to specifically improve upon the study of food breakdown in the stomach. The gastric compartment is made of latex and peristaltic contractions are applied with rollers and belts. Gastric secretions are added gradually to decrease the pH and incorporate pepsin and gastric mucin. The chyme produced is ejected in small aliquots. The system has been used to demonstrate, for example, that white rice breaks down to smaller particles than brown rice. The larger particle size of brown rice resulted in delayed gastric emptying and increased chyme viscosity (Fanbin *et al.*, 2011). This difference in disintegration and digestion may account for the lower glycemic response to brown rice.

A number of institutions have constructed their own computer controlled upper gut digestion systems. Typically they have two vessels, a stomach reactor and a duodenum reactor, with automated temperature and pH control. Peristaltic pumps feed the meal through the system to better represent the flow *in vivo* (Guerra *et al.*, 2012). They have been used to study the digestibility and characteristics of (Villeneuve *et al.*, 2013) and survival rates of probiotic bacteria (Mainville *et al.*, 2005; Tompkins *et al.*, 2011) They are also being used to simulate and compare populations with different digestion conditions, including infants (Ménard, *et al.*, 2014) and the elderly (Levi and Lesmes, 2014).

The major constraint on the use of dynamic apparatuses is cost. The complexity of engineering, operating and maintaining these systems is expensive. The systems may also be unnecessarily complex for some routine applications. For example, although dynamic *in vitro* digestion systems would be useful for determining digestibility of protein, they have been ruled out for routine use (Minekus *et al.*, 2014). To date, dynamic systems have been used relatively more often for pharmaceutical applications and less for routine analysis with foods. They are, however, moving the field towards a greater appreciation for physiological relevance, the need for feedback loops in studying gastrointestinal process as well as the need for standardization across experiments.

5.7 Limitation of *In vitro* Digestion Tests

The use of *in vitro* digestion methods has proven very useful for understanding how foods and nutrients are digested. However, they cannot accurately mimic all aspects of the human digestive system and researchers must be mindful of the limitations. In the long run, *in vitro–in vivo* correlations remain an important goal.

The human digestive system is responsive to the food ingested in ways that an *in vitro* system cannot replicate. Fluids flow in and out of the stomach and intestine to control viscosity and facilitate mixing and maintain suitable concentrations (Marciani, *et al.*, 2000). *In vitro* protocols generally add a fixed amount of buffer at each phase of digestion (Minekus *et al.*, 2014). Importantly, unless a dialysis step is included, water and products of digestion are not removed (Gauthier *et al.*, 1982), raising the potential for inhibitory feedback not observed *in vivo*. Likewise, the gastrointestinal tract has the ability control pH. However, for example, the stomach pH can range from 1–3 or even higher, depending on the composition and buffering capacity of the meal and is not constant from person to person (Bornhorst *et al.*, 2014). Some methods include constant monitoring of pH and titration to maintain predetermined pH, but most do not. The rate of release of digestive enzymes *in vivo* is also influenced by gut hormones, including CCK, PYY and

GLP-1, and hormonal responses to absorbed nutrients (Jahan-Mihan *et al*., 2011). *In vitro* methods are restricted to adding predetermined amounts of enzymes, unless manually adapted when guided by human data. *In vitro* methods can reduce the inter-individual variability, allowing investigators to focus on the characteristics of the food.

With static *in vitro* digestion models, the enzyme additions and pH changes are done all at once, rather than over time. Also, mixing is typically very different from the peristaltic motions of the intestines (McClements and Li, 2010). Conditions of mixing and shear are an aspect where dynamic models have significantly improved on physiological relevance to minimize, but not entirely eliminate, these concerns (Guerra *et al*., 2012).

In vitro digestions have limitations. However, there are instances where correlations with *in vivo* results have been observed, providing support for their utilization. Furthermore, they allow the investigation of mechanisms that could not otherwise be explored and for routine sample screening. Although differences between true *in vivo* digestion and *in vitro* testing influence the final results to some extent, they are not sufficient to disregard their value all together. Ideally, researchers would use *in vivo* results to optimize *in vitro* protocols and *in vitro* studies to better understand the chemistry and dynamics of the situation *in vivo*. Working back and forth between the two platforms will give the most complete understanding of how foods are digested.

5.8 Conclusions

Although *in vitro* digestion experiments cannot replace human studies entirely, for the study of macronutrient digestibility, they are very useful tools, enabling mechanistic investigations and comparisons between foods. For food researchers, these protocols offer a way to compare different foods and food processes relatively quickly. The ability to control enzyme concentration and physical conditions makes the results more repeatable, without the inter- and intra-individual variability of *in vivo* trials.

Over the years, a number of *in vitro* models have been developed and modified to suit different applications. They vary in terms of complexity, depending on the food being investigated, the information being gathered, and the resources available. The ideal *in vitro* digestion protocol would be accurate, rapid, inexpensive, robust, and simple (Butts *et al.*, 2012) and could be adapted to a wide range of foods and interests. In order to ensure the relevance of *in vitro* systems and comparisons between studies, researchers utilizing *in vitro* digestions should ensure thorough and accurate reporting of the protocols utilized. Efforts are being made to standardize static *in vitro* digestion methods for general use (Minekus *et al.*, 2014), although the potential of dynamic models validated against human conditions and results remains a worthy target.

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6

In vitro Approaches for Investigating the Bioaccessibility and Bioavailability of Dietary Nutrients and Bioactive Metabolites

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6.1 Introduction

The human food supply contains many thousands of chemical components. Discoveries during the 19th and 20th centuries revealed that only 42 of these organic compounds and minerals are essential for life. Research continues to define optimal ranges of these essential nutrients during periods of health and various disease states throughout the lifecycle. During the past 50 years, the health promoting properties of many non-essential compounds in foods and their metabolites have been demonstrated. In order for essential nutrients and other dietary compounds to sustain life and promote wellness, they must be transferred from consumed foods to target tissues where they may be utilized directly, converted to bioactive or inactive metabolites, and/or stored for later use. Stated alternatively, the compounds must be *bioavailable* (Holst and Williamson, 2008). This requires that the food is digested by a combination of physical, chemical and enzymatic processes that release molecules and minerals from their food matrices and convert more complex molecules into low molecular weight products. Assuming that the small molecules and minerals are water soluble or incorporated in soluble particles in the aqueous environment of the lumen, they may be

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accessible for uptake across the apical membrane of epithelial cells lining the gut mucosa, and possibly metabolized within these cells. A portion of the intracellular dietary compounds and/or its metabolites are transported across the basolateral membrane to the circulation for delivery to peripheral tissues.

Human volunteers represent the "gold" standard for examining the bioavailability of dietary compounds and orally administered drugs. Bioavailability generally is determined by quantifying the amount of the consumed parent compound or its bioactive metabolite(s) in plasma at various times following ingestion. The fraction of a compound or mineral in the consumed food that appears in plasma is generally referred to as being bioavailable. Buccal cells that line the inner cheeks and excreta may also be collected. Conducting such studies in human volunteers is labor intensive, costly and may have ethical constraints. This limits the ability to assess the effects of different varieties of plant foods, styles of processing and cooking plant and animal foods, co-consumed foods and ingredients, and the physiological and health status of human subjects on the bioavailability of compounds. The traditional view of bioavailability also fails to consider the uptake, metabolism and transport of dietary compounds and their gastrointestinal metabolites by the mucosal epithelium, especially if poorly absorbed into the circulatory system. Ingested compounds and their metabolites that are not bioavailable or retro-transported from the epithelium and excreted from the liver into bile and subsequently into the gut lumen are likely to enter the large intestine where they may be metabolized by the microbiota to products that are taken up and utilized by, and perhaps absorbed from, the colonic epithelium. One such example is soluble fiber that is fermented by the microbiota to short chain fatty acids such as acetate, propionate and butyrate (Russell *et al*., 2013). Butyrate is a major source of energy for the colonic epithelium and the absorbed short chain fatty acids have anti-inflammatory activity and participate in the regulation of gene expression. Similarly, many dietary polyphenols are poorly absorbed, but they are metabolized by the colonic microflora to phenolics that possess a variety of health promoting activities in mammalian cells (Del Rio *et al*., 2013). Some of the challenges that limit the investigation of bioavailability in human volunteers can be offset by using

animal models such as laboratory rodents, pigs and birds. However, differences in the physiology of digestion and absorption by these animals compared to humans, ethical considerations associated with the use of animals for research, and possibly costs may remain problematic.

In vitro models generally provide investigators with technically simple alternatives for examining the effects of diverse factors on the *bioaccessibility* and bioavailability of dietary components. Bioaccessibility refers to the partitioning of compounds and minerals in foods and beverages in the soluble fraction of chyme during digestion so that they are accessible for uptake by the epithelial cells lining the mucosal barrier of the upper gastrointestinal tract. Similarly, metabolism of dietary compounds by the microbiota in the lower gastrointestinal tract is generally studied by anaerobic incubation with cecal, colonic or fecal samples. Specific strengths of the *in vitro* models include cost effective screening of numerous types of foods, beverages and ingredients, well controlled testing of hypotheses, and the use of findings to generate hypotheses for the design of focused *in vivo* studies with animal models and human volunteers. *In vitro* studies also may provide insights to explain mechanisms responsible for *in vivo* observations.

The primary objective of this chapter is to review the characteristics of *in vitro* models of digestion and differentiated cultures of Caco-2 cells. Several examples of their use to address questions about the bioaccessibility and bioavailability of dietary compounds are provided. While these models have increased our understanding of the digestion and absorption of essential nutrients, attention will primarily be given to dietary phytochemicals with health promoting properties. Relevant reviews are cited when appropriate to direct the reader to informative sources for more detailed summaries of available information.

6.2 Static Models of *In vitro* Digestion

There are two general models of *in vitro* digestion, i.e., static and dynamic, designed to simulate the general process of digestion in monogastric mammals. Static *in vitro* models generally are biphasic and include simplistic simulations of the gastric and

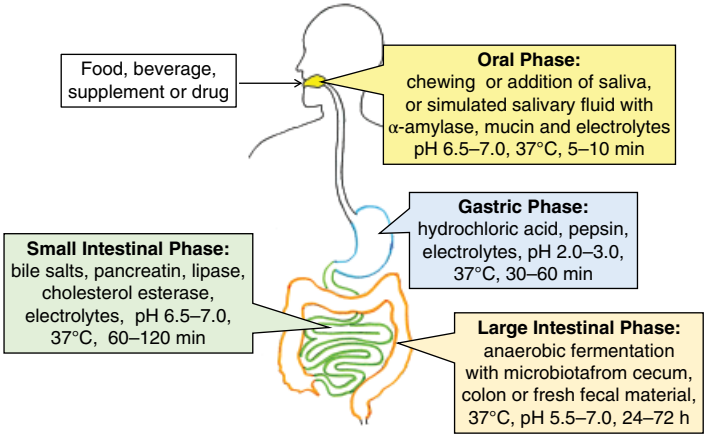

Figure 6.1 *In vitro* digestion of test foods, beverages, ingredients and compounds is designed to mimic the chemical, biochemical and mechanical characteristics for each phase of digestion. Both static and dynamic models of simulated digestion have been developed. The concentrations and activities of the various components in the simulated fluids for each phase of digestion remain constant and mechanical activity is limited to shaking in static models of digestion. In contrast, computer controlled variations in the digestive enzyme activities, pH, incubation time and peristaltic forces are modified in each compartment in dynamic models of *in vitro* digestion to better simulate physiological responses to the physical and chemical properties of consumed foods. The gastric and small intestinal phases of digestion are most often used to determine the stability and bioaccessibility of compounds in the upper gastrointestinal tract. The oral phase also can be included in simulated digestion, especially when the food has a high content of starch such as bread, cassava and potato. Dietary compounds that are poorly digested and absorbed in the upper gastrointestinal tract enter the large intestine where they may be transformed by the microflora into bioactive metabolites such as short chain fatty acids and phenolics. Such metabolites are generated during fermentation of dietary compounds added to cultures containing microorganisms present in either the lumen of the large intestine or freshly expelled feces.

small intestinal phases of digestion (Figure 6.1). Some investigators also include an oral phase of digestion, especially when working with foods containing high amounts of starch (e.g., Walsh *et al.*, 2003; Thakkar *et al.*, 2007). Others study anaerobic fermentation by colonic or fecal bacteria (e.g. Williamson and Clifford, 2010). The two phase method of gastrointestinal

digestion involves initial addition of an acidified solution containing pepsin and electrolytes to a homogenized food, an extract, a supplement or a beverage of interest. The final pH of the mixture is decreased to 2.0–3.0 and samples are incubated with continual mixing for 30–60 minutes at 37°C. Samples are then neutralized with sodium bicarbonate, pancreatic enzymes and either pure bile salts, crude bile extract or bile are added and the mixture incubated at 37°C for 60–120 minutes with continuous shaking at 37°C. Upon completion of the small intestinal phase of digestion, compounds and complexes released from the food matrix are separated from undigested materials by centrifugation or dialysis. The aqueous fraction is generally filtered to remove residual aggregates after centrifugation. Dietary components of interest in pre-digested material and chyme are quantified to assess their stability during simulated digestion. Similarly, components of interest in the aqueous fraction or dialysate are compared to that in pre-digested material to determine their bioaccessibility, i.e., release from the food matrix and solubilization in simulated small intestinal fluid. Because the quantities of enzymes, electrolytes and bile salts, as well as the pH, remain constant regardless of the composition of the test food, beverage or supplement, the model is "static". Nevertheless, this technically simple, low cost method provides the ability to screen the digestive stability and bioaccessibility of dietary components in diverse foods and to assess the influences of post-harvest processing, styles of cooking, and the presence of other foods and ingredients on the relative efficiency of their release from the matrix and potential accessibility for uptake by absorptive epithelial cells. The absence of feedback responses to the amounts of carbohydrates, fat and protein food in test foods that affect the amounts of digestive enzymes and bile secreted into the lumen, regional and temporal alterations in pH and electrolytes, transit times, and removal of digestion products taken up by the epithelium *in vivo* are all limitations of *in vitro* digestion. Moreover, specific conditions such as physical state and amount of food to be tested (e.g., particle size), biological origin, purity and activities of digestive enzymes, pH for gastric and small intestinal phases, and methods of separating compounds released from the matrix during digestion often vary across laboratories. Such differences confound comparison of

reported findings. In an effort to resolve this problem, an international group of investigators recently published a standardized static method for *in vitro* digestion (Minekus *et al*., 2014). The method is based on physiologically relevant conditions in the lumen of the human gut. Guidelines for the preparation of simulated fluids for oral, gastric and intestinal fluids, appropriate digestive enzymes for each phase and standardization of tryptic activity in crude preparations of porcine pancreatin vs. individual exocrine pancreatic enzymes are described. Despite some increases in the intensity of labor and cost of the standardized method, validation and adoption will greatly improve inter-laboratory comparisons of results.

6.3 Dynamic Models of *In vitro* Digestion

To more closely examine the complex physico-chemical, biochemical and mechanical processes during digestion, computer regulated mono-, di- and multi-compartmental models have been developed. For example, the Dynamic Gastric Model designed by Kong and Singh (2010) consists of a latex rubber vessel and external rollers that produce peristaltic waves similar to those that occur *in vivo*. Gastric digestion is initiated by placement of foods of interest in a mesh bag with 1.5 mm pores that is located within the latex vessel. Upon addition of simulated gastric juice, the sample of food and the gastric solution are mixed by the action of exterior rollers that shear and grind the food into small particles that pass through the pores of the mess bag. The mixture that passes through the pores is drawn to the base of the vessel that is connected to a peristaltic pump for continual removal of the chyme to simulate gastric emptying.

Greater complexity is offered by the TNO gastrointestinal model generally referred to as TIM-1 (Minekus *et al*., 1995). This is a dynamic multi-compartmental system that includes glass vessels lined with flexible walls to simulate the stomach and the three sections of the small intestine (i.e., duodenum, jejunum and ileum). Digestion is simulated by computer-activated gradual changes in pH, frequency and intensity of peristaltic forces, transit into and through each compartment,

sequential additions of digestive secretions and electrolytes, and passive removal of low molecular weight molecules via hollow fiber membranes. Contents diffusing into the fiber membranes are continuously collected from the jejunal and ileal compartments to determine bioaccessibility of compounds of interest. Further descriptions of these and other dynamic models of simulated digestion, specific examples of their use for investigating the digestion and bioaccessibility of nutrients, allergens and toxicants, as well as limitations and remaining challenges for dynamic models of digestion, have been reviewed by Guerra *et al*. (2012) Dynamic *in vitro* digestion models also have limitations. For example, the absence of epithelial cells and endogenous mediators that affect functions of absorptive cells preclude consideration of the intestinal cell uptake, metabolism and absorption of compounds that diffuse into the hollow fiber membranes.

6.4 Application of *In vitro* Digestion Method for Determining the Digestive Stability and Bioaccessibility of Dietary Compounds

In vitro digestion has been used to assess the influence of plant genotype, post-harvest processing, food matrix, formulation of foods and beverages, other components in the food and beverage and styles of cooking on the bioaccessibility of nutrients, phytochemicals and toxicants (e.g., Glahn *et al*., 1998; Glahn *et al*., 2002; Bechoff *et al*., 2011; Neilson and Ferruzzi, 2011; Failla *et al*., 2012; Lipkie, *et al*., 2013; Failla *et al*., 2014; Berni *et al*., 2014; Lemmens *et al*., 2014). Readers are encouraged to consult reviews that have summarized results from numerous studies using *in vitro* static and dynamic models of digestion to investigate the bioaccessibility of compounds from plant and animal based food (Hur *et al*., 2011; Alminger *et al*., 2014; Bornhorst *et al*., 2014; Carbonell-Capella *et al*., 2014). Several examples of the use *in vitro* digestion as a cost effective tool for investigating the influence of formulations of foods and beverages on the stability and bioaccessibility of specific compounds follow.

Flavan-3-ols are a class of polyphenols that are present in relatively high concentrations in some fruits, vegetables and botanicals (e.g., grapes, cocoa and tea). Epidemiological studies have suggested that the amount of foods and beverages rich in flavon-3-ols consumed is inversely correlated with the incidence of several non-communicable chronic diseases (Scalbert *et al*., 2005). These benefits have been attributed to catechins, the most abundant class of polyphenols in tea. However, the bioavailability of catechins is poor due to their instability (autooxidation and epimerization) during digestion in the small intestinal and their efficient conjugation by phase II enzymes within and apical efflux from absorptive epithelial cells. Stabilization of the catechins during digestion represents a potential strategy to increase their absorption and thus their effective concentrations in target tissues (Neilson and Ferruzzi, 2011). Various ingredients, citrus juices and creamers are often mixed with tea to improve sensory properties and increase stability of the formulated products. The effects of such additives on the stability of green tea catechins during *in vitro* digestion has been investigated (Green *et al*., 2007). Recovery of catechins after simulated gastric and small intestinal phases of digestion was markedly greater in the tea beverage containing 12 mg/100 mL ascorbic acid than in either plain green tea or tea containing other common ingredients added to foods (i.e., EDTA, citric acid, butylated hydroxytoluene, gallic acid and the vitamin E derivative Trolox) to limit degradation and development of off-flavors and colors. Similarly, total catechin recovery after *in vitro* digestion was greater in formulations of tea beverage containing 20–50% freshly squeezed citrus fruit juices and moderately improved in green tea beverages containing as high as 50% bovine, rice and soy milks. The presence of the citrus juices in the formulation stabilized epicatechin (EC), epicatechin-gallate (ECG), epigallocatechin (EGC) and epigallocatechin-gallate (EGCG) and was not solely related to ascorbic acid content. The investigators suggested that other polyphenols, proteins, sugars and fiber in the juices likely contributed to catechin stability during small intestinal digestion. Support for this hypothesis was demonstrated in a subsequent investigation (Peters *et al*., 2010). Both digestive stability and the bioaccessibility of the monomeric catechins as determined by the accumulation and retention in

Caco-2 cells was further enhanced when both sucrose and ascorbic were added to green tea.

Co-consumed fat is essential for the absorption of lipophilic nutrients, phytochemicals and drugs (e.g., Brown *et al*., 2004). This requirement is due to the need of ingested lipophilic compounds to be released from the food and capsular matrices, solubilized in lipid droplets and transferred along with lipid digestion products into bile salt micelles in order to be accessible for uptake by absorptive epithelial cells (Harrison, 2012; Borel *et al*., 2013). *In vitro* digestion has been used to study the influence of type and amount of dietary lipids on the bioaccessibility of compounds such as carotenoids and vitamin E. Such information is important for the development of dietary recommendations regarding consumption of dietary fat and for the strategic design of functional foods and beverages containing lipophilic health promoting compounds. Micellarization of carotenes and lycopene during simulated digestion was greater when the salad contained relatively low amounts (≤2.5%) of triolein rather than either trioctanoin or tributyrin (Huo *et al*., 2007). In contrast, micellarization of the more hydrophilic xanthophylls lutein and zeaxanthin was minimally affected by the addition of these triacylglycerides to the salad. The addition of common commercial lipids (3% wt:wt) to vegetable salad greatly increased the efficiency of micellarization of carotenoids and α-tocopherol during simulated digestion (Failla *et al*., 2014). Regarding the influence of type of dietary fat, micellarization of carotenes and lycopene, but not lutein, zeaxanthin or α-tocopherol, was greater during digestion of salad containing either olive oil, canola oil or soybean oil compared to salad with butter with its higher ratio of saturated vs. unsaturated fatty acids. Furthermore, the quantities of β-carotene, lutein and α-tocopherol transported across the monolayer of enterocyte-like Caco-2 cells was greater in cultures to which micelles prepared with mixtures of fatty acids that mimicked those present in the oils compared to micelles containing mixtures that mimicked butter were added to the apical medium. This difference was associated with the stimulatory effect of unsaturated fatty acids, and particularly oleic acid, on the assembly and secretion of chylomicrons that transfer bioaccessible carotenoids and vitamin E into circulation. These and other data suggest that co-consumed fat

stimulates the absorption of carotenoids, vitamin E and other dietary lipophiles by increasing both their bioaccessibility in the small intestinal lumen and chylomicron-dependent secretion into lymph following uptake by absorptive intestinal epithelial cells. Furthermore, dietary fat rich in unsaturated fatty acids enhances both processes to a greater extent than fat rich in saturated fatty acids (Goltz *et al*., 2012).

6.5 Caco-2 Cell Model

Primary cultures of cells and cell lines provide relatively simple models that allow researchers to investigate the transport and metabolism of dietary components and drugs, as well as the influence of environmental and endogenous factors on such processes. Because small intestinal absorptive cells are difficult to isolate, are leaky and rapidly lose viability, transformed cell lines provide an alternative (Frontela-Saseta *et al*., 2011). The Caco-2 human intestinal cell line is the most widely used model for investigating the characteristics and mechanisms of apical uptake, metabolism and retro- and trans-epithelial transport of compounds, their metabolites and electrolytes in consumed foods, beverages, supplements, extracts and medications. This cell line was established from a moderately differentiated colonic adenocarcinoma and is unique in that it spontaneously differentiates into a polarized columnar cell that morphologically and functionally resembles small intestinal enterocytes (Hildago *et al*., 1989; Delie and Rubas, 1997; Englund *et al*., 2006). Other characteristics of interest include the presence of intercellular tight junctions in confluent monolayers that create a barrier to the paracellular transfer of virtually all solutes, viruses and microbes, the expression of brush border enzymes that participate in the metabolism and influx of compounds and electrolytes, the expression of phase 2 enzymes and plasma membrane proteins that transport compounds and their metabolites across the brush border and basolateral membranes, and the presence of receptors for numerous hormones and immunological mediators on the basolateral membrane that modulate cellular homeostasis. Caco-2 cells also are capable of assembling and secreting chylomicrons and VLDL for the basolateral transfer of

lipophilic compounds and metabolites when cultured under prandial-like conditions (Luchoomun and Hussain, 1999). Global gene expression profiles have shown that differentiated Caco-2 cells are qualitatively similar to mature enterocytes, but that profiles for undifferentiated cells differ markedly from proliferating epithelial cells in villus crypts (Sun *et al*., 2002; Englund *et al*., 2006; Temblay *et al*., 2006). Caco-2 cells also respond to pro-inflammatory insults by secreting a variety of cytokines, chemokines and other mediators that activate other cells in the epithelial mucosa. Some studies have shown that the *trans*-epithelial transport and metabolism of nutrients, phytochemicals and orally administered drugs by differentiated cultures of Caco-2 cells is predictive of small intestinal absorption *in vivo* (Arturrson *et al*., 2001; Glahn *et al*., 2002; Chitchumroonchokchai *et al*., 2004; van Breemen *et al*., 2005; Reboul *et al*., 2006; Verwei *et al*., 2006; Bumrungpert *et al*., 2009).

Caco-2 cells can be grown in culture vessels directly adhered to the plastic surface or to a coating of the surface with extracellular matrix coating the surface (Figure 6.2A). This simple system is used to investigate the apical uptake, metabolism and apical efflux of compounds and ions. Cells also can be grown on semi-permeable membrane inserts that are suspended in wells of culture dishes to create a three-compartment system that simulates the luminal, cellular and basolateral regions of the mucosal epithelium (Figure 6.2B). Upon achieving confluency, cells begin to differentiate and after several weeks exhibit a tight barrier that separates the apical and basolateral compartments. This model facilitates investigation of apical to basolateral transfer of compounds and their metabolites, and the effects of endogenous mediators on transport and metabolism of compounds located within both the apical and basolateral compartments (Hubatsch *et al*., 2007). It is essential to demonstrate barrier integrity of the monolayer when assessing apical to basolateral and basolateral to apical flux for compounds of interest. Trans-epithelial electrical resistance (TEER) and/or flux of compounds that are transferred across the monolayer paracellularly (e.g., mannitol, inulin, phenol red and fluorescent Lucifer yellow) are commonly used to ensure integrity of the monolayer.

Despite the widespread use of Caco-2 cells for investigating the transport and metabolism of dietary compounds by absorptive intestinal epithelial cells, this model, like all cell models, has

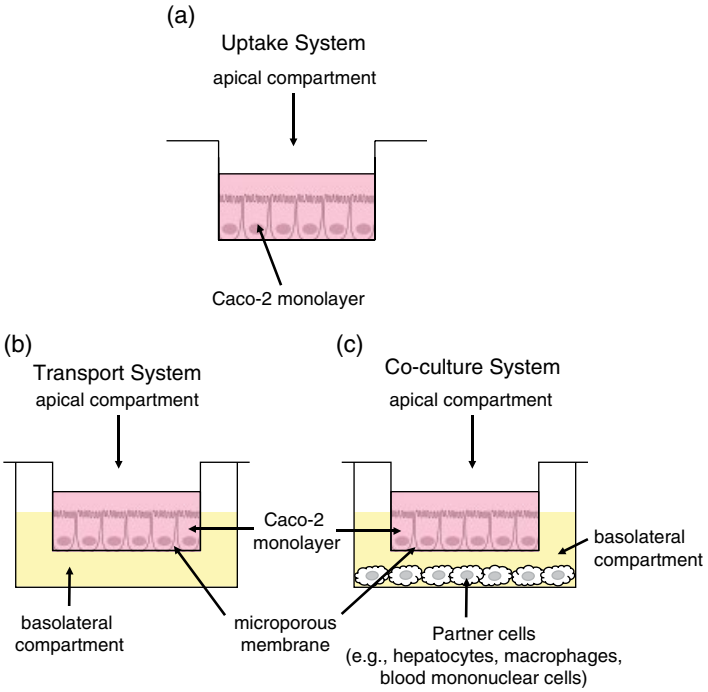

Figure 6.2 Caco-2 cell models are useful for investigating the uptake, metabolism and transport of dietary components by enterocyte-like small intestinal epithelial cells. Cells can be grown on plastic surface of wells (panel A) to study apical uptake, metabolism and apical efflux of bioaccessible dietary compounds and minerals. Cells can also be grown and differentiated on semi-permeable membranes in inserts placed in wells to create a three-compartment model to investigate the uptake, metabolism and *trans*-epithelial transport and secretion of dietary compounds and minerals, as well as the effects of compounds, extracts and digested foods on enterocyte-like cellular functions (panel B). Monolayers of differentiated Caco-2 cells in inserts may also be co-cultured with other cells types to investigate the influence of cell-cell interactions on the transport, metabolism and bioactivities of dietary compounds (panel C).

limitations. Because the parental cell line (HTB37) is heterogeneous, it is important to work with cells within a set range of passages as phenotypic characteristics of the cells change with increasing passage number and are also affected by using different types of medium and amounts of fetal bovine sera (Delie and

Rubas, 1997). Several stable clones such as TC7 have been established and some characteristics differ from both the parental cell line and normal human enterocytes (Turco *et al.*, 2011). As mentioned above, Caco-2 cells also do not synthesize and secrete mucin. Thus, compounds added to apical medium have direct access to the brush border surface without possible interactions with the mucus coating of the brush border surface that is present *in vivo*. Similarly, Caco-2 cells maintained in standard culture medium either lack or have low activities of cytochrome P450 enzymes. However, these enzymes are expressed when cells are exposed to appropriate inducers. For example, incubation of cultures in medium containing 1α, 25-dihydroxy-vitamin D_3 induces expression of CYP3A4, the most abundant cytochrome P450 in small intestinal epithelial cells (e.g., Schmiedlin-Ren *et al.*, 1997; Aiba *et al.*, 2005). Also, absorptive, metabolic and other relevant functional activities of gut epithelial cells are modulated by hormones, immunological mediators, oxidants and luminal microorganisms. Moreover, intestinal mucosa pore size in the healthy gut is 8–13A° (Fine *et al.*, 1995), but only about 5A° in confluent Caco-2 cells (Knipp *et al.*, 1997). This can underestimate paracellular flux of test compounds. The absence of endogenous factors with modulatory properties and other cell types in Caco-2 cell cultures may also confound comparison of results with *in vivo* activities of enterocytes.

6.6 Examples of the Effects of Bioaccessible Dietary Compounds on the Functions of Absorptive Intestinal Epithelial Cells

In addition to investigating the transport and metabolism of dietary compounds, Caco-2 cells also have been used to gain insights about how dietary compounds in more complex extracts from foods can influence one or more of the diverse activities of absorptive intestinal epithelial cells. One such example is the modulation of inflammatory response of gut epithelial cells to pro-inflammatory insults. The importance of such inquiry is evident as more than 1

million individuals in the United States suffer from inflammatory bowel diseases (IBD) (CDC, 2015). Crohn's disease and ulcerative colitis are the two major subtypes of IBD and their incidence in the United States has been estimated to be 201 and 238 per 100,000 adults, respectively (Kappelman *et al.*, 2007). These chronic, idiopathic conditions result from the dysfunction of complex interactions between the gut microbiota, gut epithelial cells and gut immune cells in genetically susceptible individuals. Therapeutic treatment generally consists of administration of immunosuppressive drugs and monoclonal antibodies that target tumor necrosis factor-α. Responses of individuals vary widely and are often accompanied by undesirable side effects (Fakhoury *et al.*, 2014). This situation has provided impetus to identify natural products, extracts and possibly intact foods that are efficacious and safe alternatives to prevent or attenuate the severity of chronic inflammation in the gut. Polyphenols are a diverse family of secondary metabolites in plants with anti-inflammatory and antimicrobial activities (Del Rio *et al.*, 2012). These compounds are ubiquitous in the human diet and their anti-inflammatory activities in gut intestinal cells and rodent models have been reviewed (Romier *et al.*, 2009; Martin and Boiling, 2015). The majority of studies have focused on the activities of a single polyphenol of interest. Far fewer studies have considered the effects of polyphenol-rich plant extracts as modulators of inflammatory responses in the gut. Consideration of such extracts is important as additive and synergistic effects of complex mixtures of phytochemicals in fruits and vegetables have been suggested to be responsible for health promoting activities of whole foods (Liu *et al.*, 2004).

Romier-Crouzet *et al.* (2009) investigated the anti-inflammatory activity of plant extracts on gut epithelial cells. Extracts from tissues of 74 distinct plants were pre-screened for phenolic content, anti-oxidant activity, and NF-κB activity as a marker of anti-inflammatory activity. Six promising polyphenol-rich extracts were selected for investigation using Caco-2 cells. These included extracts from grape seed, cocoa, oak, sugar cane, pomegranate and mangosteen. Caco-2 cell cultures were pre-treated with an extract before addition of one or more inflammatory cytokines to activate the cells. Pre-treatment of cells with extract from pomegranate suppressed the cytokine mediated-activation of NF-κB and ERK1/2 transcription factors and several of their target gene mediators

of inflammation, i.e., IL-8, prostaglandin E2 and nitric oxide (NO). Oak extract decreased activation of NF-κB and production of IL-8 and NO, whereas anti-inflammatory activities of the other extracts were limited. Based on these results, the investigators concluded that pomegranate was the most promising of all tested extracts for the prevention/attenuation of gut inflammation. More recently, Muto *et al.* (2015) reported the effects of pre-treating Caco-2 cells with phenolic extract from extra virgin olive oil on the inflammatory response of differentiated cultures of Caco-2 cells that had been activated with either LPS or IL-1β to simulate early and intermediate states of inflammation, respectively, within the gut. Decreased promoter activity of IL-8 gene in cells pre-treated with the olive oil extract was associated with reduced amounts of IL-8 mRNA and IL-8 protein in LPS-treated cells. This inhibition by the extract appeared to be the result of decreased activity of NF-κB which promotes transcription of the IL-8 gene. The extract also inhibited IL-1β mediated increases in activation of NF-κB and promoter activity of the IL-8 gene in differentiated Caco-2 cells. However, there was an increased level of IL-8 and mRNA in cells activated with IL-1β that had been pre-treated with the polyphenol-rich extract from olive oil compared to that in activated cells. This unexpected effect appeared to be due to activation of p38 and ERK pathways. These results suggests that IL-8 expression in enterocyte-like Caco-2-cells is differentially modulated by olive oil extract in cells exposed to early vs. intermediate inflammatory mediators by modulating transcriptional and post-transcriptional processes. In vivo studies are needed to determine the relevance of the findings of both of the above studies with Caco-2 cells. Nevertheless, these studies have provided interesting results that can be used to generate testable hypotheses in pre-clinical models of gut inflammation.

6.7 Coupling the *In vitro* Digestion and Caco-2 Cell Models

Glahn and associates (1998) pioneered the coupled static model of digestion with the Caco-2 cell model to determine iron bioaccessibility from simple solutions and foods. Chyme generated during gastric digestion in a polypropylene tube was placed in a plastic

cup with a base of dialysis membrane (molecular weight cut-off, 12,000–14,000) hanging in a well that contained a monolayer of differentiated Caco-2 cells. Small intestinal digestion then was initiated in the cup. Iron released from the matrix during a 2-hour incubation diffused through the dialysis membrane. After removal of the digestion chamber, cultures were incubated overnight. Bioaccessibility was estimated by the increase in the intracellular quantity of ferritin, the intracellular iron storage protein.

The approach was subsequently modified by Garrett *et al.* (1999) to examine the bioaccessibility of lipophilic compounds in foods. Gastric and small intestinal phases of digestion of carotenoid-rich foods were performed in sealed reaction tubes. Upon completion of digestion, the aqueous fraction of chyme was separated from non-digested materials by centrifugation with the supernatant filtered (0.2 μm pores) to yield the mixed micelle fraction containing the carotenoids and other lipophilic products of digestion. This fraction was diluted with DMEM medium to avoid adverse effects of residual digestive enzyme activity on cellular and monolayer integrity. Cellular accumulation of carotenoids from apical medium during a 4-hour incubation confirmed the accessibility of carotenoids that partitioned in mixed micelles during simulated digestion. When diluted medium containing the micelle fraction generated either during simulated digestion or mixed micelles that were prepared *in vitro* was added to mono-layers of Caco-2 cells grown on semi-permeable membrane inserts, apical uptake, metabolism and trans-epithelial transfer and apical efflux of the dietary compounds, drugs and their metabolites can be determined (e.g., Chitchumroonchokchai *et al.*, 2004; Failla *et al.*, 2014).

A novel use of the coupled *in vitro* digestion/Caco-2 cell model compared transport of catechin monomers and proanthocyanidin oligomers in grape seed extract (Wang *et al.*, 2013). Although this extract has been associated with the promotion of health, *in vivo* absorption of catechins and proanthocyanadins is very low. Consequently, a portion of these ingested compounds and their degradation products generated during transit through the small intestine enter the large intestine where they are metabolized by the microflora to phenolic acid derivatives. Several such microbial metabolites have been detected in plasma and may affect physiological functions. The investigators separately

incubated grape seed extract with samples of porcine ileal fluid and feces to examine anaerobic microbial production of phenolic metabolites and their transport across monolayers of Caco-2 cells. Two fermentation products, tentatively identified as 3-(3-hydroxyphenyl)propionic acid and 5-(4-hydroxyphenyl) valeric acid, were produced in sufficient quantities to assess transport across the cell monolayer. Both compounds were stable in apical medium and trace amounts were transported across the monolayer during a 30-minute incubation. In contrast, none of the compounds in grape seed extract added directly to apical medium of cultures of Caco-2 cells were detected in basolateral medium after similar exposure. This study supports the feasibility of coupling microbial fermentation of poorly absorbed dietary compounds with the Caco-2 and perhaps other colonic epithelial cell lines to investigate the transport and bioactivities of microbial metabolites generated in the large intestine.

As mentioned previously, one limitation of dynamic *in vitro* models of digestion has been the absence of epithelial cells to determine uptake of low molecular weight compounds released from the food matrix. It is noteworthy that Deat *et al.* (2009) combined the TIM-1 model with Caco-2 cell cultures to investigate the bioaccessibility of lycopene and α-tocopherol from red tomatoes mixed with sunflower oil. The hollow fibers and dialysis pump were disconnected and ileal deliveries were collected, diluted in medium and added to monolayers of Caco-2 cells adhered to the surface of wells. Both compounds were accumulated by the cells in a time dependent manner with the efficiency of α-tocopherol uptake exceeding that of lycopene as had been reported for *in vivo* studies.

6.8 Co-culture Models Using Caco-2 Cells

Unlike mono-cultures of Caco-2 cells, *in vivo* functions of absorptive small intestinal epithelial cells are influenced by the activities of other types of cells (e.g., immune, goblet and enteroendocrine cells) located within the mucosa, microorganisms and their metabolites present in the gut lumen, and various mediators secreted by peripheral tissues (e.g., hormones and host defense proteins). Monolayers of differentiated Caco-2 cells grown on membrane inserts have been added to wells containing other

types of cells to better simulate physiological conditions that can influence the transport, metabolism and bioactivities of dietary compounds (Figure 6.2C). For example, hepatic regulation of intestinal uptake and basolateral transport of iron has been investigated using co-cultured Caco-2 cells with HepG2 human hepatocytes (Scheers *et al.*, 2014). Both apical uptake of iron and its transfer across the basolateral membrane were greater in the co-culture model than in mono-cultures of Caco-2 cells. The increased uptake and transport of iron in the co-culture model was associated with greater expression of divalent metal transporter (DMT1) and ferroportin on the apical and basolateral membranes, respectively, of Caco-2 cells. Addition of fucoidan, a sulfated polysaccharide in brown seaweed, to the apical compartment of the Caco-2 monolayer was shown to attenuate secretion of TNF-α by RAW264.7 cells despite the absence of detectable polysaccharide in the basolateral compartment (Tanoue *et al.*, 2008). This reduction on TNF-α secretion resulted in decreased expression of IL-8 in both Caco-2 and HepG2 cell. These results suggested that fucoidan induced cross-talk between Caco-2 and the macrophage-like cells and that co-culture of these two cell types provides a useful model for the screening anti-inflammatory factors in foods. Another interesting example is co-cultures of Caco-2 cells and goblet-like, methotrexate-resistant HT-29 cells. Appropriate mixing of these two different types of cells results in a monolayer with a coating of mucin gel on the apical surface with thickness similar to that *in vivo* (Walter *et al.*, 1996). Para-cellular permeability of several drugs was greater in the co-culture model than in mono-cultures of Caco-2 cells and better correlated with the extent of absorption in humans (Walter *et al.*, 1996; Chen *et al.*, 2010). The Caco-2/HT-29-MTX model also has been used to assess the influence of the mucin coating of the cell surface on apical uptake of iron during small intestinal digestion of mixtures of highly accessible iron, e.g., $FeCl_3$ + ascorbic acid (Mahler *et al.*, 2009). Cell ferritin content, an indicator of iron uptake, was lower in co-cultures compared to the Caco-2 cell mono-culture, suggesting that binding of iron to the anionic glycoprotein gel inhibited its accessibility to the divalent metal transporter located in the brush border membrane. Additional examples of observations from co-culture systems containing Caco-2 and other types of cells are listed in Table 6.1.

Table 6.1 Examples of caco-2 co-culture models

Caco-2 partner	Cell origin	Dietary component	Outcomes	References
HT-29-MTX (goblet-like cells)	Human	Curcumin	Curcumin was stabilized in trimyristin/emulsifier nanoparticles. Some particles in apical medium adsorbed to mucin layer. Trans-epithelial transport of curcumin to basolateral compartment was not enhanced by apical delivery in nanoparticles compared to simple emulsions. Results suggested that primary limitations for curcumin bioavailability are solubilization in gut lumen and extensive metabolism by absorptive epithelial cells.	Guri *et al.*, 2013
Monocyte-derived dendritic cell (DC)	Human	Oxidized cholesterol	7-ketocholesterol (KC) decreased integrity of Caco-2 barrier even in presence of DC. KC also ↓ IL-10 expression in both DC and Caco-2 cells. ↓ IL-10 expression favors maturation of DC which can exacerbate local inflammatory responses to ingested foods.	Chalubinski *et al.*, 2014
Macrophage-like RAW 264.7 cells	Mouse	Luteolin	Luteolin aglycone added to apical compartment of Caco-2 cell monolayer ↓TNF-α, IL-6 and IL-1β expression in RAW264.7 cells and IL-8 expression in Caco-2 cells in LPS treated co-cultures. The concentration of luteolin transported across the Caco-2 monolayer was sufficient to ↓ LPS induced expression of TNF-α and activation of NFκB in RAW264.7 cells that in turn decreased IL-8 production in Caco-2 cells.	Nishitani *et al.*, 2013

(*Continued*)

Table 6.1 (Continued)

Caco-2 partner	Cell origin	Dietary component	Outcomes	References
HepaRG cells (differentiated)	Human	βC, retinol	Apical addition of micellar βC and retinol to monolayers of TC7 clone of Caco-2 cells ↓ hepatocyte and ↑medium levels of retinol binding protein 4 and ↑retinoic acid-dependent expression of CYP26A. Exposure of hepatocyte mono-cultures to concentrations of βC and retinoids transported into basolateral compartment of the co-culture similarly ↑ induction of CYP26A1.	Rossi *et al*., 2012
HepG2 liver cells	Human	Benzo[a] pyrene (BP)	BP increased CYPA1/2 activities that biotransformed BP to toxic metabolites. Caco-2 cells protected HepG2 cells by ↑ apical efflux of BP and its toxic metabolites into the apical compartment	Choi *et al*., 2004
HepG2 liver cells	Human	Grape seed extract (GSE)	Profile of flavon-3-ols in basolateral compartment differed markedly from GSE added to apical chamber of Caco-2 monolayers. Identified compounds in basolateral medium included sulphated and glucuronidated metabolites, procyanidin dimers and trimers, and unconjugated compounds. The presence of these compounds was associated with ↓ synthesis of triglycerides and cholesterol in hepatocytes.	Castell-Auvi *et al*., 2010

Umbilical vein endothelial cells (UVEC)	Human	Grape anthocyanins	Caco-2 cells transported some compounds in anthocyanin extract and pure malvidin-3-glucose (M-3-glu) added to apical compartment to basolateral compartment. Presence of these compounds and ACN degradation products in basolateral medium or direct addition of same concentrations to cultures of UVEC treated with low dose TNF-α ↓leukocyte adhesion, ↓ expression of adhesion factors, and ↓ secretion of IL-8. and IL-6, and ↓ NF-κB mRNA in TNF-α activated UVEC. Suppression of the inflammatory response by activated UVEC was not observed when the epithelial barrier was a mixture (9:1 and 4:1) of Caco-2 cells and HT-29/B6. This lack of effect was associated with inhibited transfer of ACN and M-3-glu from apical to basolateral compartments.	Kuntz *et al*. 2015
Salmonella enteritidis	Bacteria	Iron	Elevated amount of intracellular iron in Caco-2 cells ↑ bacterial invasion and intracellular survival and ↑ expression of TGFα, IL-8 MCP1 and TNF-α	Foster *et al*., 2001

6.9 Conclusions

In vitro models of gastrointestinal digestion and small intestinal absorptive epithelial cells represent useful tools for investigating the digestive stability, bioaccessibility, bioavailability and health promoting activities of dietary compounds. *In vitro* digestion provides researchers with the ability to screen the effects of post-harvest processing techniques, styles of cooking and other foods on the bioaccessibility of compounds of interest. Differentiated cultures of Caco-2 human intestinal epithelial cells are morphologically and biochemically similar to small intestinal absorptive epithelial cells and have been widely used to investigate the apical uptake, metabolism and *trans*-epithelial transport and secretion of dietary compounds. Results obtained with both the digestion and cultured cell models facilitate the generation of hypotheses to be tested *in vivo*, as well providing novel insights to explain *in vivo* results related to the bioavailability of dietary compounds. Judicious use of the coupled *in vitro* digestion and Caco-2 cell model and co-culture models of Caco-2 cells with other cell types represent useful tools for investigating the health-promoting effects of diverse foods and extracts on the functions of the gut epithelium and its interactions with other cells in the gut mucosa and peripheral tissues. The model systems should be viewed as complementary tools rather than replacements for *in vivo* testing.

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7

In vitro Models for Testing Toxicity in the Gastrointestinal Tract

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7.1 Introduction

Historically, safety evaluation has been dependent on toxicology studies in animal models Toxicity in laboratory animals has been assessed mainly by analyzing clinical pathology parameters in blood and urine and histopathological examination of altered tissue as indicators of organ damage. These adverse health outcomes are studied in animals exposed at high doses in order to identify no-observed-adverse effect level (NOAEL) or the lowest level at which an adverse effect is observed (lowest observed-adverse-effect level, LOAEL). Subsequently, extrapolations to expected human responses at much lower doses are performed. However, the current animal-based perception of "no adverse effect levels" is challenged by modern techniques (e.g. genomics, transcriptomics, proteomics, metabolomics) that are extremely sensitive and enable the detection of adverse effects at very low concentrations of a compound. Furthermore, there is a generally negative view of animal testing by the wide public. Concurrently, the regulation of chemicals and food-related compounds puts a strong focus on prevention and has become stricter in terms of the toxicological data required for

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different compounds. The Food Safety Modernization Act of 2010 has transformed the US FDA into a system that aims to prevent outbreaks instead of trying to respond to them, as it did before this new legislation came into force. In order to achieve this, more toxicity studies are requested for all compounds that may be present or contaminate food through the whole food supply chain. In the European Commission, the Regulation on Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) that entered into force in 2007 demands toxicological data for all chemicals, forcing industry into testing a large panel of potentially hazardous substances. Consequently, REACH is calling for non-animal tests whenever possible and industry requires test methods that are cheaper and more relevant than animal experiments.

Overall, scientific advances, animal welfare concerns and ethical issues associated with animal use for toxicity testing, together with a growing number of chemicals, cosmetics and pharmaceuticals that require *in vivo* testing have led to a consensus for the imperative need to replace animal testing by alternative approaches. The visional strategy for replacing animal testing by alternative approaches is based on the "3Rs principles" (Reduction, Replacement, and Refinement) which have been encouraged in particular in Europe. The European legislation (Directive 2010/63/EU) requires the Commission and Member States to contribute to the development and validation of alternative methods and intends to reduce the number of animals used for experiments by requiring that an animal experiment should not be performed when an alternative test method exists.

Non-animal test methods, including *in vitro* studies and computer-based approaches are important tools for assessing hazardous effects of chemicals on humans. *In vitro* systems are mainly used for screening and ranking chemicals as well as for generating more comprehensive toxicological profiles. *In vitro* methods have been sporadically taken into account for risk assessment purposes for certain food additives. Cell models are gaining a growing interest among the scientific research community, resulting in their wider use in the pharmaceutical and cosmetic industry in particular. This chapter aims to overview the different intestinal cell models that are used for assessing the toxicity of food-related compounds particularly. Furthermore,

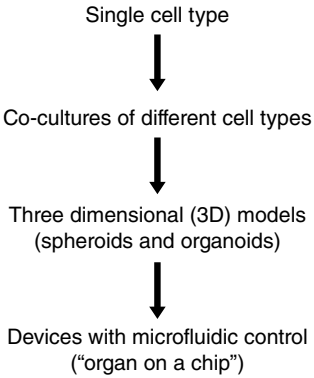

Figure 7.1 Progression toward more physiologically relevant (i.e. predictive) cell-based models.

some emerging models that have so far been used for assessing the effects of drugs to humans will be presented since it is expected that these models may also be applied in the future in food safety testing. The different models are grouped on the basis of their complexity, starting from simple models (single cell lines) to more complex and elaborate models (artificial organs) (Figure 7.1).

7.2 Advantages of *In vitro* Tests

Animal and human physiology and anatomy can be very different in some aspects, which make extrapolation of results from animal studies to the human context difficult. Being based on human-derived cells, which are more representative of the human response to hazardous chemical compounds than the animals, the emerging *in vitro* tests are considered to be more relevant than the traditional animal-based tests and are expected to increase the predictivity toward human toxicological effects (Berg *et al.*, 2011).

Furthermore, most animal tests are based on simply the observation of adverse effects caused by a chemical rather than looking at the underlying cause. The effect of a toxic chemical on a biological system in most cases is fundamentally reflected,

at the cellular level, by its impact on gene expression. Thus, measurement of the transcription (mRNA) and translation (protein) products of gene expression can reveal valuable information about the potential toxicity of chemicals before the development of a toxic/pathological response. Such information enables the in-depth analysis of mechanisms of toxicity both at the cellular and molecular level and the evaluation of both causal and adaptive responses. Enhanced understanding of the mechanism of toxicity can also lead to the identification of key molecular events that drive toxicity, enabling the development of biomarkers of effect. Such biomarkers can be used to assess the exposure of humans to the compound that caused the toxicity, biological changes or susceptibility that can be investigated directly in human population (Anadón *et al*., 2013).

Through modern miniaturization and automation techniques, cell culture models can support massive screening and cost-effectiveness in contrast to the more expensive animal trials with limited screening capacity. Additionally, cell line models simplify biological systems to an easier and more manageable platform for research and provide well defined systems. Simplicity, reproducibility, low-cost and high-throughput enable studies that are otherwise not easy to perform in animals; studies regarding structure–activity relationships and investigations of the concentration–effect relationship that can lead to the establishment of effect-specific thresholds.

7.3 Limitations of Established Cell Line Models

Most established cell-line models that are commonly used for *in vitro* studies share some common limitations. These cells are usually derived from tumors and are thus transformed. Human cells that are derived from normal tissues need to be immortalized. In other *in vitro* approaches primary cells are used. These cells can only be kept in culture for a certain time (hours to days) and in many cases will lose their differentiated properties during this culture time. These are inherent weaknesses due to the fact that, *in vitro*, the cells are isolated from their natural environment and are no longer integrated into an ordered tissue and

organ topology. This entails intrinsic limitations concerning reduced survival, imbalance of metabolic competence, and lack of cell–cell interaction, destroyed organ topology and absence of tissue communication.

There is an additional limitation that applies specifically to intestinal cell line models. The intestinal wall is composed of several cell types (mainly enterocytes) that form the epithelial barrier, accompanied by immune cells on the basolateral side of the epithelia. Therefore, an *in vitro* model should reflect both the natural responses as well as the complex physiology of the intestine.

7.4 Single Cell Lines

Primary cell cultures are rarely used in models due to limitations with repeatability and long-term studies because of their short lifespan. Most of the current cell line intestinal models are using transformed cell lines, isolated from cancer affected individuals. The two most commonly used human intestinal cell lines are Caco-2 and HT-29, which were isolated from colon adenocarcinoma (Cencič and Langerholc, 2010) (Zweibaum *et al.*, 2010). Among them, only Caco-2 has been studied in detail. Caco-2 cells are used as a model for mature human enterocytes since this cell line forms polarized monolayers in culture and differentiates into cells with high homology to small intestinal enterocytes (Rousset, 1986). HT-29 cells in culture, although essentially undifferentiated, are heterogeneous in that they contain a small proportion (i.e. < 5%) of mucus-secreting cells and columnar absorptive cells (Huet *et al.*, 1995).

A stable homogenous subpopulation of mucus-secreting cells (HT29-MTX) has been obtained from HT-29 cultures after treatment with methotrexate (Lesuffleur *et al.*, 1990). HT29-MTX cells exhibit entirely differentiated goblet cell-like phenotype secreting mucins predominantly expressed in the small and large intestine (Lesuffleur *et al.*, 1993; McGuckin *et al.*, 2011). Mucins are heavily glycosylated proteins that form mucus; a protective gel coating over the entire surface of the gastrointestinal tract. Mucus acts a selectively permeable barrier as it facilitates nutrients absorption while physically

protecting the epithelia from commensal bacteria and toxins (McGuckin *et al*., 2011).

Intestinal cell models can be applied in the risk assessment analysis of toxic substances that can be found in the food chain, such as toxins of microbial origin or chemicals. Caco-2, HT-29 and the mucus-secreting HT29-MTX were used as models to investigate Salmonella adhesion and invasion in the intestine (Gagnon *et al*., 2013). HT-29 and Caco-2 cells have been used to study the permeation and internalization of the botulinum toxin (Nishikawa *et al*., 2004; Niwa *et al*., 2007). These two cell lines were also used to study the toxicity of Ochratoxin A and Patulin as well as the interactions of these two very potent mycotoxins with the intestinal barrier (Maresca *et al*., 2001; Mahfoud *et al*., 2002). Both Ochratoxin A and Patulin are mutagenic, carcinogenic, teratogenic and induce intestinal injuries. Caco-2 cells have been used to investigate the mutagenicity of melanoidins which are products of the Maillard reaction and are formed during cooking (Glösl *et al*., 2004). Additionally, Caco-2 cells have been used to assess the toxicity of pesticide residues that can be found in food and water, such as the insecticides imidacloprid (Brunet *et al*., 2004), phenylpyrazole (Vidau *et al*., 2009) and clorpyrifos (Tirelli *et al*., 2007).

A common alternative to cancer-derived cell lines is cell immortalization by infection with viruses such as Epstein–Barr or SV40, but metabolic and morphological changes are often observed (Chen *et al*., 2004). Lately, there are also efforts to develop alternative models to cancer-derived or virus-immortalized cells. These are normal cell lines, such as the Human Colonic Epithelial Cells (HCEC) that have been developed by isolating epithelial cells from a healthy epithelium. These cells were immortalized by transfection with the non-oncogenic anti-apoptotic proteins cyclin-dependent kinase 4 and the catalytic component of human telomerase (Roig and Shay, 2010). HCEC cells are an emerging model of normal tissue, since they are not tumorigenic, do not have mutations in hot spot genes and express epithelial as well as stem cell markers (Roig *et al*., 2010). HCEC have been used for assessing the impact of bioactive food components in human colonic cells (Constantinescu *et al*., 2014).

7.5 Co-culture Cell Models

Currently, most *in vitro* intestinal models are cultivated as one-dimensional (1D) monolayers on plastic surfaces. In such models, the cells do not always establish a functional, epithelial character, and this may result in misleading results. Two-dimensional (2D) or co-culture models involve growth of cell lines in co-culture. When two or more cell types are brought together in the same culture environment they very likely interact and communicate (Hendriks *et al*., 2007). Co-culture systems mimic the natural existence of cells in an organism, so that direct effects on cells, tissues or organs of chemicals, microorganisms and their products can be tested in situ. In these models the cells are grown on microporous membranes (e.g. insert filters, permeable supports), either in direct contact with each other on the membrane or indirectly where one cell line is cultured on the apical side of a membrane and the other cell line on the underside of the membrane (basolateral) plates (Willemsen *et al*., 2002). To achieve better functionality, epithelial cells growing on microporous membranes can be co-cultured with same-species derived immune cells growing in the lower compartment or on the microporous membrane using an inverted setting (Figure 7.2). Thus, the *in vivo* basolateral feeding of intestinal epithelia can be simulated more accurately and the chemical crosstalk (chemokine and cytokine release) between the intestinal and immune cells in response of an antigenic stimulus can be studied (Westendorf *et al*., 2010). Furthermore, since intestinal epithelia is not only populated by enterocytes, they can be co-cultured with other more-specialized cell types, like mucus-producing Goblet cells (Mahler *et al*., 2009).

Co-culture models offer a clear advantage over single cell-line models since they mimic better normal organ, tissue and physiological organization in human organism. The 2D cell culture has been used conventionally for *in vitro* drug candidate testing; there are also limited applications for testing the toxicity/absorption of food-related compounds. A Caco-2/Raji B co-culture model system was developed to mimic the M cells that overlay the intestinal Peyer's patches. This model was used to assess the translocation of *Vibrio parahaemolyticus* (a bacterium

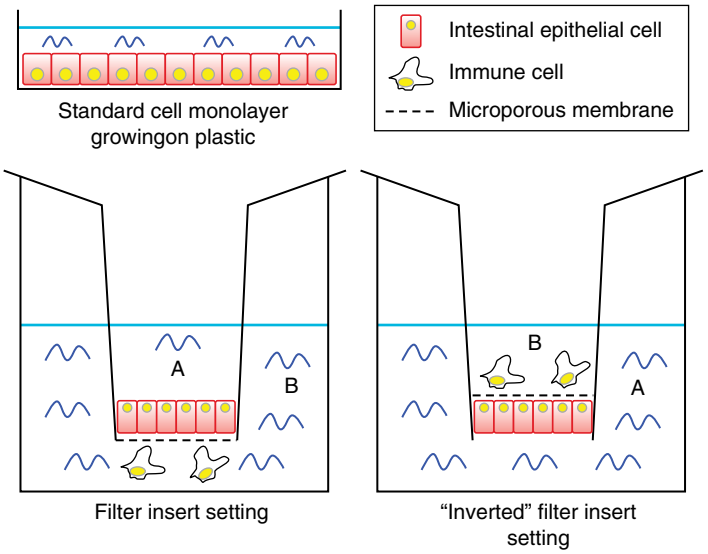

Figure 7.2 Experimental intestinal cell model settings. Epithelial cells can be grown on plastic or on microporous membranes (filter inserts) in the presence of immune cells, leading to better cell polarization and differentiation due more accurate simulation of the *in vivo* intestinal environment (A = apical; B = basolateral). Cells can be grown in a conventional manner with apical side upwards, or alternatively, the filter with polarized cells can be inverted, so that the top of the membrane is equivalent to the basolateral side. In both conventional and inverted setting co-cultured immune cells, substances and microorganisms can be added to apical or basolateral side, enabling functional studies described in the main text on both sides of the epithelia.

found in shellfish that can cause inflammatory gastroenteritis) across the intestinal epithelium (Finn *et al.*, 2014). A co-cultured Caco-2/HT29-MTX intestinal cell monolayer model was used to assess the delivery and absorption of bioactive food compounds such as flavonols (Jailani and Williamson, 2014). Co-cultures of these human cell lines, (Caco-2 and HT29-MTX) were also incorporated into an *in vitro* digestion/cell culture model; a range of different foods were subjected to *in vitro* digestion, and iron bioavailability was assessed in the co-cultured cells (Mahler *et al.*, 2009).

7.6 3D Co-culture Models

In living tissue, cells exist in three-dimensional (3D) microenvironments where they attach with each other and to the extracellular matrix they produce. They also form gap junctions which directly couple one cell to another. These junctions enable cells to communicate via exchange of ions, small molecules and even electrical currents. In 2D cell culture cells are grown on flat dishes made of polystyrene plastic that is very stiff and unnatural. The cells adhere and spread on this plastic surface forming a monolayer. When cells are grown as a monolayer there are limitations such as loss of extracellular matrix and loss or compromise of cell-to-cell and cell-to-matrix signaling that occurs in the 3D *in vivo* environment where such signals are essential to cell differentiation, proliferation and a range of cellular functions (Mazzoleni *et al*., 2009; Vanvliet, 2011). Therefore, 2D cell cultures are limited in mimicking this environment, which often makes them unreliable predictors of *in vivo* toxicity.

As a means to overcome these limitations, 3D cell culture models are being developed. This area is one of the fastest growing experimental approaches in life sciences. A 3D cell culture is an artificially-created environment in which cells are permitted to grow or interact with its surroundings in all three dimensions. In 3D cultures special matrices and scaffolds are used and the cells are usually grown in bioreactors, small capsules in which the cells can grow into spheroids, or 3D cell colonies. Materials such as hydrogels are used as scaffolds, but cells can also be grown in a scaffold-free manner with the use of low-adhesion plates, micropatterned surfaces or hanging drop techniques (Mazzoleni *et al*., 2009).

Recently, 3D gastrointestinal spheroids were produced using NCM460 and SW480 colorectal cells (Chia *et al*., 2015). These spheroids were used as a model to assess the toxicity of ZnO nanoparticles (NPs). These NPs have been incorporated into some consumer products such as dietary supplements and food packaging. Previous studies in 2D cell culture models have shown that ZnO NPs are highly cytotoxic (Setyawati *et al*., 2013). However, the toxicity studies in the 3D gastrointestinal spheroids suggest that toxicological conclusions made from 2D cell models might overestimate the toxicity of ZnO NPs (Chia *et al*., 2015).

These findings from the comparison between 2D and 3D models demonstrate that cell dimensionality plays a critical role in governing the spatiotemporal cellular outcomes like inflammatory response and cytotoxicity. This is particularly important in the case of nanotoxicity studies where scientists try to find size-related differences in toxicity; 3D cell models have tissue-like mass transfer and could show more realistically how NPs could cross the multiple layers of cells in the *in vivo* environment.

The birth of embryonic stem cells and induced pluripotent stem (iPS) cells has provided a biological opportunity to develop spheroids from an individual's own intestinal epithelial cells. Such cells can develop into complex tissues on their own. The spheroids produced by iPS are made of multiple types of cell, including mucin-secreting goblet cells and nutrient-absorbing enterocytes. Due to their higher similarity with human organs they are termed as "organoids". By isolating intestinal crypts from human biopsy samples during routine endoscopy, series of intestinal human spheroid cell lines have been established from a wide array of regions along the gastrointestinal tract and from both healthy individuals and those with gastrointestinal diseases (e.g. inflammatory bowel disease) (Vandussen *et al.*, 2015). The intestinal organoids are currently considered as a valuable tool for personalized medicine (e.g. screening for novel therapies, clinical testing to determine the efficacy of a therapy) but they may well be used in the future in toxicological studies in order to identify populations that are particularly vulnerable to specific populations food components. Figure 7.3 shows how such organoids, also termed as "mini-guts", can be developed and what are their potential applications.

3D cellular assays are considered to be more analogous to – and so predictive of – *in vivo* events compared to more simplified 2D cultures, however, the 3D models have the risk of central necrosis due to low oxygen and nutrient supply and are not always suitable for standardized (96-well plate based) assays.

7.7 Organs on a Chip

3D cell culture models have potential to mimic the physiological architecture of the human gastrointestinal tract (Sung *et al.*, 2011), however, the living intestine is a dynamic environment

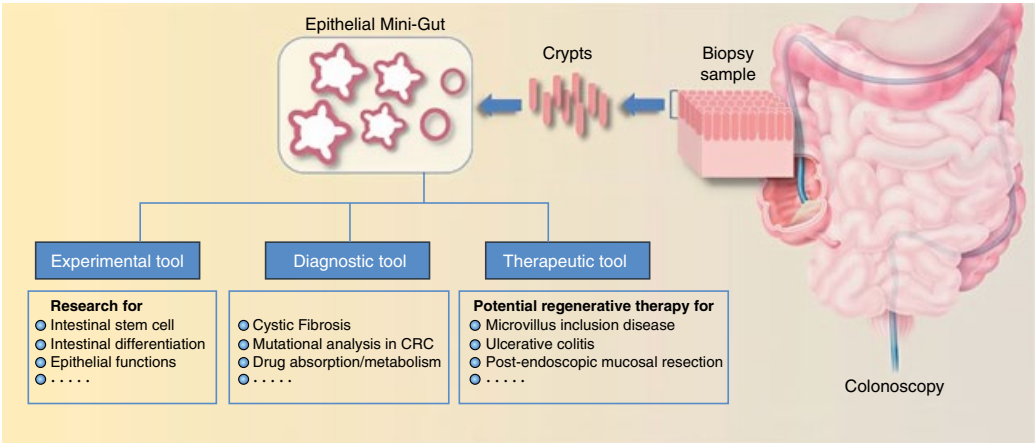

Figure 7.3 Basic and clinical applications of an epithelial mini-gut. An epithelial mini-gut is efficiently established from a single endoscopic biopsy sample. Up to ~3000 crypts can be released from a biopsy sample. An epithelial mini-gut grows logarithmically and expands 1000-fold within a month. Three applications of epithelial mini-guts are as follows: (i) As an experimental tool. Genetic manipulation, gene expression analysis, live imaging, and other standard biological analyses can be employed for normal and patient-derived epithelial mini-guts. (ii) As a diagnostic tool. Patient-derived epithelial mini-guts recapitulate *in vivo* intestinal epithelial functions and genetic signatures. Efficient expansion of pure epithelial cells provides a high-quality source for deep sequencing or functional assays. (iii) As a therapeutic tool. Epithelial mini-gut transplantation may become a feasible regenerative therapy.

that undergoes peristalsis and experiences fluid flow and none of the existing 3D intestinal models recapitulate these mechanical activities that are critical for normal organ physiology. Therefore, considerable effort has already been made to provide *in vitro* surrogates in the form of "organ-on-a-chip" devices that are designed to mimic both the physiological architecture and dynamics of human organs. These devices are microfluidic cell-based systems that contain physiologically scaled micrometer-sized chambers (Ramadan *et al*., 2013) in which gastrointestinal cells are cultured while being exposed to fluid flow rates and shear stresses that are comparable to those observed *in vivo*. The goal is not to build a whole living organ but to synthesize minimal functional units that recapitulate tissue- and organ-level functions. A "human gut-on-a-chip" microdevice was recently developed (Figure 7.4) (Kim and Ingber, 2013). This device composed of a confluent layer of intestinal epithelial (Caco-2) cells cultured on a porous membrane under peristalsis-like motions and flow mimicking the physiological conditions. Under these conditions the Caco-2 cells spontaneously regenerated 3D intestinal villi and differentiated into different types of epithelial cells and recapitulated multiple physiological functions of normal intestinal villi.

Analogous devices have been developed with co-cultured enterocytes (Caco-2) and mucin-producing cells (HT29-MTX) with the aim to develop an even more realistic model where mucus acts as a physiological barrier to toxins (Esch *et al*., 2014). There are currently efforts to develop analogous devices and use them for assessing the effects of foods in the intestinal tract (Ramadan *et al*., 2013). In the future, it is anticipated that sample treatments, such as food digestion, could be integrated in chip-based platforms. In addition to the "artificial gut", these devices will comprise multiple chambers connected with microfluidic channels each of them containing artificial juices that mimic the different steps during human digestion (saliva, gastric juice, intestinal juice).

These chip-based miniaturized devices have intrinsic potential, however accommodating new analysis and protocols into a micro-scale environment will require the tackling and solving of practical challenges, particularly those associated with the analysis of complex samples, such as food. Additionally, all

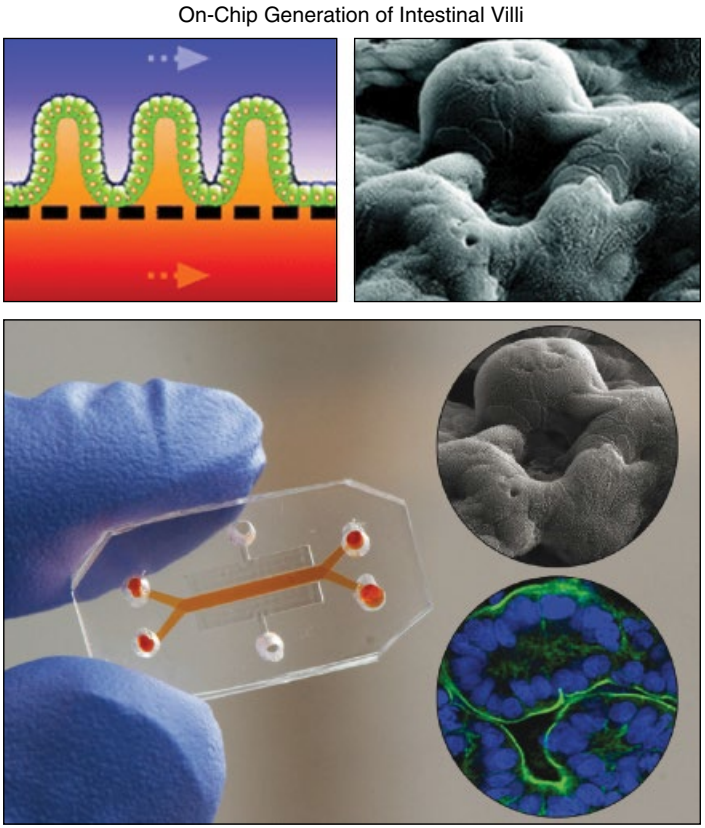

Figure 7.4 A microfluidic "Gut-on-a-Chip" technology that exposes cultured cells to physiological peristalsis-like motions and liquid flow can be used to induce human Caco-2 cells to spontaneously undergo robust morphogenesis of three-dimensional (3D) intestinal villi.

assays currently used for assessing toxicity *in vitro* have been optimized for culture and analysis of cells in the wells of plates. Due to the size of the miniaturized devices, very small sample volumes are involved; therefore fewer cells can be accommodated. As a result, some of the analytes that serve as biomarkers of toxicity may be present in very low amounts, thus elaborate methods for their collection and more sensitive assays for their analysis need to be developed.

7.8 Summary and Conclusions

Integration of *in vitro*, in silico and other non-animal-based methodologies into the process of toxicological risk assessment is expected to increase predictivity toward human toxicological effects. Being based on human biological systems, the emerging tests are considered to be more relevant than the traditional animal-based tests. The growing use of "omics" technologies (e.g., transcriptomics, proteomics and metabolomics) in combination with emerging *in vitro* test systems (co-culture cell models, human stem cells, high-throughput testing) and imaging and systems biology, allows a comprehensive analysis of the impact of a chemical agent at the molecular level and can indicate potential toxicity pathways that may lead to adverse health effects. A more comprehensive understanding of the relationship between toxicity and biological pathways will make it possible to prevent and reduce animal testing by using a combination of tests that individually represent key events of the mechanisms of action of toxicity and that allow for assessment of the potential of a test compound to affect these key events. The establishment of standardized and validated cell culture based methods for toxicology, food-borne diseases, bioavailability and metabolism studies can bring the field of food science and nutrition to a new era. All these emerging methodologies have their limitations and require further development, nevertheless have the potential to transform in the future the way toxicological risk assessment is performed.

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8

In vitro Methods for Assessing Food Protein Allergenicity

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8.1 Introduction

Food allergies are the immunopathogenic manifestations of exposure to food proteins that have antigenic properties. They cause a wide array of symptoms ranging from mild discomfort, with respiratory syndromes, enterocolitis, and urticaria, to more severe symptoms that can be life threatening and may lead to anaphylaxis and death. Because of the adverse health consequences, national and international public health authorities such as the Food and Drug Administration of the United States, European Food Safety Authority (EFSA) and the Food and Agriculture Organization of the United Nations (FAO/WHO) necessitate the identification, testing and assessment of allergens, in addition to the proper labeling of food products.

The true prevalence of food-induced allergic reactions remains debatable and highly challenging to accurately establish, mostly due to inconsistencies in study designs and methodologies, discrepancies in defining the term food allergy, variations between clinically assessed and self-diagnosed cases,

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and differences in food consumption patterns and geographical distributions (Allen and Koplen, 2012; Lack, 2012; Sampson, 1999, 2004; Sicherer, 2011, Sicherer and Sampson, 2014). The majority of food-related allergic reactions are caused by a handful of allergens known as the "The Big 8" and include allergens found in cow's milk, eggs, fish and shellfish, peanuts, tree nuts, wheat, soybean, and fruits (Ahsan *et al*., 2016; Allen and Koplin, 2012; Berin and Sicherer, 2011 Hefle *et al*., 1996). Nevertheless, this list is constantly expanding and it now includes other foods such as cereals that contain gluten, mustard, sesame seed, and lupin (Commission-Directive 2006/142/EC). Moreover, as protein-rich ingredients and foods increasingly penetrate the food market, the need to detect and establish their safety for consumers becomes crucial. This is especially true for novel alternative protein sources (such as chia seeds, rapeseed, edible insects, genetically modified foods and others) that are continuously being developed to be incorporated into food products destined for human consumption. Although such foods are considered important from a sustainability point of view, they can bring forth undesirable consequences such as the potential to elicit adverse food reactions ranging from allergic sensitization to hypersensitivity and allergic reactions. Therefore, it is important to evaluate the currently available *in vitro* analytical methods and to propose new approaches that can identify existing and novel allergens in newly introduced foods to avoid exposure to people with food allergies (Mills *et al*., 2012).

8.2 Food Sensitization, Hypersensitivity and Allergy

Allergic sensitization to foods is best described as a priming phase in which a complex interaction between an exogenous allergenic component and the human body occurs (Perry and Pesek, 2013; Sicherer and Sampson, 2014; Valenta *et al*., 2015; Van Ree *et al*., 2014). However, the reason why some individuals are more susceptible to developing allergic sensitization and adverse food reactions compared to others is still not well understood. Primary allergic sensitization typically occurs early in life. While reactions to some foods such as wheat, soy milk

and eggs have higher probability to be resolved during childhood, sensitization and allergies to tree nuts, peanuts, fish and shellfish have been shown to last longer (Burks *et al*., 2012). A number of factors have been proposed to influence the development of food sensitizations and allergies, and they can be categorized into intrinsic or host specific and extrinsic or allergen related factors. The first set of factors is host related. This group is multifactorial and comprises of various proposed predisposing factors such as the genetic make-up of the individual, atopy, gastrointestinal epithelial barrier, insufficient intake of certain vitamins such as vitamin D, the intake of antimicrobial medication such as triclosan, gender (males being more predisposed than females), and the reduced dietary intake of antioxidants, (Berin and Sicherer, 2011; Burks *et al*., 2012; Ly *et al*., 2011; Savage *et al*, 2012). Hong *et al*., (2011) attributed the development of food sensitization to an important gene-environment interaction that occurs during childhood. After following the development of food sensitization in 970 children (breast-fed and non-breast fed), Hong *et al*. reported that breast feeding increased the risk of food sensitization by a factor of 1.5 compared to children who were never breast fed. Moreover, intrinsic factors also play a major role. Genetic variations between the infants highly influenced the risk of sensitization. Children that carried the GG genotype demonstrated a much higher risk of sensitization compared to those carrying the GT/TT genotype (Hong *et al*., 2011). On the other hand, other studies have verified the importance of secretory antibodies IgA (SIgA) in preserving the epithelial barrier. Karlsson *et al*. (2010) demonstrated that mice deficient in SIgA and secretory IgM are prone to develop food allergen-induced anaphylactic shock, which was controlled by the adoptive transfer of splenic T-regulatory.

Other indirect causes of allergic sensitization may include consumption of alcohol and drugs, stress, etc. These factors tend to affect the intestinal barrier integrity, enable and even aggravate sensitization. Recently, the international symposium titled "Sensitizing Properties of Proteins" in Czech Republic, addressed another critical factor in the allergenicity of proteins, which is the complexity of the food matrix. The food matrix surrounding an antigenic protein may contain other components such as carbohydrates, fats, proteins (allergenic or

non-allergenic) and several other components such as metal ions, vitamins and phytochemicals, of unknown immunomodulatory properties and overall impact on allergenicity (Bublin *et al*., 2014; McClain *et al*., 2014).

The recent years have seen a significant interest in the physical and chemical properties of food allergens and their orchestration of allergic reactions (Poulsen *et al*., 2014; Van Ree *et al*., 2014). Hence, the second set of factors that influences the development of food sensitizations is related to the allergen itself. This includes the biochemical and biophysical properties of the allergen, in addition to its stability during thermal food processing and digestion (Sharief *et al*., 2011; Sicherer and Sampson, 2014). Nevertheless, the effect of some of these factors is not completely straightforward. For example, thermal processing can either enhance or suppress the allergenicity of proteins depending on the protein and the intensity of the thermal treatment. Proteins such as casein that have more complex tertiary and quaternary structures, can be relatively more heat stable than whey proteins, which can denature more easily upon heat treatment (Bu *et al*., 2013; Chandan and Kilara, 2011). Thermal processing can also activate the Maillard reaction, resulting in either lower allergenicity by deactivating certain epitopes through glycation and shifting the allergenic profile of foods into a non-allergenic one, or by altering specific motifs in IgE epitopes and forming neo-allergens or neo-antigens that can worsen the severity of allergic reactions in sensitive individuals. The type of thermal processing has also been proposed to influence allergenicity. Cooking foods at milder temperatures such as boiling reduced allergenicity by lowering the IgE-binding capacity of peanut allergens Ara h 1, h2 and h3 when compared to roasting (Beyer *et al*., 2001).

8.2.1 The Mechanism of Developing Food Hypersensitivities

When a sensitized individual is successively exposed to sufficient amounts of foods containing the sensitizing allergen, hypersensitivity may develop and an allergic reaction may be elicited resulting in a variety of clinical symptoms ranging from minor irritations to anaphylaxis. Hypersensitivity reactions to

foods can be divided into two broad categories; non-immune mediated (cell-mediated or type IV), and immune mediated hypersensitivity reactions (antibody mediated or types I–III). This grouping is based on the original Gell and Coombs classification of hypersensitivity reactions, which has undergone many modifications since its initial report in 1963. Moreover, it is worth mentioning that although the terms "food allergy" and hypersensitivity are often used interchangeably, the main difference between them is that food allergy reactions are IgE mediated immunologic response to a food allergens (Allen and Koplen, 2012; Gell and Coombs, 1963; Van Hengel, 2007).

Food hypersensitivities develop from an initial sensitizing encounter of dendritic cells with an allergen at an early onset in life. This constitutes the sensitization phase and is characterized by the production of Th1 memory (in cell-mediated hypersensitivities) or Th2 memory cells (in antibody mediated reactions) (Figure 8.1). These memory cells exist primarily in a dormant state and are reactivated upon the re-encounter with the antigen to proliferate and secrete various cytokines, such as interleukin (IL)-2 and IL-3, interferon (IF)-gamma, tumor necrosis factor (TNF)-beta, and chemokines such as IL-8 (in cell-mediated hypersensitivities) or IL-4, IL-5 and IL-13 cells (in antibody mediated reactions). Subsequently, these factors activate the

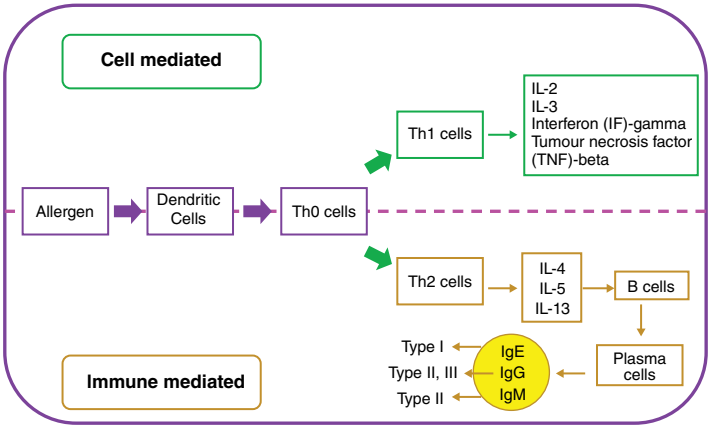

Figure 8.1 Cell and immune mediated mechanisms of allergic sensitization.

macrophages and cause an inflammatory response (Chaplin, 2010). On the other hand, class switching of the B cells produces allergen-specific IgE antibodies specific to the hypersensitivity type (I, II or III) (Murphy *et al*., 2008) (Figure 8.1). An allergic reaction is elicited when the IgE cross-link to the specific epitopes within the specific antigens activating the mast cells and basophils and triggering them to degranulate. The lysed cells release histamine and other chemotactic mediators (such as cytokines, interleukins, leukotrienes, and prostaglandins) into the surrounding tissues inducing systemic effects that involve multiple organs such as respiratory tract, nervous system and smooth muscles.

The cell-mediated hypersensitivities are relatively more common than immune mediated reactions and are considered to be the relatively milder form of the food reactions. Although extremely uncomfortable to experience, these hypersensitivity reactions are non-life threatening. They include the inflammatory disorders such as food allergen related enterocolitis syndrome, celiac disease, food allergen-induced proctitis, allergic eosinophilic gastroenteritis, allergic eosinophilic esophagitis and urticaria. These symptoms typically occur within hours of ingesting an allergenic antigen due to the interaction of a sensitized T cell lymphocyte and the specific antigen (Taylor and Hefle, 2006). On the other hand, food allergy symptoms typically develop in less than 15 minutes up to a few hours after consuming specific antigen-containing foods. Immune mediated allergic reactions can lead to rhinorrhea, itchiness, dyspnea, and even anaphylaxis (Taylor and Hefle, 2006).

8.2.2 The Exposure to Allergens

Food allergens are dietary antigens that can modulate the immune responses of T-cells and particularly those of the B-cells, eliciting the wide array of food hypersensitivity reactions mentioned earlier. In simple terms, allergens are proteins. They have varying structural and functional properties and, consequently, different allergy eliciting capacities. Some allergens are "complete allergens" and are able to induce sensitization and trigger allergic immune reactions, while others are weaker but can elicit allergenic responses by cross-reacting with

a stronger immunogen (Aalberse, 2000; Masilamani *et al*., 2012; Verhoeckx *et al*., 2015).

Allergens are food specific and are identified based on the first three letters of the genus of that specific food, followed by the first letter of its species and an Arabic number for the order that they were identified. For example, the first identified allergen from peanuts (*Arachis hypogaea*) is named Ara h1 (Table 8.1). Each antigen is characterized by specific epitopes or binding sites on its structure, which can interact with the paratope of the antibody. There are two main types of epitopes, (i) linear epitopes, which are made up of a continuous sequence of amino acids; and (ii) conformational epitopes, which are formed from folding within the secondary or tertiary structure of the protein. Whether an epitope is linear or conformational has a significant impact on its properties such as stability (against thermal processing and enzymatic breakdown), allergenicity (the ability to aggregate) or its interaction with an antigen-specific antibodies. Therefore, the ability to identify the structure and determine the structural properties of an allergen is crucial in developing ways to fully understand the mechanistic processes of antigen-antibody interaction, with the hope of preventing, managing and controlling food hypersensitivities.

Food allergens are classified into animal and plant food allergen superfamilies. Tropomyosins, β-parvalbumins and caseins along with α-lactalbumin and β-lactoglobulin are among the most common allergen families of animal origin, while the prolamin, cupin super-family and the pathogenesis-related proteins PR-10 family (Bet v 1) constitute the allergen families of plant origin. Exposure to food allergens can occur via three main routes.

8.2.2.1 The Gastrointestinal (GI) Route

The major route of exposure to food allergens is through their ingestion via the gastrointestinal tract. This category of allergens is also called the class 1 allergens and include many food allergens such as Ara h1, Ara h2 and Ara h3 in peanuts; Bos d 8 (caseins), ALA and Bos d 4 (α-lactalbumin), BLG and Bos d 5 (β-lactoglobulin) in milk (Kattan *et al*., 2011); Gal d 1 (ovomucoid), Gal d 2 (ovalbumin) and Gal d (ovotransferrin) in eggs (Caubet and Wang, 2011). Food allergy in the GI tract develops

Table 8.1 Major food allergen groups and corresponding proteins[a]

Food group	Protein family	Protein name (common name)	IUIS name[b]	Molecular weight (kDa)
Crustaceans	Tropomyosin	*Penaeus aztecus* (Brown shrimp)	Pen a 1	37
		Litopenaeus vannamei (White shrimp)	Lit v 1	37
		Pandalus borealis (Northern shrimp)	Pan b 1	–
		Metapenaeus ensis (Greasyback shrimp)	Met e 1	–
		Penaeus indicus (Indian white shrimp)	Pen i 1	–
		Penaeus monodon (Black tiger shrimp)	Pen m 1	–
		Crangon (North Sea shrimp)	Cra c 1	–
		Panulirus stimpsoni (Spiny lobster)	Pan s 1	–
		Homarus americanus (American lobster)	Hom a 1	–

		Macrobrachium rosenbergii (Giant freshwater prawn)	Mac r1	37
		Portunus pelagicus (Blue swimmer crab)	Por p1	39
	Arginine kinase	*Litopenaeus vannamei* (White shrimp)	Lit v 2	40
		Penaeus monodon (Black tiger shrimp)	Pen m 2	–
		Crangon (North Sea shrimp)	Cra c 2	–
Wheat	Profilin	*Triticum aestivum*	Tri a 12	14
	α-Amylase inhibitors	*Triticum aestivum*	Tri a 15	12
		Triticum aestivum	Tri a 28	13
		Triticum aestivum	Tri a 29, Tri a 30	13,16
	α/β-Gliadin	*Triticum aestivum*	Tri a 21	28e35
	γ-Gliadin	*Triticum aestivum*	Tri a 20	28–35
	ω1,2-Gliadin	*Triticum aestivum*	–	40
	ω5-Gliadin	*Triticum aestivum*	Tri a 19	65

(*Continued*)

Table 8.1 (Continued)

Food group	Protein family	Protein name (common name)	IUIS name[b]	Molecular weight (kDa)
Peanut	Cupin (7S globulin)	*Arachis hypogaea*	Ara h 1	64
	Cupin (11S globulin)	*Arachis hypogaea*	Ara h 3	60
	Conglutin	*Arachis hypogaea*	Ara h 2	17.5
		Arachis hypogaea	Ara h 6	15
		Arachis hypogaea	Ara h 7	16–17
	Profilin	*Arachis hypogaea*	Ara h 5	14
	Oleosin	*Arachis hypogaea*	Ara h 10	18
		Arachis hypogaea	Ara h 11	14
	Defensin	*Arachis hypogaea*	Ara h 12	5.2
		Arachis hypogaea	Ara h 13	8.4
Chicken egg	Ovomucoid	*Gallus domesticus*	Gal d 1	28
	Ovalbumin	*Gallus domesticus*	Gal d 2	44
	Ovotransferrin	*Gallus domesticus*	Gal d 3	77
	Lysozyme	*Gallus domesticus*	Gal d 4	14
	Serum albumin	*Gallus domesticus*	Gal d 5	69
Cow's milk	α-Lactalbumin	*Bos domesticus*	Bos d 4	
	β-Lactoglobulin	*Bos domesticus*	Bos d 5	18.3

	Serum albumin	*Bos domesticus*	Bos d 6	67
	Immunoglobulin	*Bos domesticus*	Bos d 7	160
	Caseins	*Bos domesticus*	Bos d 8–12	19–30
Tree nuts	Chitinase Ib	*Castanea sativa* (Chestnut)	Cas s 5	–
	Lipid transfer protein	*Castanea sativa* (Chestnut)	Cas s 8	9.7
	Bet v 1	*Corylus avellana* (Hazelnut)	Cor a 1	17.4
	Profilin	*Corylus avellana* (Hazelnut)	Cor a 2	14
	Lipid transfer protein	*Corylus avellana* (Hazelnut)	Cor a 8	9
	Cupin (7S globulin)	*Corylus avellana* (Hazelnut)	Cor a 11	48
	Cupin (11S globulin)	*Corylus avellana* (Hazelnut)	Cor a 9	40
	2S albumin	*Bertholletia excelsa* (Brazil nut)	Ber e 1	9
	Legumin like (11S-seed Storage protein)	*Bertholletia excels* (Brazil nut)	Ber e 2	29

(*Continued*)

Table 8.1 (Continued)

Food group	Protein family	Protein name (common name)	IUIS name[b]	Molecular weight (kDa)
Soy	Lipid transfer protein	*Glycine max*	Gly m 1	–
	Defensin	*Glycine max*	Gly m 2	–
	Soy profilin	*Glycine max*	Gly m3	–
	Bet v 1 family	*Glycine max*	Gly m4	–
	Cupin (7S globulin)	*Glycine max*	Gly m5	–
	Cupin (11S globulin	*Glycine max*	Gly m6	–
Fruits	Pathogenesis-related protein PR10	*Fragaria ananassa* (strawberry)	Fra a 1	18
		Malus domestica (apple)	Mal d 1	–
		Prunus persica (peach)	Pru p 1	18
	Profilin	*Fragaria ananassa* (strawberry)	Fra a 4	13
		Prunus persica (peach)	Pru p 4	14
	Thaumatin-like protein	*Malus domestica* (apple)	Mal d 2	–
		Prunus persica (peach)	Pru p 2	25–28
	Non-specific lipid transfer protein 1	*Prunus armeniaca* (apricot)	Pru ar 3	9
		Prunus persica (peach)	Pru p 3	10

[a] The following references were used to prepare this table: Matsuo *et al*., 2015; Radauer *et al*., 2014; and Roux *et al*., 2003.
[b] Allergen nomenclature was retrieved from http://www.allergen.org

as a response to a disruption in the dendritic cells residing in the intestinal mucosa, which consequently generates an immune response by releasing food-specific IgE (Steele *et al*., 2012).

8.2.2.2 The Respiratory Tract Route

Another way to develop sensitization to food allergens is through inhalation. These are the class 2 food allergens also sometimes referred to as aeroallergens, which includes the pollen allergens. The major pollen allergen in this category is the Bet v 1 (birch pollen). Most people with sensitivity to Bet V1 also tend develop allergic cross-reactions to the Mal d 1, which is the major allergen found in apples (Ahammer *et al*., 2017; Valenta *et al*, 2015).

8.2.2.3 The Cutaneous Route

The third route of allergic sensitization is the epicutaneous route. Sensitization to food allergens through skin contact is a relatively more recently explored means of developing food allergies. It is also relatively a minor means of exposure when compared to the other routes. The mechanism of cutaneous sensitization occurs when food proteins penetrate through a disrupted epidermal barrier. Animal models are important platforms to explore these types of reactions. Many studies have demonstrated that exposing allergic mice to allergens from peanuts and ovalbumin (OVA) through skin contact can lead to significant increases serum specific IgE (Valenta *et al*., 2015).

8.3 Safety Needs and Regulatory Consideration in Detecting Allergens in Food

The importance of food allergies as a public health concern has been recognized for several years. As protein rich foods progressively enter the food market, the need to establish their safety for all consumers, particularly for people with atopy, is critical. Over the past years, the need to detect allergenic ingredients in manufactured food products and to determine their safety thresholds have received notable attention from both food manufacturers and regulatory public health agencies both

at the national and international levels. To assess whether a manufactured food is safe for consumption, from allergy perspective, consumers should have access to food composition information (list of ingredients) and safe consumption limits recommended by regulatory bodies. Therefore, two important considerations include (i) whether the protein component (established or new) of the food has allergenic potential; and (ii) the minimum amount of the allergenic food that can be tolerated by the consumer without producing any adverse reactions.

The detection of known allergens in food is relatively well established. However, in the absence of solid analytical approaches that can predict the allergenic potential and safety of novel foods, other tools such as bioinformatics have been adopted. Bioinformatics uses simulations and statistical algorithms that can predict the allergenicity of newly introduced proteins by comparing their amino acid sequences to those of known allergens (Astwood *et al.*, 2003; Codex Alimentarius, 2003). If the amino acid profile of the new proteins reveals similarities to those of the known allergens, this could indicate the possibility that the new protein has the potential to generate allergic reactions in sensitized individuals. The bioinformatics tools for detecting novel allergens are discussed in detail in a later section of this chapter.

Once the allergenic potential of a protein is identified, its potential to cause an adverse food reaction necessitates the establishment of regulations to protect people with food allergies. Internationally, public health authorities and regulatory agencies, such as Codex Alimentarius Committee of the Food and Agriculture Organization (FAO) and the European Food Safety Authority EFSA of the European Union, have recognized that accurate and complete food labeling are the best means for the risk management of food allergens. Therefore, it is important that a uniform risk assessment framework is adopted to create each list. Such assessments evaluate the hazard, determine its characteristics (such as identification of minimum eliciting doses, exposure thresholds, and dose–response correlations), and set market regulations to control or eliminate exposure to the allergen. In the United States, food allergens are regulated under the Food Allergen Labelling and Consumer Protection Act (FALCPA). In 2006, The FALCPA addressed the most

common allergenic foods in the USA (milk, eggs, fish, crustacean shell fish, peanuts, soybeans, wheat and tree nuts) requiring food manufacturers to declare and identify them on the ingredient lists of their food labels, irrespective of their levels. In Europe, the EU Directives 2000/13/EC and 2003/89/EC similarly require a mandatory declaration of allergenic foods. In 2006, the Commission Directive 2006/142/EC also announced the addition of lupin and molluscs to the allergenic food list, which now contains 14 allergenic foods (milk, eggs, fish, crustacean shell fish, peanuts, soybeans, wheat and tree nuts, mustard, sesame seeds, sulfites, lupin and molluscs) (Gendel, 2012; Van Hengel, 2007; European Parliament and Council, 2000, 2003, 2006).

On the other hand, regulations regarding potential novel foods (such as insects and genetically modified foods) are much more complicated. In Europe, for a novel food to be considered for consumption, they first need to comply with the "Novel Food" definition set by the European Parliament and Council. They also need to undergo a specific pre-market risk assessment according to Commission Recommendation 97/618/EC. However, setting specific recommended intake levels for such foods can be extremely challenging, especially considering the differences in geographical food traditions and eating habits, which can affect the development of food sensitizations and the severity of allergic reactions. Eating novel allergens can either result in allergic reactions independently, or can cross-react with other allergens (Belluco *et al*., 2013). Despite the fact that food labeling regulations provide a good basis for awareness and caution for consumers, they have several deficiencies. For example, they do not take into consideration the allergen dose-risk relationship and therefore do not reliably predict the level of sensitivity to low doses of the allergenic food. The best way to determine an individual's sensitivity is through a double-blind, placebo-controlled food challenge (DBPCFC). However, the main disadvantage of DBPCFC studies is that they are rather qualitative than quantitative and provide an indication of an overall food allergy rather than determining the extent of adverse effects. At the population level, the DBPCFC is substituted with Eliciting Dose ED10, the eliciting dose predicted to incite a reaction in 10% of individuals with a specific food allergy. The threshold values that are calculated are the Observed

Adverse Effect Level (NOAEL), the Lowest Observed Adverse Effect Level (LOAEL), and the Maximum Tolerated Dose (MTDs) (Gendel, 2012; Van Hengel, 2007).

8.4 *In vitro* Analytical Methods for Testing Known Allergens

Developing reliable analytical methods for the detection and quantification of food allergens is important to ensure compliance with regulatory requirements as well as consumer safety. An optimal analytical method should provide a rapid, sensitive, selective and convenient (easy to use, cost-effective, and portable) detection of allergenic components in food products. However, developing such a method can be challenging because of the minute quantities of specific allergens in foods, the presence of multiple types of allergenic proteins in a single food, in addition to the complexity and heterogeneity of the food matrix. Currently there are two major analytical approaches used to detect and quantify allergens: the protein-based (immunological) or DNA-based (molecular) approaches (Table 8.2).

8.4.1 Protein-Based Approaches

Protein based methods comprise of a wide selection of immunological approaches to detect and quantify the allergenic protein components of foods. These immunoassays use human antibodies or antibodies produced in animals against specific food allergens. Protein-based methods mainly involve immunoassay approaches such as the radio-allergosorbent test (RAST), enzyme allergosorbent test (EAST), rocket immunoelectrophoresis (RIE), immunoblotting, and enzyme-linked immunosorbent assay (ELISA). A relatively newer addition to this list is the spectrometric techniques including proteomics that have been developed and applied to the quantitative study of allergenic components in food (Kirsch *et al.*, 2009; Lin and Alocer, 2017).

Table 8.2 Analytical methods for the detection of food allergens

Method category	Analytical technique	Method type	Mechanism of specificity	Detection	Advantages	Disadvantage
Protein-Based Approaches	Allergosorbent tests (RAST/EAST inhibition)	Immunological	Bind to allergen specific human IgE	Qualitative, quantitative	Easy to perform, rapid	Require serum from allergic humans which is not readily accessible in high quantities[a]
	Immunoblotting SDS-PAGE	Immunological	Bind to human IgE or IgG originating from animal sources[b]	Qualitative, Semiquantitative[c]	Easy to perform, Inexpensive, rapid	Not sensitive for quantitative analysis
	ELISA	Immunological	Bind to human IgE or IgG originating from animal sources[b]	Qualitative, quantitative	Rapid, consistent, easy to analyze, sensitive, and specific	Cross reactivity with similar proteins[d]
	ELISA-PCR	Immunological	Oligonucleotide primers[e]	Qualitative, quantitative	Sensitive, specific	Cross reactivity with similar proteins[d]
	ELISA-Dipstick	Immunological	Bind to IgG originating from animal sources[b]	Qualitative	Inexpensive, rapid, portable, can be used for population screening	Do not provide quantitative information, Not highly specific

(*Continued*)

Table 8.2 (Continued)

Method category	Analytical technique	Method type	Mechanism of specificity	Detection	Advantages	Disadvantage
	Lateral flow assays	Immunological	Bind to IgG originating from animal sources[b]	Qualitative, semiquantitative	Inexpensive, rapid, portable, simple to function	Do not provide quantitative information, Not highly specific
	Dot immunoblotting	Immunological	Bind to IgG originating from animal sources[b]	Qualitative, semiquantitative	Simple, inexpensive, good screening tool	Do not provide quantitative information, Not highly specific
	Rocket immuno-electrophoresis (RIE)	Immunological	Binding to human IgE or IgG originating from animal sources[b]	Qualitative, semiquantitative[a]	Inexpensive	Laborious Gel preparation and immuno-staining procedures[d]
DNA-Based Approaches	PCR	Molecular	Oligonucleotide primers	Qualitative, semiquantitative	Highly specific in detecting specific allergens in foods, not affected by harsh protein extraction processes, not affected by seasonal protein variations[b]	They are not allergen detection tools. Results may not be reliable for processed foods (false positive or negative).

	Real time-PCR	Molecular	Oligonucleotide primers	Qualitative, quantitative	Highly specific, accurate, sensitive	Require expensive laboratory Equipment, laborious
	Microarray assay	Molecular	Oligonucleotide primers	Qualitative, semiquantitative	Good screening tools for epidemiological studies	Do not provide quantitative information
Mass Spectrometry-Based Approaches	Liquid chromatography-tandem mass spectrometry	Chromatographic and spectrometric	Peptide, protein	Quantitative	Highly sensitive and specific, multi-target analysis[f]	High cost of instruments, trained and specialized personnel[f]

[a] Baumert, 2014.
[b] IgG originating from animal sources such as rabbits, goats, sheep, chicken, and mice.
[c] Poms *et al.*, 2004.
[d] Costa *et al.*, 2016.
[e] Van Hengle, 2007.
[f] Besler *et al.*, 2002.

8.4.2 Immunoassay Approaches

8.4.2.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Among the various immunoglobulin methods, the most commonly used approach by food manufactures, clinical specialists and food control agencies is the ELISA. The principle of ELISA is based on the direct or indirect detection of antigens by immobilizing the antigen or antigen-specific antibody, respectively, onto a plate surface. Afterwards, a secondary antibody conjugated to an enzyme is added followed by a substrate that elicits a chromogenic or fluorescent response. The intensity of the elicited color or fluorescence is directly proportional to the amount of primary antibody providing semi-quantitative or quantitative information. ELISA uses either food allergen specific IgE antibodies derived from human sera or IgG antibodies produced in animals directed against specific allergenic food proteins. Also, there are different variations or types of ELISA such as direct, indirect, sandwich and competitive ELISA. The selection of a particular ELISA type for food allergen detection depends on the desired sensitivity, antigen quantity, antibody and antigen characteristics and specificity.

Competitive ELISA

ELISA is considered to be the best method for quantification of small quantities of antigens. In this approach, a primary antibody is incubated with the sample antigen. The resulting antigen- antibody complex is then added to microplates, which have been pre-coated with the same antigen. Unbound antibodies are then washed off. Secondary antibodies specific to the primary antibody are conjugated with an enzyme and added into the sample. Finally, a substrate is added, and the elicited fluorescent signal is detected. This method is called competitive because the more antigen there is in the sample, the less antibody will be able to bind to the antigen in the well. The main advantage of competitive ELISA is its high sensitivity especially in complex antigen mixtures (Gan and Patel, 2013). Competitive ELISA has been used by various research groups for the detection of several different food allergens such as peanut proteins in food products (Holzhauser and Vieths, 1999), bovine milk allergens in goat and buffalo milk and cheese products (Hurley *et al*., 2004), and soybean allergens (Ma *et al*., 2010).

Sandwich ELISA

Sandwich ELISA is used to detect and quantify antigens positioned between two layers of antibodies. In this approach, the antigen measured must contain at least two antigenic epitopes for binding with the two antibodies. The well surface is prepared with a known quantity of bound antibodies and all nonspecific binding sites are blocked. The antigen-containing sample is then added to the plate followed by a specific primary antibody. Secondary antibodies conjugated with enzyme are then added to bind the primary antibody. Finally, substrate is added and color formation is measured. Sandwich ELISA is a highly sensitive and specific approach. Also, it is suitable for complex samples because it eliminates the need to purify the specific antigen from a mixture of antigens (Gan and Patel, 2013; Poms *et al*., 2004). Sandwich ELISA has also been developed and used for the detection of a wide variety of allergens in food products such as the peanut allergens in processed foods (Stephan and Vieths, 2004), bovine milk allergens in goat milk and cheese (Hurley *et al*., 2006), fruit allergens such as orange Cit S2 in fresh and processed orange products (Kiyota *et al*., 2017), and lupine protein in processed food (Holden *et al*., 2005).

Direct and Indirect ELISA

Direct and indirect ELISA are the simplest formats of ELISA. They are both similar in process with minor differences and one additional step in the indirect method. In both assays, the antigen is adsorbed onto a plate, then an excess of a protein (such as bovine serum albumin) is added to block all other binding sites. In direct ELISA, an enzyme-conjugated antibody that has specificity for the antigen is then added. However, in indirect ELISA, first a primary antibody is added followed by an enzyme-conjugated secondary antibody. In both cases, a substrate is added and the generated color is measured. Both ELISA assays have been developed to detect the presence of several allergens in fish, milk and meat (Asensio *et al*., 2008).

ELISA-Derived Kits

In recent years, ELISA has been incorporated into various rapid, affordable and easy-to-use commercial immunoassay kits for use in the food and feed industry. These kits are suitable for

screening large numbers of samples in a short time and include the lateral flow tests and ELISA-dipsticks. They use the same immunoassay principles as the classical ELISA but use nitrocellulose membrane coated with antibodies instead of microplates. Moreover, they do not have enzymes, but rather use dyes, latex beads and colloidal gold conjugates (Asensio *et al*., 2008). These kits have been developed and used for the detection of allergens such as egg proteins (Baumgartner *et al*., 2002) and peanut and hazelnuts in cookies and chocolates (Pele *et al*., 2007).

ELISA-Coupled Approaches

ELISA-coupled methods offer highly sensitive detection of food allergens. They include approaches that are coupled to the conventional ELISA test such as the ELISA Inductively Coupled Plasma Mass Spectrometry (ELISA/ICP-MS) and ELISA Polymerase chain reaction (ELISA/PCR). The ELISA/ICP-MS uses antibody labeled with stable isotopes instead of an enzyme, thereby providing highly sensitive and precise detection and quantitation of allergens (Kirsch *et al*., 2009). For example, peanut allergens were detected using this method at concentrations as low as 2.2 μg/g of food in cereal and chocolate containing snacks (Careri *et al*., 2008). On the other hand, ELISA-PCR combines the specificity of a DNA-based method with the sensitivity of the ELISA assay. In this technique, a specific DNA fragment encoding the allergenic protein is amplified and then coupled with ELISA for the determination of concentration via color detection.

8.4.2.2 Other Immunoassay-based Methods

RAST/EAST Inhibition

Other protein-based methods include the enzyme allergosorbent test (EAST) and the radio allergosorbent test (RAST). These two immunoassay approaches detect allergen specific IgEs in the human sera. Although they are rarely used as a quantitative allergen detection method, they can be used to detect a wide variety of food allergens in peanut and soybean, tree nuts, birch pollen, codfish, and others (Besler *et al*., 2001). Both methods use solid phase-bound allergens in microplate wells. In the RAST test, the secondary antibody comprises of a radioactive isotope and hence quantification is performed with a gamma

counter. In the EAST test, the absorption of the colored product is inversely proportional to the quantity of allergen in the sample. Although these two assays are relatively simple and can detect IgE binding capacities of both crude and purified food, their major disadvantage is their specificity, considering that commercial solid phases of food allergens can vary in their IgE binding capacities. Moreover, their standardization depends on the use of human sera.

Basophil Activation Test

Another immunochemical methods is the basophil activation test (BAT) also knowns as flow-cytometric allergen stimulation test (FAST). This test detects the activation of basophils by specific food allergens and measures immunochemical markers such as histamine and IL-4 and IL-13 (Sanz *et al*, 2001; Kirsch *et al*., 2009; Monaci and Visconti, 2010; Van Hengel, 2007).

SDS-PAGE Immunoblotting

Another widely used immunochemical approach is the SDS-polyacrylamide gel electrophoresis (PAGE) which detects allergens by separating the food proteins (including targ*et al*lergens) by their molecular weight on a 1D or 2D-SDS-PAGE. However, such techniques are not applicable for rapid and routine analysis and rely on the use of human sera similar to the EAST/RAST assays (Poms *et al*., 2004). SDS-PAGE/immunoblot has been used for the identification and confirmation of various food allergens such as Ara h1-h4 in various snacks (Schäppi *et al*., 2001) and Bet V1 and Ara h1 in different foods (Pastorello and Trambaioli, 2001).

Rocket Immunoelectrophoresis

Rocket immunoelectrophoresis detects food allergens based on their antigenic specificity and electrophoretic mobility. In this assay, food antigens in a sample migrate through a gel containing the antigen-specific antibodies, forming rocket (cone-shaped) bands (Besler *et al*., 2002; Poms *et al*., 2004). This approach has been used for the determination of various food allergens such as egg ovalbumin, hazelnut corylin, milk caseins and peanut proteins in processed food products (Malmheden Yman *et al*., 1994).

Dot immunoblotting

Dot immunoblotting is a simple and inexpensive screening assay for food allergen. Samples are spotted onto a nitrocellulose or polyvinylidene fluoride membrane strips and incubated with enzyme-labeled antibodies. A substrate is then added and the color intensity of the spots is measured. Dot immunoblotting has been used for the detection of allergens such as peanut proteins in various foods (Blais and Phillipe, 2000).

8.4.3 DNA-based Approaches

DNA-based methods provide an indirect method of analyzing allergenic components in foods (Besler *et al.*, 2002). They are based on the amplification of specific genes encoding an allergenic protein using polymerase chain reaction (PCR). There are different PCR methods ranging from conventional (qualitative) PCR to PCR coupled to ELISA and real-time PCR, which provide semi-quantitative or quantitative assessment of allergenic protein concentrations in foods based on their genes (Besler, 2001; Kirsch *et al.*, 2009; Monaci and Visconti, 2010; Van Hengel, 2007).

8.4.3.1 Real-Time PCR

Unlike regular PCR, real-time PCR can be used for both qualitative and quantitative analysis of food allergens. In real-time PCR, the amplification of a specific gene is measured as the reaction progresses with product quantification after each cycle. It uses a target-specific oligonucleotide probe with a reporter and quencher dye attached producing a measurable signal proportional to the amount of specific PCR product (Poms *et al.*, 2004). Various real-time PCR methods have been developed for the detection of food allergens such as lobster allergens (Eischeid, 2016), peanut allergens (Scaravelli *et al.*, 2008; Stephan and Vieths, 2004), and celery, mustard and sesame allergens in processed foods (Mustorp *et al.*, 2008).

8.4.3.2 Microarray Assay

A relatively recent DNA based assay for analyzing allergenic food ingredients is the microarray assay. This method was first applied to the study of allergens in the early 2000s. The assays use preactivated reactive coated glass slides, which acts as a solid phase

antigen, to bind allergen-specific antibodies such as IgEs from the serum of patients. This approach provides a reliable screening tool in epidemiological research for determining food allergy sensitization profiles of populations (Hamilton, 2017).

8.4.4 Mass Spectrometry-based Approaches

A relatively recent approach for detecting food allergens is the use of mass spectrometry-based techniques such as liquid chromatography-mass spectrometry (LC-MS) or tandem mass spectrometry [LC-MS/MS, quadrupole time-of-flight MS/MS (LC-qTOF-MS/MS) and matrix-assisted laser desorption ionization-time of flight (LC-MALDI-TOF-MS)]. These analytical methods are used in the new omics platform of detecting food allergens known as allergenomics (Di Girolamo *et al*., 2015). Allergenomics is considered a powerful tool for comprehensive food determination of protein allergens allowing not only the detection, quantification and characterization of allergens, but also the collection of information on various allergenic proteins and peptides within a food sample in electronic databases. This facilitates and allows the rapid detection of multiple food allergens in a single analysis in a highly sensitive and selective way. Moreover, this approach can be used for analyzing the entire food proteins and/or peptides. Allergenomics has been used for the detection of several allergens in wheat, wine, soy, milk, egg and other sources by various research teams (Monaci *et al*., 2013, 2014, 2016; Uvackova *et al*., 2013; Cryar *et al*., 2013; Flodrová *et al*., 2015). Mass spectrometry-based approaches have many advantageous in terms of sensitivity and selectivity; however, generated data can be very difficult to analyze and interpret due to frequent superimposition of spectra, resolution and ion suppression issues. Moreover, MALDI, LC-MS/MS or qTOF-MS/MS instruments are very costly, require highly trained personnel, and are not easily accessible for routine analysis.

8.4.5 *In vitro* Cell-based Methods for the Prediction of Food Allergenicity

New methods of food processing and the use of genetically modified raw materials in foods may lead to the emergence of new allergens (Jank and Haslberger, 2003; van Putten *et al*.,

2006). The aforementioned methods of detection of known allergens are ineffective for evaluation of potentially new allergens, necessitating the development of new approaches with predictive power. However, potential immunogenicity predictions can be difficult at the population level due to many interacting factors that contribute to allergic responses to food proteins at the individual level. These factors include pattern of proteins digestive breakdown, dose of exposure, effect of other food ingredients and food matrix, and presence of multiple allergenic proteins (Takagi *et al.*, 2003; Peyron, Mouécoucou *et al.*, 2006; Polovic *et al.*, 2007; Mohan *et al.*, 2015). Animal models used for immunogenicity prediction aim at overcoming these challenges, but they suffer from limitations such as difficulty to achieve statistical significance in the results, lack of interlaboratory reproducibility or lack of diversity of animal strains to mimic the human immune system (Bussiere, 2003).

Simple *in vitro* digestion assays have been used to predict the potential allergenicity of novel food proteins. The assays are based on the persistence of proteins, especially the allergenic epitopes, after simulated gastrointestinal digestion (Bannon *et al.*, 2002; Goodman *et al.*, 2008). However, as pointed out by Schnell and Herman (Schnell and Herman, 2009), the resistance of proteins to digestion is only an indicator of possible allergenicity since peptide fragments of digested protein allergens can also trigger allergic reactions (Van Hoeyveld *et al.*, 1998; Yi *et al.*, 2009). These limitations, added to the difficulty to assess dose exposure and food matrix effects, make the use of solely *in vitro* digestion methods a poor predictor of the allergenic potential of novel foods. Therefore, *in vitro* models that utilize human cell cultures have been developed to enhance immunogenicity prediction. These models have been largely developed to evaluate the potential immunogenicity of therapeutic proteins (Johnson and Jiskoot, 2012; Jawa *et al.*, 2013) and can be adapted to food products. The procedures generally consist of (1) subjecting food samples to *in vitro* digestion; (2) adding the resulting digesta to cultured human peripheral blood mononuclear cells (containing T-cells and dendritic cells); and (3) measuring CD4+ T-cells activation.

T-cells activation can be measured by assessing the proliferation of T-cells. This can be performed by scintillation where radioactive thymidine is incorporated into the DNA of the proliferating cells and measured by scintillation (Broman *et al*., 1996; Hamza *et al*., 2013). A simpler method involves the decrease of fluorescence response of labeled T-cells by flow cytometry as the fluorescent dye is diluted with cells proliferation (Sathiyaseelan and Baldwin, 2000; Brenchley and Douek, 2004). T-cells activation can also be measured by assessing the secretion of target cytokines (Hamza *et al*., 2013). Classical ELISA or flow cytometry methods are used to measure cytokines secreted by culture T-cells upon their activation by potential immunogenic proteins (Jung, Schauer *et al*., 1993; Carson and Vignali, 1999). Cytokines as diverse as IL-2, IL-4, IL-5, IL-10, IL-13 and IFN-γ can be detected and quantitated. However, T-cells are not the only cells that can secrete cytokines, which can lead to the overestimation of T-cell activation (Whiteside, 2013). The removal of the other cytokine-secreting cells (NK-cells or CD8+ cells) can affect the response of the CD4+ T-cells themselves, thereby underestimating T-cell activation (Fort *et al*., 1998). Despite these limitations, commercial T-cell-based tools such as EpiScreenTM (Antitope Ltd, Cambridge, UK) have been developed to assess immunogenicity of biopharmaceutical protein formulations (Choi, 2015).

These methods measure the ability of proteins to trigger immune responses, thus they have limited predictive power. Therefore, Strickler *et al*. (2004) suggested a method to rank proteins according to their potential allergenicity using positive and negative controls (proteins known to be immunogenic and tolerizing) on CD4+ T-cells, for which proliferation was measured upon exposure to various proteins. Whereas this method does not claim to measure potency of immunogenic, it can be used to identify protein with reduced allergenic risk. Applied to food products, it may enable the screening and selection of formulations and ingredients based on their risk of triggering allergic reactions. Finally, a general limitation of these methods is the difficulty to establish a physiologically relevant ratio of digesta quantity over number of T-cells that would enable a realistic simulation of the immune response to a certain food.

8.4.6 *In Silico* Methods for the Prediction of Food Allergenicity

A relatively recent and alternative approach for determining the allergenic potential of novel food proteins involves the use of *in silico* methodologies and online databases to assess the inter allergen similarities between newly introduced and previously characterized proteins. The FAO/WHO scientific advisory panel first introduced the integration of bioinformatics approaches in novel allergen determination in 2001 (FAO/WHO, 2001). The approach relies on using public search engines (such as FASTA or BLAST) to perform a global sequence similarity search between the novel protein and known allergens accrued in databases. According to the FAO/WHO *in silico* protocol guidelines, if a novel protein contains a peptide of six consecutive amino acids or has more than 35% homology over 80 amino acids of a known allergen, it can be considered to be allergenic or to have the ability to cross-react with another allergen (Björklund *et al*., 2005; Hayes *et al*., 2016). Although the FAO/WHO recommendations are currently used in predicting the allergenicity of novel proteins, over the past years, several improvements to these protocols have been proposed and applied to enhance the precision and specificity of this predictive approach. For example, *in silico* methods have been designed to compare amino acid sequences and structures of potential allergens with IgE epitope data, which enhances the precision of the method (Sharma *et al*., 2013). Other studies have improved allergenicity prediction by using larger datasets and more elaborated algorithms (Soeria-Atmadja *et al*., 2004), while others have developed motif-based approaches to be used along with the existing sequence similarity search methods (Li *et al*., 2004).

An important factor for conducting structural comparison is the availability of representative protein databases developed from large-scale DNA sequencing studies. Currently, there are various allergen databases and allergen prediction servers that contain and offer information on International Union of Immunological Societies (IUIS) or non-IUIS recognized allergen proteins with IgE binding capacity. These databases differ from each other especially with the various allergen features presented (Table 8.3). Some of the databases [for example, All

Allergy (http://allallergy.net) and IUIS (http://www.allergen.org)] are simple compilations of allergenic proteins providing general information such as nomenclature and Genbank accession numbers. Moreover, others [for example, Allergome (http://www.allergome.org] and Structural Database of Allergenic Proteins (SDAP) (http://fermi.utmb.edu/SDAP)] provide additional details such as allergen motifs, protein sequences, IgE-binding B-cell epitope collections, and links to sequence and structural databases (Table 8.3), which facilitates the comparison of the molecular properties of proteins and their epitopes to existing allergens. Once a database is selected, assessment of allergenicity or cross-reactivity of a protein then depends on determining the overall sequence similarity to known allergens by using tools such as FASTA or BLAST. For example, FASTA can be run from any sequence file in a database and generates a table that lists all similar allergens in that database along with the statistical significance (or the E-value) of each result (Schein *et al*., 2007). This method provides comparative information on the resemblance of one protein to another in terms of their molecular sequence; however, in order to establish (with confidence) its functionality as an allergen, additional information should be used and *in vitro* and *in vivo* testing should be conducted. Recently, Hayes *et al*. (2016) proposed the integration of a four step extensive guide for *in silico* methods that provide a better prediction of allergenic and cross-reactivity potential of proteins. In this scheme, *in silico* methods of both T-cell and B-cell epitope identification is suggested, followed by a protein sequence structure and pattern analysis (by selecting comprehensive databases and algorithms), and finally a study of immunogenicity providing empirical validation of allergenicity (Hayes *et al*., 2016). A similar multistep approach was also proposed by Jawa *et al*. (2013) and consisted of integrating sequence-driven assessments, obtained using *in silico* algorithms, with *in vitro* assays and *in vivo* models (Figure 8.2) (Jawa *et al*., 2013).

In conclusion, *in silico* tools serve as powerful high throughput screening methods that can compare a large number of proteins in a short period of time, with low cost, hence reducing the need for *in vitro* testing on large sample sets (Jawa *et al*., 2013). However, the approach has several limitations as follows:

Table 8.3 Major allergen databases and their functional features

Allergen Database	URL	Hosting country	Functional features
ABCpred	http://www.imtech.res.in/raghava/abcpred/	Institute of Microbial Technology, India	Predicts B cell epitopes in an antigen sequence using datasets of fixed length patterns.
AlgPred	http://www.imtech.res.in/raghava/algpred/	Institute of Microbial Technology, India	Predicts allergens using combined approach comprised of Support Vector Machine (SVM) module + IgE epitope mapping + allergen-representative peptides BLAST + MAST.
AllAllergy	http://www.allallergy.net	–	Provides general information on allergens. allergens are listed by category for each food group.
AllergenOnline (FARRP)	http://www.allergenonline.org	University of Nebraska-Lincoln, USA	Provides comprehensive information on allergens including names, Genbank accession numbers and thresholds. It is used to assess the allergenicity and cross-reactivity potential of novel proteins. It incorporates protein sequence searchable tool and FASTA search algorithm.
Allergome	http://www.allergome.org	Italy	Provides a searchable list of allergens and sequence information. It also provides links to related research on PubMed.
AllerHunter	http://tiger.dbs.nus.edu.sg/AllerHunter/	National University of Singapore, Singapore	Provides cross-reactive allergen prediction using a combination of combining SVM and pairwise sequence similarity.
Allermatch	http://www.allermatch.org/	Wageningen, The Netherlands	Used to compare protein sequences of novel proteins with known allergens. It provides UniProt and WHO-IUIS databases sequences of known allergenic proteins.

AllerTOP 1.0 and 2.0	http://www.pharmfac.net/allertop/	Medical University of Sofia, Bulgaria	Uses autocross covariance which is a protein sequence mining method. It transforms protein sequences into uniform equal-length vectors.
Biopep	http://www.uwm.edu.pl/biochemia/index.php/en/biopep	University of Warmia and Mazury, Poland	Provides a list of allergeic protein names, sequences, and IgE epitopes.
Food Safety Centre Allergen Bureau	http://www.allergenbureau.net	Australia	Provides information and guide on the presence of allergens in foods.
InformAll	http://research.bmh.manchester.ac.uk/informall/allergenic-foods/	University of Manchester, UK	Lists the allergen in different foods and provides related clinical and biochemical information allergens, epitopes, sequences, links to literature.
IUIS (International Union of Immunological Societies)	http://www.allergen.org	–	Provides a comprehensive list of allergen names, Lineage, biochemical name, and Genbank accession. It also provides some information of allergenicity of each allergen.
Pfam	http://pfam.xfam.org/	Harvard University, USA	Contains sequence information on a large collection of protein families and indicates which other allergens a unknown protein resembles.
Structural database of Allergenic proteins (SDAP)	http://fermi.utmb.edu/SDAP	University of Texas, USA	A comparative server that integrates a database of allergenic proteins with various computational tools. It Provides allergen sequences, IgE epitope collections, and links to sequence and structural databases such as SwissProt, PIR, NCBI, PDB.
UniProt	http://www.uniprot.org/	–	Uses BLAST and Clustal Omega program to align and compare sequences from peptides and proteins

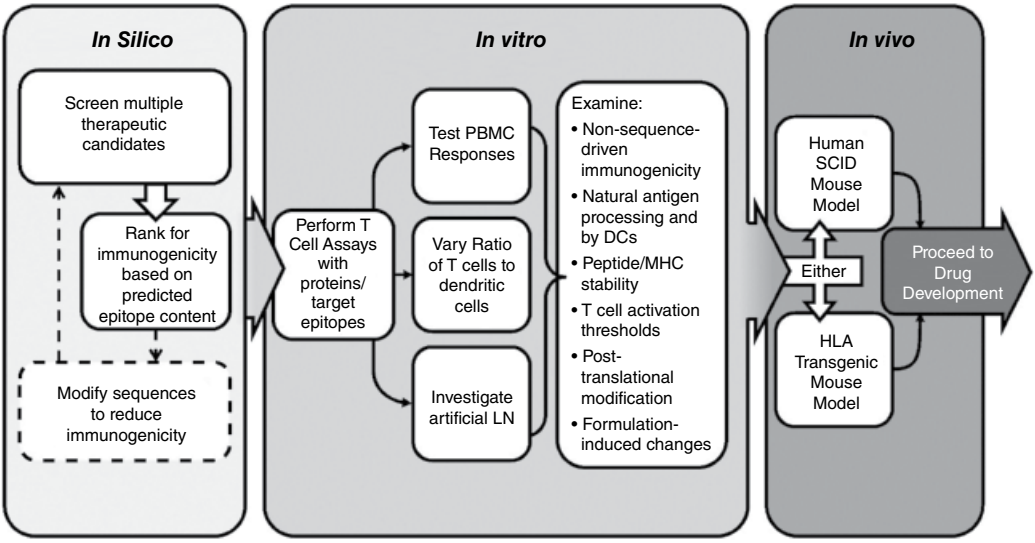

Figure 8.2 Overview of steps for immunogenicity prediction highlighting the *in silico, in vitro* and *in vivo* evaluation methods (Reprinted from Jawa *et al*., *Clinical Immunology*, 2013; 149: 534–555, with permission from Elsevier).

- *In silico* methods do not provide a conclusive proof on the presence of allergens but rather provide a prediction of potential allergenicity.
- *In silico* predictions made using allergen databases depend upon the datasets, and on the update and maintenance of the databases.
- *In silico* methods do not use experimental tools to validate the 3D structure of a novel protein but derived the structures from linear epitopes, which do not always depict the accurate structure of a protein.
- *In silico* methods may not be able to predict whether allergenicity will be altered during food processing.
- Computational methods for allergenic protein detection may not be able to accurately discriminate between an allergen and a non-allergen, thereby potentially providing false positive results.

Therefore, *in silico* methods need to be validated using *in vitro* and *in vivo* testing. Moreover, based on the merits and limitations of each method, we recommend the use of a combination of several methods for accurately detecting and quantifying existing and new allergens in food products.

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9

Challenges of Linking *In vitro* Analysis to Flavor Perception

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9.1 Introduction

When consumers are asked for their opinions on food and drink, flavor is often mentioned as one of the key properties that determine acceptance and palatability. The role of flavor not only contributes to a pleasurable eating experience, but provides the identity of food and drink. Describing the quality of a flavor can be difficult to articulate for most people due to the subjective nature of the subject. The most common approach to understand flavor involves sensory and consumer studies, which helps standardize vocabulary (lexicon) and apply statistical rigor; however, this is usually in isolation to chemical compounds that stimulate sensory perception. The use of analytical tools should provide objectivity to determine key flavor component(s) that contribute to flavor. Flavor analysis requires careful consideration of extraction, detection and expert data interpretation to understand flavor perception. The interpretation of flavor analysis data requires the appreciation of the complex relationship between chemical stimuli, the biological receptors and sensory perception as detailed Chapter 3. Decades of flavor research has resulted in a multitude of approaches to develop *in vitro*

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techniques to attempt to tackle the analytical challenges. Many are still fundamental, but as yet no single method can be depended on to explain the full impact of flavor.

This chapter introduces the reader to the complexity of flavor and techniques of flavor analysis. An overview of techniques is presented, from established and current research methods, to new and emerging *in vitro* techniques. The chapter highlights existing issues to current "gold standard" methods such as gas chromatography olfactometry, and how new *in vitro* methods have to overcome the challenges of linking chemical analysis to flavor perception.

9.2 What is "Flavor"?

Flavor perception is a result of chemical, physical and visual stimuli and their interactions, at the receptor and neural level. The reader is encouraged to read Chapter 3 "*In vivo* Foundations for *In vitro* Testing of Functional Foods" in tandem for further background. The dominant features of flavor are "taste" and "aroma" as a result of "non-volatile" and "volatile" chemical stimuli respectively. The sense of taste can be simply categorized into sweet, sour, salty, bitter and umami. In contrast to taste, categorizing aroma into distinct groups is not so simple. Aroma descriptions can generate a vast list of descriptors. Aroma wheels can be used to deconstruct all perceivable sensations into general categories and then sub-divided further into very specific odor descriptors. This was first widely applied to wines as an approach to bridge generic terms to more defined sensory descriptors and create a visual aid called a "wine flavor wheel" (Noble, 1987).

9.2.1 Flavor Analysis Overview

Flavor was once a subject that was studied in isolation to the other senses and initially approached as a targeted and systematic analytical exercise to uncover key flavor impact compounds. Taste compounds have typically followed a classical wet chemistry approach due to their non-volatile chemistry, though the search for new and novel compounds has led recent research

into taste receptor and cell model techniques. These are at early developmental stages and are used to screen compounds based on ligand-binding studies, between the chemical stimuli and taste receptor (the reader is referred to Chapter 3 and section 9.7 of this chapter).

Aroma analysis is a multi-step procedure due to the large range of physicochemical properties of compound volatility and polarity. This generally falls into three main steps: isolation, separation and detection. Sample preparation, specifically "flavor isolation", is a major topic that cannot be fully covered in this short chapter and the reader is referred to texts by Marsili, 1997; Parliment, 1997; Morello and Mussinan, 1998; and Reineccius 2006. As with any analysis, the end goal or objective will drive the selection of the most appropriate approach and may require a combination of analytical tools to understand the flavor of foods and beverages from a consumer point of view.

9.2.2 Significance of Aroma Compounds

Flavor development is significantly influenced by aroma chemistry through chemical, thermal and enzymatic reactions. Reactions such as lipid oxidation, Maillard browning (i.e. baking and roasting) and enzymatic release (i.e. fruits and vegetables) produce significant contributions to the flavor profile. The contribution of aroma compounds to food flavor is generally greater than that of taste compounds. This can be demonstrated in fruits where many fruits share common taste compounds, e.g. sugars and acids, though it is the aroma compound profile that drives our perception of different fruit flavors. The proportion of aroma compounds relative to each other is a significant factor in defining flavor character. Similarly across meat products where taste compounds are shared, the specific lipid profiles for each meat source generate the identifying aroma and define the meat flavor. Taste can dominate when it is overpowering, e.g. sweetness or sourness, or unpleasant such as bitterness or saltiness.

To demonstrate the power of aroma on the perception of flavor, an example of drinking a fresh cup of roasted coffee brew is given. The taste profile of coffee is considered complex. When placed in the mouth specific descriptions such as bitter, sour, hot, smooth, are easy to verbalize. The taste profile of coffee also

includes a slight sourness from carboxylic acids, body from polysaccharides, proteins and lipids; as well as slight astringency from chlorogenic acids (Buffo, 2004). The effect on the taste profile is fairly predictable when the composition of taste compounds is measured. However, when people are asked to describe in detail the aroma of coffee, the complexity is much greater as most people struggle to really explain beyond the taste quality of bitterness in any detail. When analyzing coffee aroma composition (which currently stands at over 900 identified compounds generated via biochemical reactions and formation mechanisms such as the Maillard reaction during roasting, carotene degradation and phenol degradation) the analyst has to consider the entire flavor composition to assess flavor quality.

In the early years before such information was known, it was generally assumed that one or two abundant compounds provide the aroma impact character of a flavor. However this is not the case, as the top 20 most odorous compounds in coffee range in various sensory descriptions such as "toasted", "fruity", "nutty", "potato", "plastic", "green", "cotton candy" with no one compound being described as "coffee" (Czerny *et al.*, 1999; Mayer *et al.*, 2000). Even with recent advancement in analytical and data processing techniques, it is difficult to explain how 900+ different aroma compounds can be recognized to be a pleasurable coffee aroma by the olfactory and neural system.

9.2.3 Challenges of Food Flavor Compounds

Any chemical analysis technique is usually designed for the target chemical, and typically *in vitro* techniques are designed to be highly specific. However, the chemistry of flavor compounds cover the broadest range of organic compounds. Taste compounds are easy to characterize and group into the five basic tastes, but they span diverse chemistries including minerals, sugars, peptides, acids to proteins. Aroma compounds are small molecules (<300 Daltons) with a vast majority formed by just carbon, hydrogen, and oxygen, with thermally or enzyme generated compounds also containing nitrogen or sulfur. There are reported to be up to 10,000 aroma compounds of which only ~2,600 are found in food and culinary applications (Fisher and

Scott, 1997). Aroma compounds include a wide range of chemical classes: aldehydes, ketones, esters, alcohols, terpenes, pyrazines, sulfur compounds etc, and structural forms: acyclic, cyclic, bicyclic etc. The combination of chemical and structure possibilities results in a wide range of physicochemical properties. The two most important physicochemical properties from a flavor chemistry point of view are solubility (the ability to dissolve in aqueous or lipid phases) and vapor pressure (the tendency of particles to escape into the gas phase). Vapor pressure of aroma compounds can range from virtually always a gas to semi-volatile compounds.

A classification system to group flavor compounds by chemistry or physicochemical property would help simplify analysis and provide dedicated *in vitro* techniques. This can be loosely applied to taste compounds, for example, sugars impart sweetness and organic acids impart sourness. However, chemical or functional classification cannot adequately predict aroma compound odor quality as there is no obvious relationship with the chemical structures. A single class of aroma compounds will likely impart a wide range of aroma sensations as the example given in Figure 9.1 in which the four aldehydes are perceived to be drastically different to one another. Hence there is no chemical based categorization of aroma compounds. As described in Chapter 3, the natural olfaction system can detect volatile molecules and

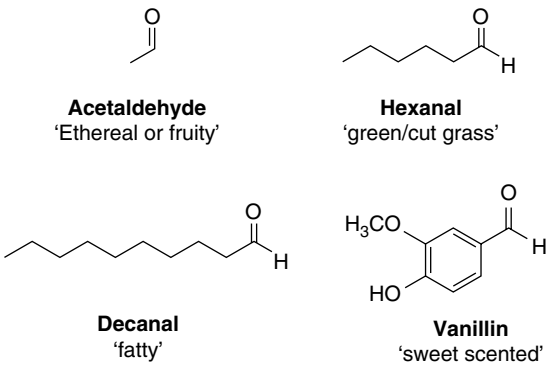
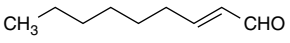


Figure 9.1 Four aldehydes with very different odor sensory properties. These descriptions can vary depending on the ability of the person to recall or associate words to scents. (Adapted from Fisher and Scott, 1997.)

discriminate odor quality. Therefore the ideal *in vitro* olfactory system would require a chemical or signal processing step, in order to evaluate aroma quality and link it to a describable "flavor label". As of yet, there is no current understanding of the odor recognition system, other than controversial theories such as vibrational theory (Franco *et al*., 2011) and shape theory (Dyson and Bell, 1928) both of which do not satisfactorily explain the receptor–aroma interactions for recognition.

Furthermore, structural rearrangements can form stereoisomers, which are mirror images of one another, like our right and left hands. These subtle difference can be perceived as different aroma sensations. As an example, carvone is a natural compound which can be found as two different enantiomers. Carvone can be found as either R-(–)-Carvone (sweet spearmint) or S-(+)-Carvone (caraway), imparting quite different aroma sensations.

Most common taste compounds are often present at readily detectable levels from mg/L to percentage levels. Aroma compounds can contribute to flavor at much lower levels, down to sub parts per billion and even parts per trillion for some sulfur compounds. The aroma quality or description of some aroma compounds can also vary with concentration (Figure 9.2) for the same chemical as in the example of trans-non-2-enal (Forss, 1981). This type of effect can be seen for many different compounds and will depend on their odor threshold. The odor threshold is the concentration at which an aroma compound is detected (Van Gemert and Nettenbreijer, 1977), which varies substantially between compounds. Some caution is required when using odor thresholds as the values will differ depending on the food matrix. Depending on the solubility and vapor pressure, the values will significantly differ in water, oil or air based systems. As most food matrices (except simple beverages) are not simple mono systems, the matrix composition will affect the release of



1000 ppb In water at 1000 ppb it can impart a strong cucumber note.

8 ppb Above 8 ppb it imparts a fatty note until becoming unpleasant at 30 ppb

0.1 ppb At just above typical detection level of 0.1 ppb it imparts a woody character

Figure 9.2 The change in aroma quality of trans-non-2-enal at increasing concentrations (Forss, 1981 and adapted from Fisher and Scott, 1997).

aroma and therefore, the amount of aroma reaching the nose. This will have a significant perceptual impact.

Similarly, taste compounds from the same chemical class can differ in their sensory qualities. For example the perception of saltiness is defined by the salt character of sodium chloride, yet salts within the same chemical series such as potassium and magnesium which can impart saltiness, also impart non-desirable sensations such as metal or bitter to different degrees. This is a challenge for *in vitro* analyses when screening for salt substitutes as not only the salt level intensity is required, but also the quality of the "salt" taste profile i.e. without other aftertastes.

9.3 Overview of Flavor Analysis Techniques

In order to isolate a flavoring from a culinary preparation or biological material, an appropriate technique is required in order to extract the representative flavor. Most food products are mixtures of carbohydrate, proteins and fats as mixed phase systems unlike simple model food systems often studied in research. The high extraction efficiency is required to overcome the trace level and contend with the large range of chemistries and physicochemical properties of aroma and taste compounds to derive the flavor composition. Thus it is clear that a range of isolation techniques are required to tackle these challenges. The analyst should be aware that the matrix will affect the degree of isolation (Maarse, 1991).

9.3.1 Key Isolation Techniques

It becomes evident that in order to perform an extraction the technique employed must consider the end objective of the analytical exercise. Is the objective, to extract and identify every compound? Or is there a need to identify a specific compound, such as a taint? Or, to establish the difference between two similar products that are perceived differently? In which case the full profile will require investigation to determine how different the profiles are and for which components. An important consideration to highlight is do we require absolute quantities present in the food matrix, or should we measure the actual amount of flavor compounds that is released and delivered to the sensory receptor?

9.3.2 Taste Compound Isolation

A wide range of chemical and physical techniques can be used to isolate and concentrate flavor compounds ready for analysis. Taste compounds are more commonly found in higher abundance and therefore pose a lesser problem when compared to aroma compounds. Taste compounds are non-volatile polar compounds that are stable under most conditions and therefore pose less challenges than aroma compounds. Most taste extraction techniques use the following steps:

- Homogenization to produce a representative sample.
- Steeping and extraction (aqueous, alcohol or other polar extraction solvent).
- Physical filtering to remove unwanted materials.
- Chemical separation to isolate the taste compounds from other components.
- Detection.

Manual techniques developed over the years have been updated with automated techniques, however some analyses are still labor intensive due to the different steps involved.

9.3.3 Aroma Compound Isolation

The selection of the isolation technique is critical and should be considered along with the analytical objective. Aroma isolation steps are critical as different methods will be required due to the diversity in physicochemical properties of the aroma compounds, as well as the challenges of trace level quantities. This section provides an introduction to the three most common approaches used in food aroma extraction and isolation which is a critical step often overlooked when evaluating total flavor.

9.3.3.1 Solvent Extraction

Solvent extraction is a simple and straight forward technique. The sample is submersed into a solvent, agitated for a period of time, centrifuged to separate the solvent from the sample residue, before recovering the solvent for analysis. Typically non-polar solvents such as hexane or dichloromethane have been used, but, other methods can also utilize more polar solvents, such as ethanol and methanol depending on the food matrix and

compound polarity. This approach suits odorous samples with high aroma concentrations. However, the solubility of compounds will differ depending on solvent properties, and differences in recovery will distort flavor profiles. Simple aqueous beverages or solids without lipids can be extracted this way. This technique is not suitable if other matrix components are extracted which may interfere with analysis such as lipids and proteins, for these samples a distillation is required.

9.3.3.2 Distillation

The volatility of aroma compounds makes them suitable for distillation. The principles of distillation are based on boiling point and vapor pressure which can be used to isolate volatile aroma compounds from the non-volatile solid or liquid food matrix. This is achieved by heating the sample in the presence of water to increase the efficiency of extraction. The combined effect of the heat and the steam lowers the boiling point of the aroma volatiles via the process of co-distillation. The steam distillate is collected by re-condensation via a cold trap. The aqueous distillate is recovered and then extracted with a non-polar organic solvent which is then dried with anhydrous salts before concentration. An improvement in the basic distillation apparatus was made by combining the steam distillation with organic solvent extraction (Nickerson and Likens, 1966). The initial aqueous distillate would condense and be simultaneously extracted by the organic solvent. Careful set-up and skill in performing this technique is required to prevent distortion or artefact formation within the aroma profile.

9.3.3.3 Headspace

The headspace (the airspace directly above the sample) is often sought for analysis as this represents the typical aroma profile composition being delivered to the nose for smell. The principle behind headspace technique is to place a known amount of sample in a sealed flask. The aroma of the sample is then allowed to come to equilibrium by allowing it to stand for a period of time specific for the sample. During this time, the volatile aroma compounds redistribute between the sample and headspace, in part determined by the partition coefficient (Gretch, 1995) of each aroma compound. The headspace extraction can be

enriched by forced purging, also referred to as dynamic headspace in which greater release of aroma is encouraged. The aroma can be continuously purged and trapped on to an absorbent material such as activated charcoal or a polymer for off-line analysis. Another critical parameter to control is sampling temperature which will invariably affect the volatility of compounds and can significantly change the headspace aroma concentration. This approach is biased in that semi-volatiles higher boiling point compounds may not be readily extracted.

9.3.4 Taste Compound Detection

Salts and acids can be isolated and concentrations determined by electrodes such as conductivity sensors for salts and pH meters for indication of acids, although the titratable acidity (hydrogen ion concentration) provides a better correlation to acidity levels. Taste compounds such as sugars, acids, tannins, polyphenols and peptides are commonly analyzed and quantified on liquid chromatography instruments with a suitable spectroscopy detector. For low concentration compounds adequate selectivity and sensitivity is required. Recent advances in liquid chromatography (LC) resolution and mass spectrometry (MS) detection levels allow analysis of previously difficult separations and low concentration compounds.

9.3.5 Aroma Compound Separation and Detection

Before the aroma isolate is detected, the critical step of sample separation is required by way of gas chromatography (GC). GC is the current standard aroma analysis method, in which a compound's boiling point and affinity to the non-mobile phase of the column is used to separate mixtures prior to detection. Capillary columns for gas chromatography were a significant advancement in the systems of gas chromatography that impacted on all aspects of volatile analysis. These columns are long (25 m or more) thin (0.25 mm) tubes with a stationary phase coating the inner wall. Compounds adsorb into the wall coatings at low temperatures and leave the coating as temperatures increase.

The most widespread general detector for aroma was the flame ionization detector (FID) due to its relatively simple use

and robustness. In FID, ions are detected upon the combustion of the volatile organic compounds, generating a signal. This strength of the signal is based on the quantity of the organic compound and not related to the aroma strength. No further information is provided by the FID detector and no differentiation is achieved between individual compounds.

Mass spectrometers (MS) provide more sensitive and selective detection of volatile compounds and the parallel development of long thin columns were easy to interface with MS detectors. Compounds introduced into the MS enter intact until reaching the ionization chamber where the compound is bombarded with high energy electrons (electron impact, EI) which fragment the compound into distinct ion species. These species make up a spectral profile based on the mass of the fragmented species called a mass spectral profile. This spectral profile is effectively a "fingerprint" of the compound which can assist in identification by comparing the spectral profile to that of a known chemical standard.

9.4 Further Developments in Aroma Analysis

9.4.1 Gas Chromatography–Olfactometry

The analyst can be confronted with chromatograms containing many peaks for different compounds, none of which may smell exactly like the original foodstuff. It is typically their combined action at the olfactory epithelium in the nose that creates that sensory impression. Consequently, the development of improved gas chromatography column and detection systems could leave the analyst wondering, which out of a forest of peaks were the most important compounds to measure? Gas chromatography combined with an odor port, gas chromatography–olfactometry, or GCO, was developed as a method to try and overcome this issue.

In GCO the sample extract is injected into the gas chromatography system and the compounds are separated on a column as they would be in a regular analytical run (Figure 9.3). The difference occurs post column. Here the flow from the column is split (Figure 9.3 (3)). Part of the flow is directed to a detection system,

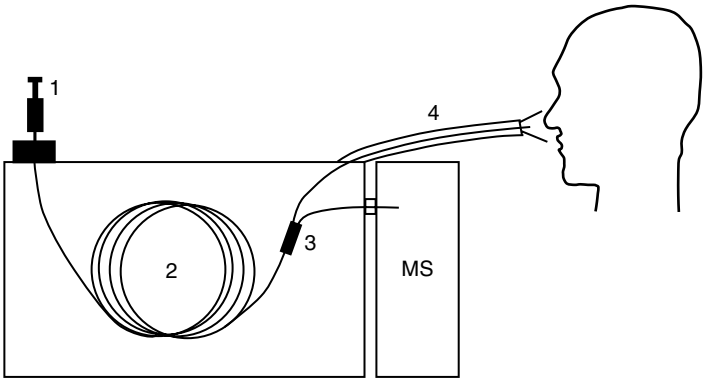


Figure 9.3 GCO schematic. The compounds are injected into the gas chromatograph (1), separated on the column (2) as it heats up within the GC oven, a splitter (3) then divides the flow, part enters the mass spectrometer detector (MS) to generate a chromatogram, the remainder exits through a heated transferline (4) where compounds can be inhaled and detected in the nose.

such as a mass spectrometer; the remainder flows out through a heated transferline where the compounds exiting the column can be inhaled and detected by a human nose. This basically allows an individual to sniff their way through all of the compounds of a chromatogram, to determine which ones are odorous and to evaluate their odor attributes.

9.4.2 Interpretation of GC–Olfactometry Data

The objectives of GCO can be targeted or broad. A typical example of the targeted approach would be to search for a contaminant, such as a taint. This would profile the contaminated sample and the standard food sample for comparison. Here the panellist would be focusing on compounds arriving at the odor port which had attributes characteristic of the problem odor. This can simplify the process of contaminant detection. The broad approach, would be to evaluate all compounds arriving at the odor port, to gain a complete profile of the odiferous compounds that are separated by the GC column. One of the main problems in GCO studies is the use of human assessors and their differing sensitivity to aroma compounds. Several

GCO methods have been proposed to try and overcome this limitation.

Two of the methods, CHARM (Acree *et al.*, 1984) and AEDA (Ulrich and Grosh, 1987) rely on the panellist sniffing a series of GCO runs with the sample at different dilutions. This is achieved by using a solvent to extract the aroma compounds from the food matrix; this is then diluted by a factor of × 2 at each step. The compounds detected in the GCO run of the most diluted sample extract are considered to be the most potent odorants associated with that sample. The output of the GCO analysis is an aromagram to complement the chromatogram. The data from the panellist sniffing a series of GCO runs are stacked up resulting in the aromagram. The height of the signal indicates the intensity of the aroma (Figure 9.4). In the AEDA approach,

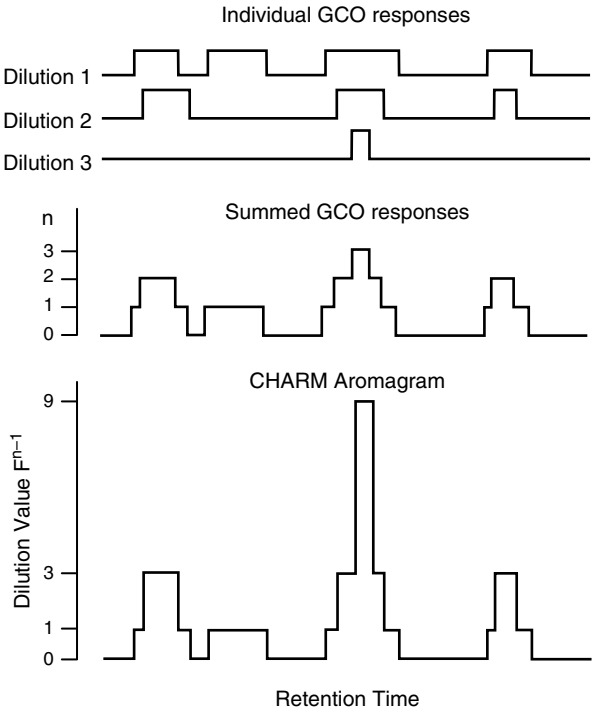


Figure 9.4 The production of a CHARM aromagram from the summation and scaling of individual GCO response traces.

the aromas are simply scored as present when they are detected by the panellist. These scores are converted into an aromagram, consisting of a series of vertical lines along the aromagram. The height of the line is used to represent the intensity of the aroma with the dilution factor on the y-axis.

Other methods such as OSME, proposed by McDaniel and co-workers (1990) generated aromagrams using four assessors, each performing four replicate runs. The assessors in this case registered their perception on a 15 cm sensory scale. Intensity scores were built into the overall average if they were detected at least 50% of the time. Consequently, this approach used a combination of sensory scaling and frequency of detection to profile samples.

Pollien and co-workers (1997) used a simple frequency detection method to generate their aromagrams. They used headspace samples at one concentration which were evaluated by a minimum of six panellists. The signal for the six individual GCO runs was then summed, to give a final aromagram. The use of several panellists, each performing one run, minimized the potential for any one individual's olfactory variation to significantly influence the aromagram. Whilst performing, as few as six runs was one of the simplest methods to generate the aromagram.

Many groups have used variants of these four main methods to generate aromagrams. It is however important to remember the limitations of such data. One of the key issues is the result of the GC separation itself; this delivers individual compounds to the panellist for detection. Consequently, a series of compounds that would be sensed together in a mixture as in a real food system (or would stimulate the olfactory epithelium in a similar manner), such as the fruity esters, will lose their additive effect when delivered separately. In addition, the aromas are sensed orthonasally in the gas phase; the context of the taste, or other attributes of the food matrix, which might influence perception are not present. However, the main limitations are the time it takes to perform the GCO runs, which is a very laborious task, and issues concerning the preparation of the sample for analysis.

Despite its limitations, GCO is a valuable tool in the analytical laboratory and can reveal compounds of key aroma significance that are present in the chromatogram that may appear as only

minor peaks. These may be overlooked by simply using the output of a physical detector, such as the mass spectrometer, since this does not have the same functionality as the human nose.

The key use of GCO data, is to use it as a filter to determine the most odiferous components of a flavor system and to potentially pay lesser attention to the magnitude of the aromagram values. At the 2014 Weurman Flavor Symposium, there were several examples presented where compounds with lower impact scores in the GCO profile were significant players as key aroma compounds for particular flavor systems. For example EZ and EE 2,4-decadienoate were found to be key to the aroma quality of pear brandy, but, were not considered the most intense aroma components of the sample. These were termed "specialists" of the aroma profile, key compounds that were of high character importance to the flavor of a foodstuff. In contrast were other compounds described as "generalists" which formed the broad backbone of the flavor for these compounds to rest on.

9.4.3 Recent Advances in Aroma Extract Preparation

GCO directs the analyst towards the compounds that can be measured. Thereafter there is the issue of how to routinely measure aroma compounds of the sample. One of the main factors that will affect the analytical approach, is the number of samples that need to be processed. The issue of how best to capture the total aroma profile has led to techniques that try to address this by advances or adaption to traditional headspace and solvent techniques.

9.4.4 Solid-Phase MicroExtraction

Solid-Phase MicroExtraction (SPME) is a simple headspace system that is readily automated and ideally suited for use with modern GC hardware and capillary GC columns (Pawliszyn, 1999; and also see the Supleco website for documentation). The key feature of the system is the absorptive fiber. The SPME fiber coating absorbs volatile compounds in much the same way that

compounds stick to the inside of a capillary GC column when it is cold. Consequently, when the fiber is exposed in the headspace above a food sample the volatiles are trapped, or, adsorbed onto the fiber surface due to the low temperature environment (Figure 9.5). When the fiber is exposed to a higher temperature environment, such as the injector region of a GC, the volatile compounds leave the fiber and re-enter the gas phase.

The system does, however, have its limitations. The sample profile is dependent on volatiles partitioning from the matrix, into the gas phase and then onto the fiber. This makes it very difficult to achieve any realistic quantification of the aroma content of most foods. Equally, matrix effects, such as fat will affect volatile partitioning into the gas phase. However, for simple, high throughput sampling, it is easy to use, and sample preparation can be no more complex than to place the sample in the vial and crimp the cap on.

New developments in solid-phase extraction are the use of solid bar sorptive extraction systems (SBSE) (Baltussen *et al*., 1999). This works on a similar principle to SPME. However, the

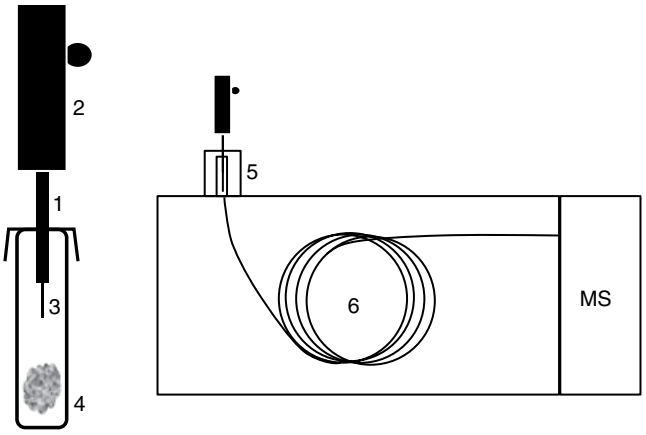


Figure 9.5 SPME sampling; the needle (1) mounted on the SPME fiber holder (2) enters the sample vial, the fiber is exposed (3) and adsorbs volatile compounds from the sample (4) headspace. The fiber is then exposed in the hot injector liner (5) and the compounds pass on the GC column (6) for chromatography.

device is larger and of similar size to a small stir bar with a thicker coating. The greater loading capacity potential of the thicker film coating, allows for longer sampling time for low concentration compounds, whilst minimizing skewing of the profile for the more abundant compounds in the same headspace. Additionally, the bar can also be used for "clean" matrix liquids although most food systems are multi phased, which can contribute to artefact formation in the GC injector.

9.4.5 Advances in Solvent Assisted Flavor Extraction

Distillation can be used to separate volatile compounds from non-volatile components, but there is the potential to damage or distort the volatile profile. One system that has attempted to solve this problem is Solvent Assisted Flavor Evaporation or SAFE (Engel *et al.*, 1999). The SAFE apparatus consists of an evaporating flask maintained at 30°C and a collecting vessel for the distillate at –196°C. The apparatus is used whilst under vacuum, which aids the volatilization of the solvent extract. This gentle distillation reduces artefact formation whilst providing an extract more representative of the total aroma.

SAFE analysis has been combined with the use of stable isotope internal standards to provide one of the best methods for accurate quantification of all of the key components of a sample. The GCO data guides the analyst to the most important odorants present in a sample. Once these have all been identified, isotopically labeled analogues of each compound can be synthesized. These can then be spiked into an extract of the sample, to compensate for any losses of each individual compound during sample work up and chromatography. A comparison of the chromatographic signal for the known amount of labeled compound spike, vs. the signal for the unlabeled compound from the sample, allows accurate quantification compound by compound. To further confirm that the compounds are of genuine significance to the aroma of the product, Odor Activity Values (OAV) can be calculated and recombination studies carried out (Grosch, 1994).

9.4.6 Challenges of Single Aroma Compound Data Interpretation

OAV are calculated by dividing the in-sample volatile concentration by its odor threshold. If the OAV is greater than 1, then the compound is considered to have olfactory significance during consumption. The larger the OAV the more it is likely to have importance for the foodstuff. Such an approach relies not only on accurate quantification of the compounds present in the sample, but, also on the accuracy of the odor threshold of the compound itself. However, a limitation of this approach is due to the psychophysical laws and the assumption that volatile delivery for all compounds *in vivo* is equal. The relationship between volatile quantity (dose) and perceived intensity is not linear but in fact sigmoidal with an initial phase during which there may be changes in concentration, but the stimulus is too weak for perception. This is followed by a near linear phase, after which increasing the dose has less and less impact on perceived intensity (Figure 9.6). This is an important consideration when interpreting *in vitro* assay data as the mechanistic relationship between aroma dose and perceived intensity is not yet fully understood.

Recombination studies have also been carried out, partly due to the limitations of OAV, and also to evaluate the potential additivity of sensory stimuli. Foods are consumed by human assessors, as no *in vitro* technique can describe flavor quality in descriptive terms. These studies involve re-creating the mixture of flavor compounds in the original product from their synthetic counterparts. This is possible due to the quality of the quantitative data available from the GCO, SAFE solvent extract and stable

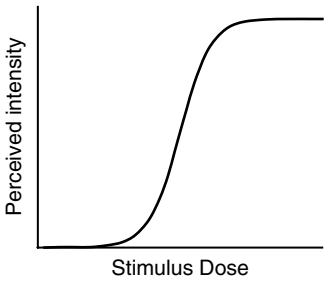


Figure 9.6 The sigmoidal nature of the stimulus dose–perception intensity relationship.

isotope quantification approach. The recombined flavor is then subjected to a sensory leave-one-out and re-evaluate approach. If a compound is left out of the mixture and there is little perceived difference, then the compound is of minor significance to the overall flavor. If its removal has a major impact, then its significance is demonstrated. This too, is where the specialists and the generalists of aroma are observed. Removal of a generalist, may simply weaken the overall aroma, whereas removal of a specialist, will impact more specifically on the character, or trueness to type of the mixture. There is no *in vitro* method now or in the near future that could replace this approach.

9.4.7 Correlation of the Sensory Experience with GC Data

Overall, analytical approaches via GC with GCO, quantification, OAV and recombination studies reveal a significant amount about the key drivers of a flavor system. There are however limitations in its contribution towards our sensory understanding of flavor. Components of the flavor mixture will match to sensory attributes; aldehydes and alcohols to green notes and esters to fruity dimensions. However, the recombined sample is just one sample and the variation in many samples needs aligning with descriptive sensory studies to determine the relationship between analysts and perception.

These methods also work best on simple food systems, such as liquids, where there are minimal matrix interactions and the system is easy to re-create. Consequently, it is easier to analyze and re-create a strawberry, since the aroma compounds are effectively held in the fruit and released into the juice when it is crushed during eating.

There are yet more factors that may combine to make it difficult to establish the link between the compounds present in a food matrix and perception. One example was presented by Imre Blank at the Weurman Flavor Symposium in 2014. He showed that furfuryl thiol, which has a burnt rubber aroma, and diacetyl, which is buttery, could combine sensorially to give a coffee-like aroma. Separate analyses of these compounds which most *in vitro* methods follow, lose the ability to detect the additive nature of such sensory integration.

The analytical procedure can be a complex process. This makes it difficult to accurately quantify the aroma in a large number of samples. In combined sensory and analytical studies, often the analytical component relies on simpler methods to process the number of samples required. This is then followed by statistical analysis, such as principle component analysis (PCA) to deconvolute the large amount of data acquired. Without multiple profiles of the composition of multiple samples, correlations between the odor active components of the aromagram and sensory descriptors is a challenge. As the techniques mature, hopefully there will be a better integration of the most advanced analysis of samples and sensory descriptive studies.

9.5 Recent Advances Developing *In vitro* Flavor Analysis Tools

The first sections of this chapter introduced the reader to the definitions, terminology and concepts of flavor and its analysis. The classic techniques remain to this day the work horse in flavor analysis and research. Advances in technology and biology as well as the coming together of different disciplines have provided the opportunity to explore brand new techniques. This section provides an overview on a range of modern day *in vitro* methods.

9.5.1 Electronic Devices for Flavor Assessment

In the era of space exploration, advances were made to identify and assemble sensors which could react to gases and other volatile molecules and in turn generate a signal to signify the presence or absence of the species being monitored. A spin-off idea arose for sensors that could detect specific compounds in industrial applications such as mining and water treatment. The sensors were found not to be highly specific with regards to detection. However, the way in which volatile compounds interacted with the sensor material to produce a signal, did appear to differ between compounds. This led to the theories on how the human brain may use the odor receptors in nose and process signals to discriminate between odor profiles (Persuad and Dodd, 1982).

9.5.2 eNose

The idea for a device to mimic the nose was conceptualized as long ago as the late 1970s/early 1980s. However, unlike the relative simplicity in industrial applications where a single or few compounds are monitored, flavor analysis requires greater specificity to measure aroma profiles. To mimic the olfactory system is truly challenging (see earlier chapter on sensory receptors). To tackle this challenge, instead of using a single specialized sensor, systems are based on an array of sensors grouped together in a single device to broaden the detection range. The idea was to assemble an electronic nose device that could be broadly responsive to all food and beverage aromas and produce sufficient discrimination (Figure 9.7).

The eNose devices are based on sensor technology which in basic terms, are materials that change in physical properties such as electrical conductivity or vibrational range and can therefore be translated to provide a signal output. Sensors can range from the early metal oxide sensors to the more recently developed polymeric conductors. Metal oxide sensors are based on the principle of a redox reaction occurring between the metal catalyst and reacting volatile at a particular temperature. Advances in producing intricate and sensitive electronic components have been combined with quartz crystals that are surface coated with chemically active materials such as polymers. The principle is that as volatile molecules are absorbed to the surface, this will change the mass causing a shift in the resonant frequency of the crystal. A variation of the crystal sensor is one which applies the principle of frequency shifts when the presence of a volatile compound causes a change to the normal frequency, also known as the surface acoustic wave.

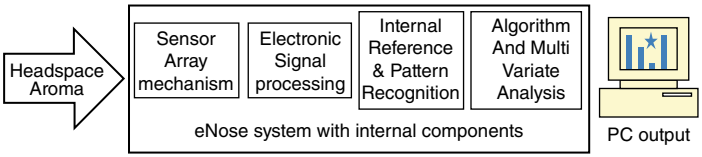


Figure 9.7 Schematic of the basic principle of the eNose.

All of these sensors cannot provide the required specificity to discriminate aroma compounds within a flavor mixture. Reported values of sensitivities are usually based on introducing a highly responsive single component, which is typical for any detector industry to showcase sensitivity. However when complex mixtures are presented all at once as in the case with flavor applications, the signal response becomes difficult to interpret.

eNose instruments with an array of sensors, need to be trained (statistical algorithm) and calibrated on the typical aroma profile that is acceptable, as well as the examples of non-acceptable, or out of specification aroma profiles. In effect the eNose is set-up to monitor for a difference or outlier based on a known reference comparison. This is acceptable for most non-food samples where the profile is relatively simple and devoid of any other influential vapors. Foods and beverages with complex aroma profiles will have many aroma compounds some of which can easily exert the same signal response due to the lack of specificity of the sensor. Significantly, the most common vapor that causes issues is water which is present in all wet foodstuff and especially so in beverages. In alcoholic beverage applications, carbonation and ethanol dominate the response signals, masking any impact of any possible aroma components. This can add confusion to signal patterns which often track changes in these major vapors rather than the aroma related species.

As technology and computer processing power has increased, it is now possible to analyze the multiple signals of responsive sensors using multi-dimensional or multi-variate data analysis and use pattern recognition in an attempt to differentiate or match aroma profiles. Although the concept is sound and has found application where a simple check is needed to determine a product is within or out of specification. There are limitations that one should be aware of as discussed above. The use of eNose instruments are not suitable for flavor research, or analysis, but may find limited quality related screening applications (Wardencki *et al*., 2013).

9.5.3 eTongue

The original intention of eTongue instruments was to characterize solutes in beverages such as ions, minerals and to measure pH as part of routine quality checks. This would improve the

efficiency of qualitative testing and reduce operator time by integrating a carousal system for automation. Various systems for an electronic tongue have been developed to screen solutions based on combining a range of established sensors into one instrument. However these are not particularly well suited for taste characterization in a flavor sense. A study of such a system used five different metal electrodes (Winquist *et al.*, 1997) to measure differences in conductivity to determine milk quality. The principle was based on different electric potential patterns for solutions with different mineral, or organic acid composition. This generated data that would be analyzed by multivariate statistics, such as principle component analysis. These systems are only appropriate for use as very basic quality control devices since they cannot be used to identify the solute components.

9.5.4 Further Developments in Electronic Flavor Devices

There is now some recognition by manufacturers of the specific issues with using eNose devices for food and beverages. Such that some are offering easy to use headspace sampling (e.g. SPME) combined with compact fast GC using two columns, with a traditional detector such as an FID as the "sensor". The data is plotted in terms of intensity and elution time on both columns. This is then cross analyzed using multivariate analysis to produce a reference fingerprint of acceptable and non-acceptable aroma profiles against which new samples are screened. It seems that the complexity of the food market has led eNose manufacturers back to traditional technology, once thought of as too tedious and specialist. Advances in capillary technology, fast GC, hardware compaction, automation and user friendly software is providing a quality control application for such instruments. However, as before, these tools are only providing chemical profiles and careful data interpretation is still required.

The recent innovation in nanotechnology is feeding into the design of next generation sensor devices based on advances in materials and manufacturing. The manufacture can be akin to electronic industry in which micro circuits have enabled faster and more productive chips. Silicon microchip technology

is gradually increasing with cost reduction of technology and advanced in chip engineering. The chips are being incorporated into headspace sampling devices and instruments to aid diagnoses of the gases sampled across medical and industrial fields. The idea for the electronic nose senor of the future, is to build nanostructures in arrays and embed them directly onto microchips. The nano-engineering would enable a vast array of sensors to be used to profile volatile compounds. The chip would then be trained by exposure to required smells or vapors to build a memory of the vapor profile. The idea to translate the concept from industrial and chemical vapor to food aroma seem straight forward, as was the case with the early eNose devices, however, the challenge will still be the ability to interpret a mixture of volatiles which vary subtly between natural foods. The ability to differentiate between smell perception remains to be fully tested and validated.

9.6 Model Mouth Systems

In order to control the inherent variability between people when studying flavor release during eating, mouth analogues, or model mouth systems have been developed to study eating effects under symmetric and mechanical control. This approach allows the study of parameters such as mouth cavity volume mastication, temperature, saliva flow and sample volume (van Ruth *et al.*, 1994; Roberts and Acree, 1995). This has been used to study the kinetics of flavor release. In order for the taste compounds to reach and be detected by the taste receptors, taste molecules must enter and pass though the saliva phase. A comprehensive review by Salles *et al.* (2011) provides an excellent overview of the complexities of in-mouth processes which can be used to develop model mouth systems for *in vitro* analysis. The use of an artificial or *in vitro* model mouth should consider the objective of the analysis and take into account that there are reported to be over 1000 protein peptides and 30 enzymes in saliva that could differ between a real and model system (Huq, 2007). Below are two different examples of how *in vitro* assessments can be used to study in mouth flavor processes that interact with saliva.

A chemical method for the taste attributes of astringency was investigated by Rodriguez *et al.* (2003) using the biopolymer chitosan. The impact of astringency development in an acidic medium was measured by turbidity development when chitosan interacted with saliva. A comparison with sensory tests showed a high correlation among the judges as astringency intensity increased when solution pH decreased. Chemical tests measured turbidity development, which also increased as pH decreased demonstrating that the relationship between oral astringency and turbidity could be related to salivary protein precipitation.

A study to understand the effect of sugar on aroma release using an *in vitro* approach to avoid human saliva sampling, found three different effects on volatile compounds partitioning from artificial saliva preparations. Of the volatiles studied, there was either no change, reduction in portioning or a variation in reduction dependent on the preparation of the artificial saliva matrix (Friel and Taylor, 2001). The artificial saliva consisted of mucin (salivary glycoprotein) the largest component of saliva and salts to which sugars were added – it was observed that the order of combining components results in competition effects with aroma molecules. This highlights an important consideration often overlooked with using model systems for *in vitro* analysis in that the artificial preparation of even simple systems can introduce differences that may not be representative under *in vitro* conditions.

9.7 Real Time Studies of Flavor Delivery

In addition to the question of what are the compounds in food that give it aroma, there is also the question of how does the aroma get from the food to where it is sensed in the nose during eating. How much, and how fast? It was considered that headspace analysis may generate volatile profiles, closer to those delivered *in vivo*, than those obtained by solvent extraction. Headspace analysis, however, typically involves sampling compounds from the equilibrium partitioning state and it too, may not truly reflect the *in vivo* state. Consequently, methods were developed to study the release of compounds *in vivo*.

Initially, breath volatile profiles were obtained, by trapping volatile compounds from the breath onto Tenax traps and analyzing them by GC-MS. But, the construction of the temporal profile was very time consuming, with individual chromatograms corresponding to one or two exhalations. Parallel to the interest in the real time delivery of aroma, there was a drive to develop real-time volatile measuring equipment for environmental monitoring, military and medical applications. This stimulus resulted in a range of approaches to the design and development of equipment.

The key issues for the systems were sensitivity, speed of data acquisition to capture the process with high temporal resolution, and the need to interface people with analytical instrumentation. The latter issue meant that the equipment would have to measure aromas in moisture laden air, which effectively directed the choice of instrumentation.

The three systems that emerged were Selected Ion Flow Tube – Mass Spectrometry (SIFT-MS; Spanel *et al.*, 1997), Proton Transfer Reaction – Mass Spectrometry (PTR-MS; Lindinger and Hansel, 1997) and Atmospheric Pressure Chemical Ionization – Mass Spectrometry (APCI-MS, Linforth *et al.*, 1996). Although the three systems were different from each other, they all used the basic principle of charge transfer from ionized molecules, typically protonated water H_3O^+, to ionize the aroma compounds (Eq. 1):

$$H_3O^+ + \text{Aroma} \rightarrow H_2O + \text{Aroma} - H^+ \qquad \text{Eq. 1}$$

There were differences in how each instrument achieved this. In APCI-MS, there was an electrical discharge, in air, at atmospheric pressure, that formed the protonated water. PTR-MS, has a separate chamber that ionizes water, which is then introduced into a low pressure region, where the ionization occurs. Whilst the SIFT, generated reaction ions in one region, then selected the reaction ions to use in the ionization reaction using a quadrupole mass spectrometer. These were introduced into the source, to ionize the sample gas. APCI-MS was simple and crude, it was restricted to just using water to ionize compounds, PTR-MS and SIFT-MS could however, utilize other ions to carry out the charge transfer reaction. Following ionization, the outcome was again similar, a charged molecule that remained

largely intact during the ionization process. Typically gaining a proton. As a result, a molecule entering the MS source with a mass of 116 would be detected at m/z 117, and could be monitored at that mass in real time.

Because the presence of air and water in the ionization region, was part of the design of all three systems, they could draw air in from the atmosphere and analyze its volatile content. Resolution of compounds, was typically by mass alone, which imposed limitations on the number of compounds that could be detected at any one time. The systems offered little information to identify compounds, since the ionization process was a simple low energy event.

Figure 9.8 shows a schematic diagram of the APCI-MS system. The air (breath in the case of *in vivo* studies) is drawn from the sampling point, down a deactivated fused silica tube, into the source of the mass spectrometer. The tube is kept warm to avoid volatile compounds, or, moisture condensing in the tube. Once in the source, the molecules entered the ionization region. This consists of a corona pin which has 4 kV applied to it, and a sample cone. The electrical discharge generated the protonated water, which ionized the compounds. These then passed through the sample cone, from the atmospheric pressure area of the source, into the vacuum region of the mass spectrometer analyzer.

Once developed, these systems could investigate the process of volatile delivery. There were many papers and models predicting how flavor was delivered *in vivo*. This was the opportunity to test them. In addition, there were claims that differences in sensory perception were directly attributable to differences in flavor delivery, caused by processes such as salting-out effects, where non-volatiles promote the volatility of aroma molecules. However, up until this point what happened in reality was little more than a black box.

One of the first findings, was that when foods were consumed a lot of the compounds that were expected to give a signal in the breath, were not detected, because their concentration was too low to measure. This was surprising, because the systems had been developed to detect aroma compounds at the low parts per billion gas phase concentration, and the vast majority of compounds have odor thresholds above this level (Devos *et al.*,

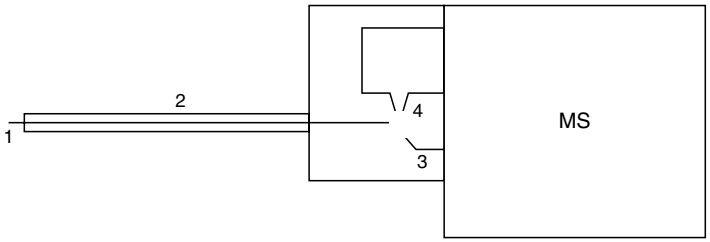


Figure 9.8 Schematic diagram of APCI-MS. 1 sampling point, 2 heated transferline, 3 corona pin, 4 sample cone.

1990). At the time, it was thought a compound had to be present, at or above its odor threshold in the breath to be detected. In addition, tables of OAV data, showed that many compounds should be present at levels 10, 100 or even 1000 fold higher than their odor threshold (Grosch, 1994).

In vivo delivery of aroma, was clearly different from what flavor scientists expected. However, in hindsight, a sensory experience produced by a large number of compounds, all arriving at the olfactory epithelium, at levels substantially above their odor threshold, may be considered more of a sensory overload than a perceptual stimulus. This finding leads to the concept of broad integration of stimuli, at potentially the receptor and the cognitive level, to generate the overall sensory construct, with all stimuli combining together, many at sub-threshold concentrations. This phenomenon was demonstrated for a combination of 26 taste compounds. These were all simultaneously tasted at sub-threshold concentrations and perceived as an overall taste (Stevens, 1997), thereby showing that broad sensory integration of different stimuli was possible. In addition, Dalton and co-workers (2000) showed that integration of a sub-threshold taste stimulus, with a sub-threshold aroma stimulus, could result in a perceivable stimulus. Consequently, the lack of detectable signals for aroma compounds during *in vivo* analyses was consistent with these other results. Many compounds deliver in concert at low levels and there is integration of the overall neural response.

Further *in vivo* studies focused on other issues. One problem for the flavor industry was how to make chewing gum with a longer lasting flavor. Breath analysis of people chewing gum revealed that their breath still contained high levels of mint aroma

molecules, long after the flavor had disappeared (Davidson *et al.*, 1999). The loss of flavor followed the taste (in this case sugar) release curve. Leading to the conclusion, that the taste–aroma interaction was important for overall perception. In this case, it could also be argued that the panellists may have adapted to the aroma over time. However, other examples showed that in-nose aroma could be delivered, and not perceived, over much shorter timescales.

These experiments studied the effect of adding thickeners to flavor systems. The addition of thickeners to flavors in water, was known to decrease the overall perceived flavor (Baines and Morris, 1987). This was attributed to the thickeners decreasing aroma delivery as a function of viscosity. In-nose measurement however, showed that there was no difference in aroma delivery to the nose as the thickeners were added to the flavor systems at higher and higher concentrations (Hollowood *et al.*, 2002). What appeared to be driving the change in perception, was either the texture of the thickener, or, its impact on tastant delivery to the tongue, disturbing the taste-aroma balance. In this instance, the lack of perception is from a stimulus delivered over a few seconds, eliminating the potential for adaptation. These studies highlight the difficulty in taking a set of analytical data and producing a perceptual construct. In terms of compound concentrations and OAV, the systems would have been the same, but, their perception was not.

To further confound the analysts attempts to correlate flavor perception with analytical composition, there are examples of the perception of aroma in its absence. One of these was demonstrated by Hort and Hollowood (2004). In these experiments, panellists rated aroma over time. The sample was a liquid which was continuously pumped into the mouth. Initially, it consisted of taste and aroma compounds, however, after a short period of time the aroma component was removed (and it was shown to have gone from the breath using *in vivo* breath measurements), many of the panellists did not notice, and still thought the aroma was present. An illusion, generated by the continuity of the taste stimulus. The same effect can happen with the consumption of boiled sweets, here the delivery of aroma to the nose happens infrequently over the eating time course, but, our experience is one of a flavor continuum.

We therefore have a situation in that the aroma of a product can be present and not be sensed, be absent and we think it is present, and where taste-aroma interactions are necessary features of product perception. The chemical composition of a food is important for its perception, but, many other factors will also impact on our final flavor evaluation. This is a fact long established by our response to orange juice that is colored to look like blackcurrant juice, or when we try and eat with a blindfold on.

It might be thought of as inefficient, for our brains to ignore so much sensory input and simply continue with a sensory illusion. An alternative view could be, that leaving a lot of our perception to run on unconscious thoughts, triggered by color, or other limited stimuli, rather than focusing on evaluating sensory input, may free up mental resources for other tasks. We do not function like computers, which cannot evaluate what to focus on and what to ignore, and we need to remember that tables of analytical data, can be more like the computer's view of the world. Flavor chemists have looked for hard rules of causal aroma perception relationships, and ultimately entered the domain where our conscious perception interfaces with our unconscious neural processing.

9.8 Future Direction of *In vitro* Flavor Studies

9.8.1 Taste Research

Taste and tastants have taken on greater significance recently, and this area of research will develop in the future. This is partly through the drive to understand perception of basic tastes, such as salt (NaCl) and sugar (sucrose), as we seek to decrease the levels of these compounds in our diet, and partly through the discovery of new taste modalities. In addition to the traditional main tastes, salt, sweet, sour and bitter, other new tastes have emerged and are emerging. Umami, was initially proposed as a taste in its own right in 1908, but, not fully accepted as such until 1985. It was only at the beginning of this century that a glutamate receptor was discovered (Chaudhari *et al.,* 2000) finally confirming *in vivo* recognition of this taste. Umami,

consists of compounds such as the amino acid monosodium glutamate, and the 5'-ribonucleotides, such as guanosine monophosphate (GMP) and inosine monophosphate (IMP). Note that the ribonucleotides have a synergetic and amplification effect thereby enhancing the perception of glutamate which is not fully understood at the time of writing. Key findings that helped to establish such molecules as tastants in their own right are the discovery of receptors that respond to MSG, IMP and GMP. Indeed, our capacity to find, clone, express and demonstrate the functionality of receptors, will be a key area of flavor research in the future.

A further set of tastants have been proposed under the term kokumi. These compounds do not have tastes of their own, like the main set of salty, sweet, sour and bitter. Instead, they are proposed as taste enhancers, working in synergy with a flavor system to enhance the richness, roundness and depth of flavors typically in savory products. The range of kokumi molecules includes peptides and some fatty acids. Their effective dose rate is typically low. Tri-peptides based on leucine and glutamic acid such as Leu-Glu-Glu, or, other arrangements of these three amino acids, are considered for use in savory foodstuffs at levels of 0.3 mM. Another example is the so called beefy meaty peptide, composed of the 8 amino acids Lys-Gly-Asp-Glu-Glu-Ser-Leu-Ala. This was originally isolated from beef soup and is thought to enhance its meaty nature.

Many of the new tastes are slow to be recognized and are greeted with considerable skepticism. However, it is logical that they should exist. They are likely to reflect our need to perceive molecules indicative of dietary requirements. If the parallel case of the cat is considered, the principle is clearer. As a strict carnivore, the cat has specific nutritional needs (which we now know through the studies of the petcare industry) and the cat has a strong link between its food intake and its dietary needs. Eating for a cat, can be more about the chemistry, and the perception of the chemistry of a food, than it is an issue of calories. People as omnivores are in a slightly different situation; we have more diverse diets, and consequently we may have lost some of our chemical sensing of foods and tend to focus more on the calorie component. This may also reflect the fact that humans have effectively been domesticated for far longer than the cat. However,

our capacity to perceive umami and kokumi may be vestiges of this long lost past, triggering receptors that once evaluated their content in the foodstuff.

9.8.2 Taste Cell Model Systems

As introduced in Chapter 2, "*In vivo* Foundations for *In vitro* Testing of Functional Foods" the understanding of molecular biology has increased over the last decade. Advances in identifying taste receptor cells is helping cell biologists to develop cell model lines with the aim of creating a predictive sensory system. The reader should note that the stimulating activity of compounds does not equate to flavor quality and it is this challenge that will demand the greatest understanding to achieve this breakthrough. Research using cell model lines has been adapted from cellular biology where it is helping understand biochemical signal and control into the flavor area. The current focus has been to develop high-throughput cell model lines, by which compounds are screened on the basis of eliciting a response or signal through compound–receptor interactions. The mechanism of transducing a signal from the interaction of a taste compound and its receptor cell varies depending on the stimulus as described in Chapter 2. Typically the signal generated is only an indication that there is a molecular interaction between the host (receptor cell) and guest (flavor compound). The uses for screening of compounds have been useful for identifying possible bitter blockers, i.e. compounds that bind to bitter taste receptors such as hTAS2R31 (Li *et al.* 2014). However these compounds also require sensory assessment in order to ensure they do not impart bitterness. The quality of flavor i.e. "how does it taste?" is not captured as this aspect cannot be derived from the interactions or signal response. It remains unclear to how the nerve fibers which transmit signals from the taste bud to the brain convey the quality of the taste. A few discussions suggest possible theories of how taste quality is generated between taste-receptor interaction; the nerve route of signals that travel to the brain, the interaction of taste bud on the tongue that combine signals together and timing of taste compound interaction with the receptor (Chaudhari and Roper, 2010).

In the previous examples of taste compounds that impart savory and umami character, the relationship between compound structure and taste receptors to signal transduction and neural processing highlight the level of understanding required to develop, use and interpret cell model systems. Given the combination of evolutionary development and neural processing of signals, the ability of *in vitro* taste cell receptors to identify and qualify ingredients with the same effect and impact as glutamate and ribonucleotides challenging.

In summary, *in vitro* cell model lines are screening systems that are still developing as research has accelerated from the early 2000s. A combination of techniques, discovery of further taste receptors and interest in new or alternative taste compounds both academically and commercially are driving this research. Further advancements are likely to occur as the early model systems are tested and refined year on year.

9.8.3 Odor Receptors

In addition to the cloning of taste receptors, there have been significant advances in the cloning and expression of odor receptors. Early work was pioneered by Zhang and Firestein (2008) and there are now clones of 391 different aroma receptors available. The clones are fundamental tools for the development of our understanding of aroma receptor function, an area which is beyond the scope of this chapter. The receptors may however, not just simply broaden our knowledge and understanding, but they may prove to be a useful tool in their own right.

With an array of 391 clones of odor receptors, it is possible to determine which receptors bind to different odorants. The receptor-ligand interaction will demonstrate the same sensitivity and selectivity as that of the olfactory epithelium itself. Consequently, it will exhibit responses to both the optical configuration, as well as the chemical features of compounds.

As technology advances it may be possible to utilize *in vitro* cultures of receptor cells as detection systems for volatile compounds. Advancements in the system, to increase the range and sensitivity of aroma compound detection, could result in a full, or partial, artificial olfactory epithelium. There is the potential

for a culture of cells to replace dogs and other sensors at airports, or, mass spectrometers on gas chromatography equipment in the laboratory.

In addition to the receptors provided by nature, we have the capacity to modify the genetic sequence of receptors *in vitro*. This would allow us to engineer new and more specific receptors for niche applications. However, although this would offer the potential to increase the selectivity and sensitivity of aroma compound analysis. The construct of what the consumer perceives will be missing unless data analysis can be programmed to mimic the neural sensory workings of the human mind.

9.8.4 Sensomics Approach

A relatively recent and combinational approach for flavor compound assessment uses *in vitro* chemical and sensory methods combined to bridge the gap between analytical data and sensory determination. An example is from the research group of Thomas Hoffman at the Technical Institute of Munich who utilizes a combination of specialized techniques such as liquid chromatography, fractionation, sensory, high resolution mass spectrometry, NMR and stable isotope markers for identification. Organic synthesis is also used to create compounds for flavor recombination and spiking into real foods systems. Even with the latest techniques and highly refined methodology, the final assessment is conducted using human assessors. This disciplined and methodical approach of using multiple refined techniques with sensory guidance has resulted in advancing key taste substances that would not be captured by isolated techniques. The use of taste receptors especially for saltiness, sweetness and bitterness are being combined with taste biology concepts to develop high-throughput bioassays to screen potential taste compounds. Screening tools such as taste receptor assays are still under development with more advancements made as each year passes (Miyano, 2014). The final taste characteristics of new taste compounds still requires validation by sensory assessment as the *in vitro* assays are only indicative of compound-receptor association and certainly not the perception of taste quality.

9.8.5 Interaction Effects and Multi-modal Perception

It is apparent that the term "flavor" is more than just the interaction between aroma (volatile) and taste (non-volatile) components. Additional factors, or stimuli also contribute to the overall flavor experience such as tactile (mouth-feel), irritant (pain or heat and cooling), auditory ("crunchiness") and visual (pre-conceptions from appearance and color) (Matheis, 1998). These stimuli are called modalities in psychophysics and impact on the neural interpretation of multi-modal perception (Auvray and Spence, 2008). The ability to measure cross-modal interactions is not easy in analytical terms, due to the necessity of the brain to "process" signals from sensory receptors into perception. It is clear that the study of flavor perception is the relationship between (i) stimuli – the physical flavor compounds; (ii) human senses – receptor activation; and (iii) the brain – neural processing (Coren *et al*., 1999). Studies have shown links between taste and aroma compounds using analytical and sensory studies to demonstrate the interaction effects (Hort and Hollowood, 2004).

9.8.6 Brain Imaging by fMRI

In addition to fundamental academic understanding, increased commercial interests in the understanding of more exotic and subtle flavors in food and beverage applications has opened up the field to connect with research experts outside traditional area of food chemistry. Although further work is required to link the analytical data with human perception the foundations have been laid using the analytical tools of neurosciences to study food stimuli (Marciani *et al*., 2006, Small and Prescott, 2005).

The main objective of functional magnetic resonance imaging (fMRI) scans of the brain has been to demonstrate the association of the stimulus i.e. aroma or taste to cortical areas of recognition (Eldeghaidy *et al*., 2012). The aim is to understand positive, or negative associations such as satiety or displeasure and how different stimuli may be used to replicate (positive) or avoid

(negative) associations in areas such as salt, sugar or fat reduced products by use of congruent aromas for example. In addition, functional MRI is starting to be used to investigate the effect of aroma and taste interactions. The challenges are the noise created by the subject and resolution required to pinpoint the subtleties of flavor differences. Development of high resolution fMRI could be used in the future to explore multi modal taste –aroma, texture, trigeminal sense as a total sensory integration. Increased instrument resolution, would enhance our mapping potential, to determine key neural associations. This technique would still require human subjects and need specificity and selectivity to interpret the findings.

9.9 Summary

The concept of *in vitro* flavor analysis whereby a scientific instrument, or cell model system, can replace the human nose and tongue to return meaningful sensory data is a long way from being achievable. *In vitro* analysis to determine flavor is currently suited to quality control and potentially a loose prediction of the quality of the flavor in certain cases. The examples presented in this chapter demonstrate the challenges and reasons for why this is the case. The *in vitro* techniques should be considered and treated as specialized tools to aid flavor research which address specific objectives in flavor experimentation. The diverse physicochemistry of flavor compounds causes limitations in conducting total flavor analysis at each key step: extraction, isolation, separation and detection. Furthermore, the sensory impact relationship between chemical stimuli and biological receptors cannot be adequately predicted or determined. Interaction effects can be of any number of types, e.g. synergistic, masking or even a change in overall flavor impression.

The real time dynamic nature of flavor delivery, from the food matrix or beverage to the olfactory systems plays a significant role in establishing flavor quality. Differences in the food matrix will impact the quality of flavor even if the gross composition of the flavor profile is the same. In order to capture and study *in*

vivo flavor delivery new techniques such as APCI-MS or PTR-MS provide an approach to study the dynamic nature of flavor. However, it is recognized that these are only individual tools that still require an array of complementary analytical tools as well as sensory or consumer studies to interpret the data.

This multi-disciplinary approach is forming the beginning an exciting period for flavor research to help bridge the gap between instrumental analysis and perception. In addition, some research is also taking place with non-traditional researchers such as culinary chefs using real complex foods rather than traditional model systems in order to understand holistic multisensory interaction effects. Interdisciplinary articles are emerging on flavor, its generation and perception, and its influence on behavior and nutrition as well as articles on the psychophysical, psychological and chemical aspects of flavor including those which take brain imaging approaches. The reader is referred to conference proceedings of the Weurman symposia and those of Wartburg dedicated to flavor research as well as international peer reviewed journals such as *Chemical Senses*.

The challenges of linking the stimuli of flavor to human senses and perception can now be explored to a greater degree with the advent of relatively recent technological advances and multi-disciplinary approaches in the fields of physical chemistry, psycho-perception including emotions and neurology. Multi-modal analysis to investigate interactions that have a synergistic, or antagonistic effect, are now forming the basis for cross-modal understanding. New and more accessible techniques from the medical field such as fMRI may start to play a greater role in helping to elucidate the interactions of aroma compounds with each other and with other stimuli such as taste, texture, trigeminal and tactile (heat and carbonation) and vision in relation to brain patterns during flavor perception.

A complete assessment of flavor by instrumental or receptor cell model analysis is a misnomer due to the fact that humans are required as part of sensory exercise to validate a given flavor of product. Although advances in both instruments and understanding have come a long way since the mid to late 20th century, it is not currently practical to understand flavor perception by means of *in vitro* flavor analysis in isolation from sensory or consumer perception studies.

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Index

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d

e

f

g

l

m

n