Ourania Gouseti · Gail M. Bornhorst
Serafim Bakalis · Alan Mackie *Editors*

Interdisciplinary Approaches to Food Digestion

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Foreword

Better human nutrition is now openly declared as one of the United Nation's Development Goals. Specifically, the objectives are "To promote healthy food systems and increase the focus on nutrition, with multiple implications for diet quality, vulnerable groups, and informed choice". We now see the rise of the obesity epidemic in all nations, even to the point where overweight parents can have stunted children. At the same time, and in different locations, macronutrient levels remain critically low in some diets.

Historically, the digestive tract has been regarded as a "black box", where the inputs are dietary components providing calories and essential nutrients whose levels can be calculated from chemical analysis of individual food inputs. The outputs are measured by various biological criteria such as weight gain, metabolites and markers in blood, urine and faeces. To achieve the new objectives, the mechanisms within the "black box" of individual human subjects will need to be identified and modelled. This is not easy, since unlike most man-made processing equipment, the human alimentary canal cannot be disassembled into its separate unit operations, and each one is likely to be different, because of individual genotype and phenotype and any and all dietary history.

This book presents a review of what we know and how we can plan to fill in the gaps by future research. One thing is for sure, the challenge is enormous and cannot be solved by the application of any one discipline.

While the development of high-throughput "metabolomics" is providing information at a massive scale and rate, the engineering approach to the digestive tract as a series of bioreactors and the physical tools to measure (non-invasively) the processes in real time add focus to the mechanism themselves. Furthermore, the material science of food is clarifying the origins of bioavailability, relating to its microstructure, and also identifying the taste and texture stimuli which cause us to prefer types of foods and choose a diet which is not always in the best interests of our long-term health. Evolution has driven the development of a digestive system that is highly effective at maximising nutrient intake and absorption from a limited supply of poor-quality food. In an environment of highly available food and nutrients, this is causing health problems. Social sciences also remind us that food is part

of everyone's culture. What we choose to eat is not simply determined by our individual nutritional requirements. Our choices of diet are strongly linked to our personal culture and environment.

The challenge is enormous, but as this volume shows, so are the opportunities to obtain evidence-based solutions. This is a fast-moving area of scientific enquiry. This book represents the state of the art but will probably need a second edition within 5 years.

Birmingham, UK Peter Lillford

Preface

Some of the questions this book aims to address are:

How can we quantify food digestion and what tools are available for measuring it?
Why are digestive processes so complex to understand and model?
What is the relevance in designing foods with specific behaviour during digestion?

Over the last two decades, there has been an increasing demand for foods that deliver specific nutritional benefits. In addition, the dramatic increase of food-related non-communicable diseases (e.g. obesity) requires development of novel food products that control satiety, glycaemic response, etc. Overall, digestion studies have gained increasing attention in recent years, especially as the link between diet and health/wellbeing becomes more evident. However, the link between digestion and health is complex, process involving a wide range of disciplines, including medicine, nutrition, chemistry, materials science and engineering. While a significant body of work exists within each discipline, there is a lack of a multidisciplinary approach to the topic, capable of providing a more holistic view of the process. To this end, a platform that brings together expertise to study how foods are disintegrated during digestion is the INFOGEST network (initiated in 2011 by COST FA 1005).

This book describes a *multidisciplinary* approach to food digestion studies. We first put food digestion in context presenting relevant phenomena, challenges and limitations in different approaches. We then focus on *quantification* studies aiming to describe food digestion and tools that are available for this quantification. *A case study* further puts theoretical knowledge in context and demonstrates ways digestion studies can be used to develop food products. Overall, we aim to produce a helpful companion and a reference book to a diverse audience throughout their journey in understanding digestion.

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Part I
Introduction to Digestion Studies

A Short History of Digestion Studies

Ourania Gouseti

Probably among the earliest signs of human's interest in understanding digestive processes is evidenced in the descriptive writings of ancient languages developed about 3000 years ago. For example, the stomach in ancient Chinese is depicted as a bag-shaped character, while the intestines in Sumerian and Egyptian show as snake-like shapes. We now know that the human stomach is schematically well represented by a j-shaped bag, while the intestines are essentially long tubes well folded to minimise the space they occupy.

Some centuries later, about 500 BC, medics and practitioners in ancient Greece considered digestion as a vital part of good health, governed by the balance between the four body humours (in this case humor = fluid, from the Greek word "χυμός" for juice), that is, blood, phlegm, yellow bile, and black bile. Any diversions from healthy digestion were treated such as to restore balance between the fluids (this practice is known as humorism). The actual process of digestion from then up until the nineteenth century, was thought of as the result of different elements: cooking (e.g. heat); mechanical action (e.g. grinding); chemistry (e.g. acid action); fermentation (used in a broad definition); putrefaction (decay); or a combination of those. Each theory had supporters and opponents throughout history and times of thriving and decline.

In Ancient Greece, the pre-Socratics (sixth century BC) considered digestion as a result of Heat (a physical quality) and/or Trituration (a mechanical feature) (Bloch, 1987). The word "*pepsis*" was introduced by Hippocrates (ca. 460 BC–ca. 370 BC, Fig. 1a) to describe the process of digestion, which he considered as an internal Cooking mechanism of food that produces body Heat (Bloch, 1987). Aristotle (ca. 380 BC–ca. 320 BC, Fig. 1b) expanded the Cooking theory and described digestion as a Heat-driven process that transforms Food into Blood in four stages, with the

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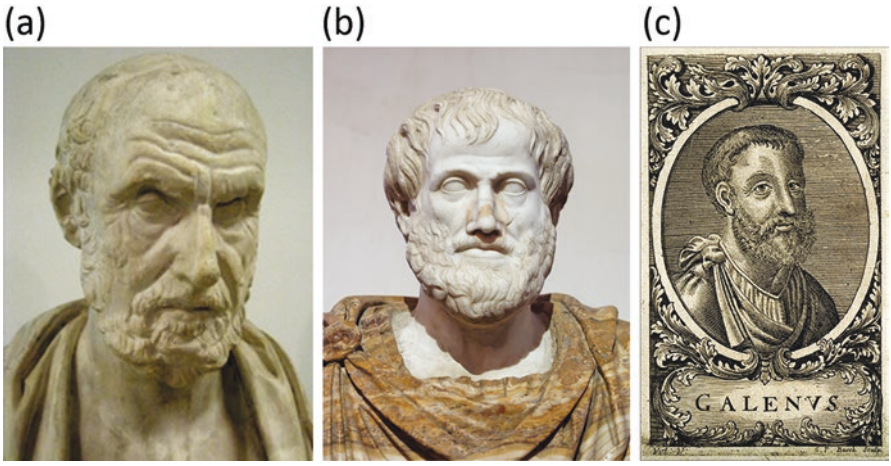

Fig. 1 Greek philosophers and scientists (**a**) Hippocrates (photo ©shako/own work); (**b**) Aristotle; (**c**) Claudius Galen

stomach, liver, and heart playing key roles (Boylan, 1982). Few decades later, Herofilos from Chalkedon (ca. 355 BC–ca. 280 BC) introduced the term "*duodenum*" to describe the short part (with length equal to twelve fingers, duo = two, deka = ten in Greek) of the small intestine immediately after the stomach (Sródka, 2003). At about the same time, Erasistratus from Keos (ca. 310 BC–ca. 250 BC) complemented and contradicted the dominant Heat theory by emphasising the importance of mechanical action in digestion (Edwards et al., 1970). It was few centuries later that Claudius Galen (ca. 130–ca. 210, Fig. 1c), a prominent Greek philosopher and physician of ancient times, combined existing theories and described digestion as the process of mincing in the stomach, decomposition in the bowel, and conversion into blood in the liver that feeds the blood circulatory system (Sródka, 2003). Interestingly, Galen associated longer intestines with higher beings.

The theories of Aristotle and Galen prevailed throughout the Middle Ages (fifth to fifteenth century) with little further scientific advancement (Bloch, 1987). Probably the most significant work of this period in the area of digestion is that of Avicenna (980–1037), a Persian medical philosopher with interests in understanding gastric illnesses, who among other observations recognised that "mental excitement or emotion; vigorous exercise; these hinder digestion".

Renaissance (fourteenth to seventeenth century) signalled scientific rising and the formation of Academies, including the UK's Royal Society (founded in 1660). It is the time when alchemists are looking for the elixir of immortality and the transformation of metal to gold. In the meantime, there is significant progress in knowledge of human anatomy, with Leonardo da Vinci (1452–1519) being granted permission to conduct human dissections, which were illegal at the time for non-physicians (he dissected about 30 bodies in the years between 1489 and 1513). The outcome is his well-known series of anatomical drawings, including a detailed realistic study of the digestive system (Jones, 2012).

Fig. 2 (**a**) Paracelsus; (**b**) Jan Baptiste van Helmont

In Renaissance, the science of chemistry is thriving, giving rise to iatrochemistry, an amalgam of chemistry and medicine. Life itself seemed to depend on the balance between acids and alkalis (Manz, 2001). Physiology is now linked to chemistry, and digestion is viewed from a chemical/biochemical perspective. A distinguished figure of the period, the Swiss Paracelsus (1493–1541, Fig. 2a), paralleled (healthy) digestive processes to a well-equipped, efficiently functioning chemical laboratory (Bloch, 1987; Manz, 2001). About a century later, Jan Baptiste van Helmont (1580–1644, Fig. 2b) proved the acidity of gastric fluids, yet he realised that acid on its own is insufficient to digest food and he thus introduced the concept of "ferments" that are responsible for specific digestive actions (Bloch, 1987; Gillette, 1967). Van Helmont hypothesised that full digestion occurs in six fermenting steps. The first step occurs in the stomach and causes transformation of food into acid chyme. In the intestines, acid chyme is neutralised into alkaline chyme (second step), while undigested material is fermented into faeces (third step). Chyme exiting the intestines then travels to the liver to be converted into blood (fourth fermentation), and is further transferred to the heart, where it is transformed into "vital spirit" (fifth fermentation). This spirit is distributed to the body's organs for a final, individual (sixth) fermentation by the organs according to the body's needs (Bloch, 1987). A follower of the fermentation theory, Franciscus de la Boe Sylvius (1614–1672), recognised the role of acids and alkalis in digestion and the importance of intestinal secretions (bile and pancreatic) in transforming acid to alkaline chyme (Bloch, 1987; Manz, 2001).

Some interesting experiments are reported in this period. For example, Regnier de Graaf (1614–1673, Fig. 3a), a student of Sylvius, was the first to taste (a dog's) pancreatic juices, which he found acid to taste. At the age of 23, de Graaf surgically inserted one end of a quill into a living dog and managed to keep the dog alive for long enough (7–8 h) to collect (and taste) the juices from the other end of the quill (Bloch, 1987; Ragland, 2008). This was the pioneering work of surgically altering the subject of a study to fit experimental purposes, a technique that was revived later by Claude Bernard (1813–1878) and was further heavily used in Ivan Pavlov's

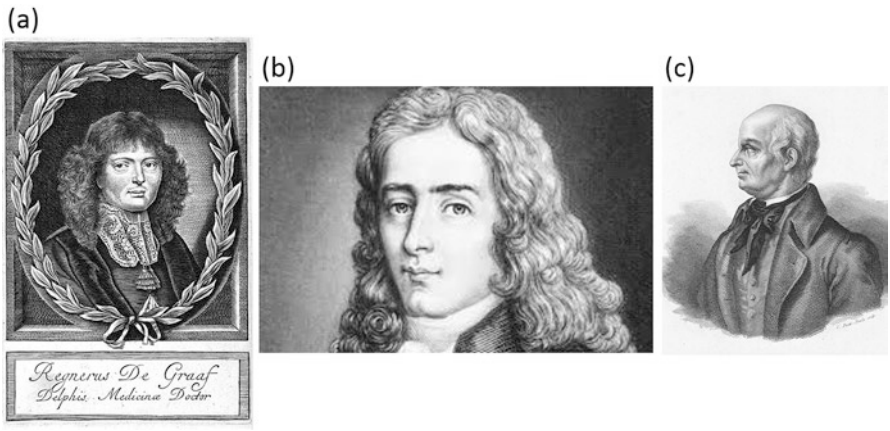

Fig. 3 (**a**) Regnier de Graaf (**b**) Reaumur (**c**) Spallanzani

(1849–1936) laboratory to produce his so-called "dog technologies" for his dog experiments (Bloch, 1987; Creager, 2002; Todes, 1997).

Few years after de Graaf sampled intestinal juices, René Antoine Reaumur (1683–1757, Fig. 3b) used a different technique to collect and taste gastric juices of a tame buzzard (or a kite according to others), which he found salty and acid to taste (Bloch, 1987). The selected birds have the convenient habit of vomiting any undigested material from the stomach. Reaumur fed the bird with hollow metallic tubes containing sponges or foods. As the bird vomited the tube, Reaumur retrieved its contents. Using sponges, he collected the gastric juices, while using foods he studied gastric digestion. He further attempted to conduct in vitro experiments by immersing foods in the collected gastric juices, only to observe inactivity outside the body. This was the first time that a similar observation was noticed, and it was later attributed to the absence of temperature control (Gillette, 1967). With his digestion experiments, Reaumur attempted to test the various popular theories of his time. For example, he hypothesised that the rate of digestion in the tubes would depend on the relative importance between the effects of the gastric juices, reaching the food through the tube's holes, and those of trituration, which was obstructed due to the presence of the tube. He noticed that trituration was more important in some foods, such as grains, than in others, such as meats and bones, and that in some cases, the dissolving effect of the gastric fluid predominated gastric digestion. His observations led him to question the theories of fermentation and putrefaction (Bloch, 1987; Gillette, 1967; Peescott, 1930). However, digestion still remained a mystery, as very wittingly William Hunter (1718–1783) remarked: "Some physiologists will have it that the stomach is a mill, others that it is a fermenting vat, others again that it is a stew pan, but in my view of the matter, it is neither a mill, a fermenting vat, nor a stew pan, but a stomach, gentlemen, a stomach" (Bloch, 1987).

It was Lazzaro Spallanzani (1729–1799, Fig. 3c) who first conducted extensive investigations of human digestion on a living body: himself. He swallowed food

contained in metal tubes, wooden spheres, or linen bags and observed the contents after digestion (Peescott, 1930). Using a similar method to Reaumur, he also attempted to collect his own gastric juices, but he soon realised that swallowing sponges in tubes and subsequently trying to vomit them is unpleasant and after few trials he declared his repulsion to this process greater than his curiosity, so he abandoned these efforts (Peescott, 1930). Experimenting with different animals, Spallanzani showed that trituration results from peristalsis of gastric muscles. He proposed the importance of mastication on human digestion and observed that gastric trituration could be less important in humans than in other animals, as masticated food was fully digested in the stomach even if contained in perforated wooden spheres (Peescott, 1930). Unlike Reaumur, Spallanzani anticipated the importance of body temperature on digestion and performed in vitro experiments with temperature control, in which food was successfully digested (Peescott, 1930). He noticed that digestive secretions differ in different species, though animals would adapt to new diets if hungry. He further noticed that digestion rate was proportional to the gastric juice surrounding the food and attributed faster in vivo digestion, compared to in vitro, to juice renewal in the body (Peescott, 1930). He classified gastric juice as "antiseptic" due to its ability to destroy putridity of slightly putrid meat. However, he failed to detect the acidic nature of gastric juice, despite van Helmont's observations a century and a half ago, and was unable to explain its effect on the foods. (Peescott, 1930). When detected, he attributed the acidic nature of the gastric content to the food rather than the gastric juice, as he could not detect acidity in fasted stomachs (Peescott, 1930). Following van Helmont's observations, the nature of gastric acidity was actually a topic for debate for almost two centuries, after which William Prout (1785–1850) established muriatic (hydrochloric) acid as the (only) cause of gastric acidity (Bloch, 1987; Gillette, 1967; Prout, 1824; Rosenfeld, 1997).

A landmark of digestion studies was William Beaumont's (1785–1853, Fig. 4a) "Observations on the Gastric Juice, and the Physiology of Digestion", published in 1833 (Beaumont, 1833). This work summarises the observations of 11 years of

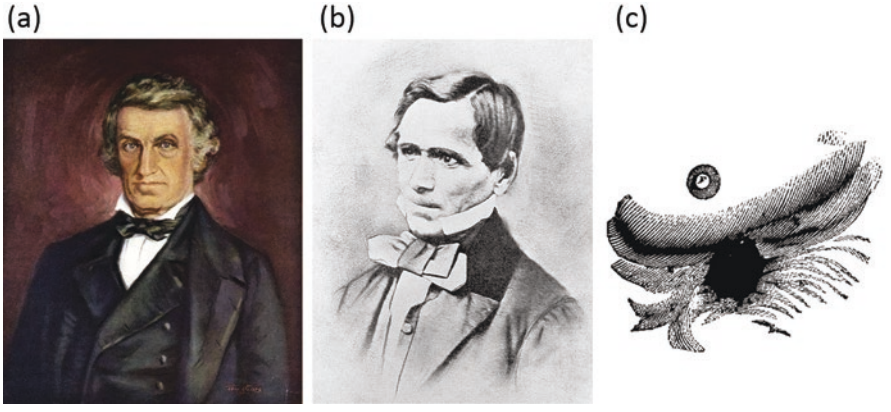

Fig. 4 (**a**) William Beaumont; (**b**) Alexis St Martin; (**c**) fistula of the stomach of Alexis St Martin

experiments that William conducted with (or rather on) his patient Alexis St Martin (1802–1880, Fig. 4b). William was the second of nine children. He was born in Lebanon Connecticut, and left for Champlain in 1807 to study medicine by reading and apprenticeship, as it was common at the time. He was appointed a surgeon and physician in 1812, and in 1822 he was a US Army surgeon on Mackinac Island (Myers & Smith, 1997). Alexis was a Canadian voyageur who on 6th June 1822 was unfortunate enough to get accidentally shot while at a fur trading post on Mackinac Island. He was treated by William and fortunately survived the shot; however, he was left with a permanent fistula (Fig. 4c), allowing direct access into his stomach (see Fig. 4). Initially, food from the stomach was leaking through the aperture; however, with time the body wrinkled in the area and food was kept inside. William, after realising the permanence of the fistula and his inability to cure it, seized the opportunity to investigate gastric digestion in-situ by tying foods onto a string, inserting them into Alexis' stomach, and retrieving the digested material at desired time intervals by pulling the string. The process was described as relatively gentle for the patient, who also apparently signed a contract with his physician "submitting" himself to science. For the record, Alexis outlived William and died at the age of 78 after having 4 children. William conducted 283 experiments in total and his observations have significantly contributed to our understanding of gastric digestion. Beaumont observed (among others) that mastication is not essential in digestion and finely chopped food directly inserted in the stomach is digested the same well (Myers & Smith, 1997). According to Sir William Osler (1849–1919) the important contributions of Beaumont were: (1) a more accurate and complete description of gastric juice; (2) confirmation of the previous observation that hydrochloric acid was the important acid of gastric juice; (3) recognition that gastric juice and mucus were separate secretions; (4) establishment of the influence of mental disturbance on secretion of gastric juice and digestion; (5) a more accurate and fuller comparison of the action of gastric juice inside and outside the stomach; (6) refutation of many erroneous opinions; (7) the first comprehensive study of motions of the stomach; and (8) a table of the digestibility of different articles of diet (Roberts, 1990).

Another significant contribution in our understanding of digestion, which resulted in a Nobel Prize in 1904, was that of Ivan Pavlov (1849–1036), who paralleled the GI tract with a chemical factory (Creager, 2002). Pavlov was the eldest of seven siblings. He showed interest in physiology very early in life and pursued studies of Medical Surgery. In the 1890s he became director of Russia's largest and best equipped physiological laboratory, where he (and his team) conducted experiments on dogs, often surgically altered to suit experimental needs (Creager, 2002; Todes, 1997). Having been called Russia's first factory physician, Pavlov managed centrally his laboratory, in which over 100 people worked between 1891 and 1904 mostly in temporal positions (Creager, 2002; Todes, 1997). In his Nobel Prize lecture, he claimed that the GI tract "consists of a number of chemical laboratories equipped with various mechanical devices". He acknowledged the importance of

mechanical, chemical, and enzymatic action on digestion. He recognised that different foods produce different responses during all stages of digestion, including oral, gastric, and intestinal, thus establishing the close link between ingested food and digestive processes and secretions. He further attributed control of digestive processes to the nervous system, which was later elaborated by his co-worker Boris Petrovich Babkin (1877–1950) (Beck, 2006).

Parallel to progress in understanding digestion, the eighteenth century also signalled the beginning of nutritional studies. In the 1760s, a Mr Grosse suggested the first classification of foods into five categories from substances indigestible or hard to digest (bone, fats, nuts, cherry stones, etc.) to substances less indigestible (pork, egg yolk, raw vegetables, etc.), easily digestible (e.g. meat of young animals, milk, fish, potatoes, etc.), foods facilitating digestion (salt, spices, wine, cheese, etc.), and foods retarding digestion (e.g. hot water, acids, oils, and employment after a meal) (Gillette, 1967). He acquired knowledge of gastric digestion by experimenting on himself: he mastered the art of vomiting on desire and he examined the contents of his ejecta after vomiting at predetermined time points of gastric digestion (Gillette, 1967). Work on the importance of the different nutritional components (sugars, carbohydrates, vitamins, amino acids, calorific value, etc.) peaked in the nineteenth century. In the early 1800s, François Magendie (1783–1855) established the importance of dietary nitrogen in maintaining life, probably using techniques that would be forbidden with current legislation and ethics: fed with nitrogen-free diets, he found that healthy dogs died in about a month (Shils & Shike, 2006). Few years later, in 1829, the word "protein" was introduced to describe complex food compounds containing about 16% nitrogen (Manz, 2001; Shils & Shike, 2006). The first nutritional recommendations came at about the same time, suggesting that an adult man between 70 and 75 kg should receive 3000 kcal (1 kcal equals the energy required to increase 1 kg of water by 1 °C), 118 g protein, 56 g fat, and 500 g carbohydrates per day (Karl von Voit, 1831–1908; Max Rubner, 1854–1932) (Manz, 2001; Shils & Shike, 2006). Today this has changed to 2000 ckal, with 50 g protein, 70 g fat, and 260 g carbohydrates; however, with the prevalence of malnutrition, obesity, and other diet-related diseases the numbers may further be revisited. The notion of what is now emerging as "personalised nutrition" suggests that supplementary to existing generalised nutritional guidelines, dietary requirements should be assessed at an individual level.

In recent years, studying the digestive system has received a lot of attention. Yet the digestive machine is still not fully understood and it remains an active (and attractive) area of research. Since the increase in in vitro studies in the 1990s, research in the area has escalated. It has been acknowledged that digestive processes govern both food and drug passage through the GI tract, which has further boosted interest in digestion studies. Furthermore, the multidisciplinary nature of digestion has prompted individuals from different backgrounds to focus efforts on this fascinating area of research: understanding complex digestive processes.

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The Digestive Tract: A Complex System

Alan Mackie

1 Introduction

The digestive tract in humans and other mammals has evolved to maximise the nutrients and bioactive compounds extracted from the food we eat while at the same time protecting us from pathogens and toxins that may be contained within it. As a result, the gastrointestinal (GI) tract is highly complex with multiple layers of control involving four distinct compartments, namely the mouth, stomach, small intestine, and large intestine. The first of these compartments is the mouth or oral cavity where food is first admitted into the body. This is where the most significant sensory appraisal of the food takes place as rejection of the food is still possible. It is also where the first preliminary steps in digestion occur as solid food is chewed to reduce particle size and saliva containing amylase is added. The saliva also provides lubrication to allow the food to be swallowed and passed to the second compartment, the stomach. The stomach acts as a reservoir for the food until it can be passed into the intestine for further digestion. This is not to say that digestion does not occur in the stomach but certainly it is limited. During the gastric phase of digestion, hydrochloric acid is added along with protease (pepsin) and gastric lipase.

From the stomach food passes through the pyloric valve into the small intestine, where the pH is increased towards neutral. Pancreatic proteases, lipases, and amylase are added along with the endogenous surfactant bile and the whole system is well mixed. Chyme is passed along the whole length of the small intestine where most of the nutrients are absorbed. The small intestine comprises the duodenum, jejunum, and ileum and chyme passes from one to the other until it finally passes through the ileocaecal valve into the large intestine. In the large intestine, some of the remaining undigested food (dietary fibre) is fermented by bacteria into absorbable

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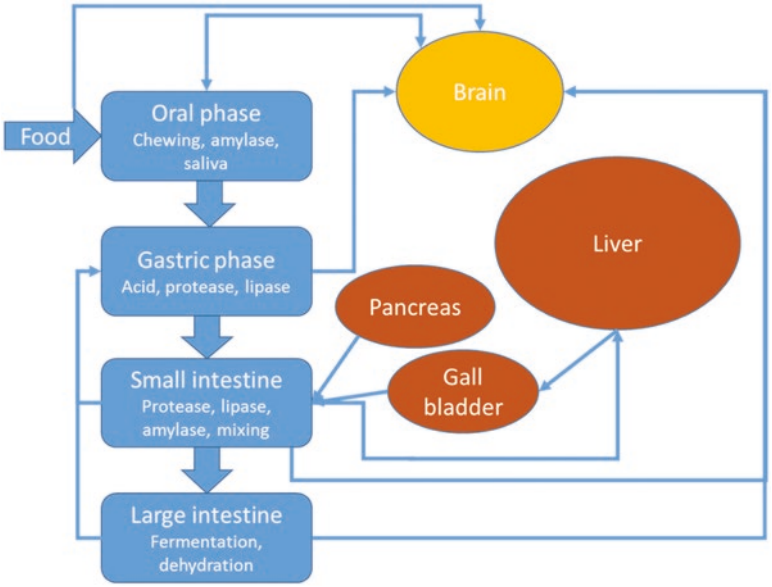

Fig. 1 A schematic diagram of the processes described in this chapter

compounds such as short chain fatty acids and most of the remaining water is removed before being excreted from the body. The basics of this complex system are illustrated in Fig. 1. In this chapter, the main processes controlling digestion and absorption in these different compartments will be described as well as how they are interlinked with one another and other organs in the body.

2 Oral Phase

Digestion is considered to start in the oral phase where food is chewed and mixed with saliva, allowing for sensory exploration of the oral contents in terms of taste, texture, and smell (Chen, 2009). The mouth represents not just the start of the digestive process by which the body gains nutrients but also the place where we gain pleasure from food. Because it is the pleasure aspect of food that drives the choice of which foods to consume, there has been significant research into factors such as texture perception (van Aken, 2010) and aroma release (Trelea et al., 2008). However, in this section only the features of oral processing that relate to digestion will be discussed although one of the confounding factors is the large variability between individuals and food types. For liquid food, the oral phase plays a very limited role in digestion but for solid food it can be critical.

After the initial bite, solid food is repeatedly chewed and manipulated between the tongue and palate until the particle size is reduced and the food has been hydrated

sufficiently to form a bolus. During this time a number of different stages can be identified depending on the food involved (Van Vliet, Van Aken, De Jongh, & Hamer, 2009):

- manipulation of the food before it is brought into the mouth
- ingestion/biting by the front scissors
- chewing of hard foods by the molars
- deforming of softer semi-solid foods between the tongue and palate
- wetting by saliva
- enzymatic breakdown
- manipulation of the food by the tongue to form a bolus at the back of the oral cavity
- swallowing

In order to complete all these stages particles should be smaller than ~2 mm although soft particles that are not liable to injure the upper digestive mucosae can be larger. This suggests the existence of a threshold for swallowing modulated in particular by food bolus consistency (Jalabert-Malbos, Mishellany-Dutour, Woda, & Peyron, 2007). In addition to lubrication, saliva also introduces amylase into the bolus and performs many other functions in the mouth. Salivary flow rate in healthy adults is ~0.3 mL/min unstimulated and 1–2 mL/min when stimulated (Sreebny, 2000) and the daily flow lies somewhere between 0.5 and 1.5 L in healthy individuals. The amylase content of the saliva is also variable but seems unaffected by stimulation and is normally ~45 U/mL (Neyraud, Palicki, Schwartz, Nicklaus, & Feron, 2012). Because of its multifunctional nature the overall composition of saliva is complex (Humphrey & Williamson, 2001). It contains a complex range of ions and also found in saliva are proteins including immunoglobulins, enzymes and mucins, and nitrogenous products, such as urea. These components function in the following general areas: (1) bicarbonates, phosphates, and urea modulate pH and the buffering capacity of saliva; (2) proteins and mucins serve to clean, aggregate, and/or attach oral microorganisms and contribute to dental plaque metabolism; (3) calcium phosphate and proteins work together to modulate dental demineralisation and remineralisation; and (4) immunoglobulins and enzymes provide antibacterial action.

The importance of oral processing has been highlighted in a number of studies showing the ability of cellular structures to retain nutrients if left intact. In particular, work on almonds (Grundy et al., 2015) has shown that following mastication, most of the almond cells remained intact with lipid encapsulated by cell walls. Thus, most of the lipid in masticated almonds is not immediately bioaccessible and remains unavailable for early stages of digestion. The lipid encapsulation mechanism provides a convincing explanation for why almonds have a low metabolisable energy content and an attenuated impact on postprandial lipaemia. Similar results have also been shown for other plant foods such as carrot (Tydeman et al., 2010). Carotene bioaccessibility was found to be greater from raw samples than heated samples of the same size. This is because heating increases the propensity for intact cells to separate, effectively encapsulating the carotene. Although the gross structure of the tissues was found to be relatively unaffected by in vitro digestion, at the

cellular level some cell-wall swelling and cell death were observed, particularly close to the surfaces of the tissue. This study suggests that cell-wall rupture prior to digestion is an absolute requirement for carotene bioaccessibility in the upper GI tract and that heating does not enhance carotene release from intact cells.

A related area of research is the issue of poor dentition in the elderly, which can also have an impact on nutrient release and digestion rate as well as food choice (Jauhiainen et al., 2017). In a study by Laguna et al., fracture mechanics of 15 commonly consumed food products of fruits, vegetables, and dairy origin were analysed using penetration test. Food score difficulty showed that high breaking forces of food products were related linearly with perceived difficulty ($r = 0.729$) and with higher oral processing time ($r = 0.816$). Other food breakdown characteristics such as the number of peaks and gradient of the penetration curves showed linear correlation with mastication time ($r = 0.830$, $r = 0.840$) and number of chew cycles ($r = 0.903$, $r = 0.914$) (Laguna, Barrowclough, Chen, & Sarkar, 2016). Regardless of the number of chewing cycles, once the bolus has been formed it is swallowed and passes down the oesophagus into the stomach.

3 Gastric Phase

The stomach is a food storage vessel where the process of food hydrolysis starts and acidic conditions start to kill bacteria present in the food. In general, in its rested state the stomach has a small volume (10–50 mL) and contains a limited volume of highly acidic secretions. The arrival of food into the stomach immediately stimulates the secretion of pepsinogen and gastric lipase by chief cells and hydrochloric acid by parietal cells. The pH sensitivity of all of the enzymes present in the stomach is key to what digestion occurs. Immediately after consumption of a meal the gastric pH is dominated by the pH and buffering capacity of the food. Thus, the pH can easily be above 6 initially and only gradually drop below 2 after several hours (Malagelada, Go, & Summerskill, 1979; Sams, Paume, Giallo, & Carriere, 2016). As the contents of the main body of the stomach is not well mixed there can be localised differences in pH that have the potential to affect the local rates of hydrolysis (Nyemb et al., 2016). There is a certain amount of evidence that gastric mixing, which relies on gastric muscle tone can decrease in the elderly. This is likely to affect not just gastric mixing but gastrointestinal motility more generally (Levi & Lesmes, 2014). In the presence of acid, pepsin is formed from the zymogen pepsinogen. Pepsin is active over a wide range of acid conditions between 1.5 and 5 with a maximum at pH 2 (Piper & Fenton, 1965). Pepsin is an aspartic protease that preferentially cleaves after the N-terminal of aromatic amino acids. However, hydrolysis depends on accessibility to the substrate and that depends on the secondary and tertiary structure of the protein as well as its aggregation state. Bovine milk contains proteins at both extremes of the pepsin susceptibility spectrum. The whey protein β-lactoglobulin has been shown to be largely pepsin resistant, while the

caseins are highly susceptible to pepsin hydrolysis under fixed conditions (Mandalari, Adel-Patient, et al., 2009). This difference is due to the differences in protein structure as β-lactoglobulin is a globular protein with its secondary structure held together by disulphide bonding while the caseins have little secondary structure and this is clearly a result of their biological functions (Sawyer & Holt, 1993). Thermal and other types of processing are known to alter protein structure, so it is not surprising that it can also alter susceptibility to proteolysis (Macierzanka et al., 2012). Heating can often lead to the irreversible unfolding of protein. However, this can also expose more hydrophobic regions that can lead to aggregation. Finally, it is worth mentioning in relation to these two groups of protein that despite the pepsin susceptibility mentioned above, casein is known for its slow digestion while whey is known as a fast digesting. This is because the combination of low pH and limited pepsin hydrolysis leads to the irreversible coagulation of the casein and subsequent slow gastric emptying (Boirie et al., 1997).

The other digestive enzyme secreted into the stomach is gastric lipase, which is active between pH 3 and 6 but has an optimum between 4 and 5. This acidic lipase preferentially cleaves triacylglycerides at the sn-3 position (Sams et al., 2016). Typically in healthy adults the lipolysis catalysed by gastric lipase reaches 10–20%. Although this limited gastric lipolysis may not be quantitatively important in healthy subjects, the action of gastric lipase may be qualitatively important by initiating fat digestion and facilitating some fat emulsification in the stomach and thereby facilitating the action of pancreatic lipase in the duodenum (Armand et al., 1995).

The final enzyme present in the gastric compartment is salivary amylase, much of which has already been intimately mixed with the food during oral processing and is indeed continuously added to the gastric content. The α-amylase in saliva, also known as ptyalin has a pH optimum between 6.7 and 7.0 but it can still be active in gastric conditions down to a pH of ~4 (Fried, Abramson, & Meyer, 1987). As has already been stated, the pH during initial stages of gastric digestion can often remain relatively high meaning that the amylase can be active for a prolonged period. It should also be noted that the loss of activity is a combination of degradation by pepsin and loss of conformation due to pH. In addition to hydrolysis, there is also some specific physical processing in the gastric antrum that can further decrease the size of particles and other structures (Marciani et al., 2000, 2012). However, in the main body of the stomach there is very little physical motion meaning that phase separation of different components in the food can occur. For example lipids, having a lower density than water, can float to the top of the stomach leaving a more aqueous phase below it, or denser particles can sediment to the bottom of the stomach (Mackie, Rafiee, Malcolm, Salt, & Van Aken, 2013; Marciani et al., 2009). Both of these possibilities can alter the composition of the chyme being emptied from the stomach at any given time.

Although there are no endogenous surfactants secreted into the gastric compartment as part of the digestion process, it is clear that both bile salts and phospholipids are likely to be present in the antrum at least. The bile is present as a result of reverse transport/retropulsion from the duodenum. An additional source of phospholipid is

from the cellular debris produced as a part of the normal turnover of the gastric epithelium. In both cases the concentrations are relatively low and highly variable but the presence of such surfactant may have an impact on protein hydrolysis by altering the accessibility of potential cleavage sites as shown in vitro (Mandalari, Mackie, Rigby, Wickham, & Mills, 2009).

In addition to the biochemical degradation of food, the stomach also erodes the food bolus and shears it into smaller particles. This process has been described in some detail using computational fluid dynamics (CFD) (Ferrua & Singh, 2010). Using this approach, the authors were able to show that in agreement with the classical description of gastric function, the strongest fluid motions were predicted in the antropyloric region. A significant degree of recirculation of gastric contents from the fundus towards the antrum was also identified. However, for a given motor response of the stomach, the viscosity of the gastric digesta significantly affected the local flow behaviour and pressure gradients that developed within the stomach. This runs somewhat counter to the idea of rapid and complete homogenisation of the meal. Indeed, gastric contents associated with high viscous meals seem to be poorly mixed. The diminution of food in the stomach is thought to involve two main flow patterns, the retropulsive jet-like motion and eddy structures where relatively high shear rates may be generated. However, this study found that increasing the viscosity of gastric contents significantly diminished the flow, while predicting a significant enhancement of the pressure field.

During the digestion of food there are two modes of gastric emptying. Firstly by eroding the solid bolus of food in the stomach from the outside, where the food has been most exposed to acid and enzymes. The chyme may then be squeezed through the pylorus into the duodenum if the particle size is sufficiently small (Marciani, Gowland, Fillery-Travis, et al., 2001; Marciani, Gowland, Spiller, et al., 2001). When the gastric contents are more fluid or semi-solid (e.g. soup or porridge), emptying occurs primarily during periods of quiescence in antral pressure activity and, by implication, in antral contractile activity (Indireshkumar et al., 2000) and thus may empty from the centre of the stomach, a zone that has not been subjected to significant pH change or exposed to gastric enzymes (Pal, Brasseur, & Abrahamsson, 2007). In the antrum, selective "sieving" permits the rapid passage of liquids and smaller food particles while the larger particles are retained for further processing, although this is effected by the viscosity of the gastric contents (Marciani et al., 2012). The size cut-off means that particles larger than about 3 mm (Kong & Singh, 2008) tend to be retained longer, although not indefinitely (Stotzer & Abrahamsson, 2000). The rate at which food is emptied from the stomach depends on a number of factors but one is the energy density of the food (Hunt, Cash, & Newland, 1975; Hunt & Stubbs, 1975). As far back as the 1970s it was shown that energy density has an inverse effect on gastric emptying. However, in addition, the rheological properties of the gastric content play an important role on gastric processing (Ferrua & Singh, 2010) and emptying rate. Although both are important, increasing the viscosity is considered less effective than increasing the energy density in slowing gastric emptying (Camps, Mars, De Graaf, & Smeets, 2016).

4 The Small Intestine

Once the chyme has been emptied into the small intestine from the stomach it undergoes an increase in pH and is mixed with pancreatic enzymes and bile. This opens a new round of hydrolysis in which salivary amylase and gastric lipase may also play a role, although pepsin will be inactivated. Pancreatic enzymes fall into three groups associated with different macronutrient substrates. The proteases present are primarily trypsin and chymotrypsin as well as elastase, carboxypeptidase, and other peptidases. Trypsin is a serine protease activated from the proenzyme trypsinogen. It is primarily active against the C-terminal side of the amino acids lysine and arginine. Chymotrypsin preferentially cleaves the C-terminal side of hydrophobic amino acids (e.g. tryptophan, tyrosine, and phenylalanine). It can also cleave other peptide bonds but at a lower rate.

Starch in the chyme is further hydrolysed by pancreatic amylase. Indeed, for the most part starch is hydrolysed in the small intestine by pancreatic amylase. There has been a significant amount of research into factors that affect rates of starch hydrolysis (Dona, Pages, Gilbert, & Kuchel, 2010; Lehmann & Robin, 2007). Starch is a complex polysaccharide comprising two different polymers, namely amylose and amylopectin that are both formed from linked glucose molecules. Amylose is a linear polymer, while amylopectin is branched and the ratio of the two has a significant effect on digestibility, with high amylose starches being less digestible. The degree of crystallinity and the extent of gelatinisation, both of which can be effected by the thermal history of the starch, can have a significant impact of digestibility. The role of resistant starch as a form of dietary fibre is still under investigation. Starch is divided into three fractions depending on digestibility and are defined as:

Rapidly digestible starch (RDS): amount of glucose released after 20 min,
Slowly digestible starch (SDS): amount of glucose released between 20 and 120 min hydrolysis, and
Resistant starch (RS): total starch minus amount of glucose released within 120 min hydrolysis

The final group of pancreatic macronutrient hydrolysing enzymes are the lipases. Although there is a limited amount of lipolysis in the gastric phase of digestion (10–20%) as indicated above, the majority takes place in the proximal small intestine. Lipolysis is a complex process because both the substrate and products involved are only sparingly soluble in an aqueous environment while the enzymes need to stay in that aqueous environment. This means that the hydrolysis occurs at the interface and is strongly affected by interfacial area and interfacial composition, both of which evolve with time. The only exception to this is with very short chain lipids such as tributyrin that are primarily hydrolysed from solution. This is why tributyrin is often used as a substrate for determining lipase activity. Non-polar lipids are generally present in food as triglycerides, which can be hydrolysed to monoglycerides and free fatty acids with diglycerides as an intermediate. Thus each molecule of

triglyceride will result in one molecule of monoglyceride and two molecules of free fatty acid. The specificity of pancreatic lipase is for the sn-1 and sn-3 positions of the triglyceride meaning that the monoglyceride is normally left at the sn-2 position. The rate of hydrolysis also tends to be faster for the shorter chain fatty acids than the long chain molecules. The hydrolysis also takes place in the presence of bile that has been secreted from the gall bladder as a result of cholecystokinin (CCK) mediated contraction along with the pancreatic secretions. Bile is important for the removal of lipid hydrolysis products from the triglyceride interface through the formation of mixed micelles (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011). In the presence of bile, the interfacial tension is lowered sufficiently that the adsorption efficiency of the lipase is decreased. This is not an issue in the small intestine as the more amphipathic protein, co-lipase can still adsorb to the triglyceride interface. Once adsorbed, lipase can bind to the co-lipase, anchoring it to the substrate and allowing hydrolysis to continue. Phospholipids are also hydrolysed in the small intestine by pancreatic phospholipases such as phospholipase A1 (PLA1) that cleaves at the sn-1 position to release a fatty acid and a lysophospholipid (Carriere et al., 1998).

Bile contains a number of endogenous surfactants but is primarily composed of bile salts, phospholipids and cholesterol. The other major component in bile is bilirubin. Bile is a potent surfactant but has an unusual structure as it is a cholesterol derivative in which one side of the sterol is more hydrophobic than the other (Maldonado-Valderrama et al., 2011). Thus the aggregation behaviour with other more conventional surfactants such as phospholipids and fatty acids leads to the formation of rather disc-shaped mixed micelles. The composition of bile can vary between individuals as bile acids are recirculated and can be modified by exposure to bacterial enzymes. Bile is formed from cholesterol in the liver and is conjugated with the amino acids glycine or taurine. In humans, the main bile salts are cholate and chenodeoxycholate with the glyco-form being more widely present than the tauro-forms. These bile acids are different to those found in other animals often used to study digestion such as pigs or mice. About 95% of the bile secreted into the duodenum is reabsorbed in the distal ileum by the apical sodium-dependent bile acid transporter (ASBT). The bile is then secreted from the basolateral side of the enterocytes via the OST α/β transporters. The amino acid conjugated bile is largely deconjugated by bile salt hydrolase secreted by gut microbiota, primarily in the large intestine, where some may be absorbed.

Although bile is secreted into the duodenum from the gall bladder, it originates in the liver and is simply stored in the gall bladder ready for use. The multistep enzymatic conversion of cholesterol into bile acids confers surface active properties that are crucial for their physiological functions in hepatic bile formation and absorption of dietary lipids and fat-soluble vitamins from the small intestine. The immediate products of bile acid synthesis are referred to as primary bile acids. In humans these consist of cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid) and chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid). Although both mice and pigs are often used as models for human digestion, murine and porcine bile compositions are very different to that of humans.

Transport of digestion products from the lumen to the epithelium of the small intestine does not rely on diffusion but on the efficient mixing of enzymes, bile and chyme. A simple calculation of the diffusion of for example, glucose will show that if we assume the intestinal diameter to be 1 cm, then the cross-sectional area of the intestine would be 7.8×10^{-5} m^2. The glucose molecule is about 1 nm in radius, the viscosity of the chyme at the very best case is equal to water but let us approximate that to 0.001 Pas and of course the temperature is 37 °C. From the area we can set the mean square displacement of the glucose at 7.8×10^{-5} m^2 and the diffusion coefficient is thus 2.27×10^{-10} m^2/s. From these values we can calculate the time taken for the glucose to diffuse from the centre of the lumen to the gut wall would be ~24 h. Because of this there is significant interest in factors that affect the efficiency of mixing such as the viscosity of the chyme or the presence of particulates (Gouseti et al., 2014; Lentle & de Loubens, 2015). The work of Gouseti et al. involved in vitro digestion studies using a dynamic duodenum model to simulate intestinal motility. The work illustrates the importance of mass transfer on simulated glucose absorption by using a range of food hydrocolloids. Addition of guar gum, CMC, and pectin showed a reduction in glucose bioaccessibility by up to 30% compared with aqueous solutions. The work suggests an explanation for the significant delay of in vivo postprandial blood glucose observed by the addition of hydrocolloids. Although there are indications that luminal viscosity can affect mass transfer, the luminal contents are often non-Newtonian in their rheological behaviour and shear regimes are poorly defined. Thus the effect of hydrocolloids or insoluble fibre on mixing in the small intestine is still poorly understood. The evidence presented by Lentle et al. suggests that the small intestine does not function to optimise mixing within a minimal length of small intestine or within a minimum time. Rather, it appears that postprandial contractile activity disperses chyme along the length of the small intestine and facilitates mixing within the bulk of the luminal space by kneading and folding. The resulting, relatively slow, rate of mixing within the bulk of the lumen allows for the slower rate of enzymatic diffusion into, and digestion of, nutrients within the complex milieu of the chyme. Establishing mixing throughout the lumen of the small intestine also maximises the surface area of mucosa that is available for absorption at a given time. Finally in addition to the mixing of material in the lumen, there is a boundary "unstirred" layer between the lumen and the intestinal epithelium that comprises for the most part intestinal mucus. Nutrients must diffuse through this layer before they are absorbed by the enterocytes.

An additional factor that effects transport and mixing is the architecture of the gut. There are significant differences in this between the proximal and distal ends of the small intestine. The highest rate of absorption is in the duodenum at the proximal end and this is where the surface area is maximised by the maximal length of the villi at this point. The villi then become progressively shorter towards the more distal parts of the small intestine. The impact of the villi on mixing has been investigated by Lentle et al. (Lentle & de Loubens, 2015; Lim, de Loubens, Love, Lentle, & Janssen, 2015) with the conclusion that laminar eddies at the edges of the groups of villi and augmented mass transfers in the radial direction between the inter-

villous space and the intestinal lumen improved the absorption of nutrients and mixing at the periphery of the lumen.

5 Large Intestine

The movement of material from the small intestine to the large intestine is controlled by the ileal-caecal valve. The large intestine comprises the ascending, transverse and descending parts of the colon followed by the sigmoid colon and the rectum. By the time that chyme reaches the large intestine, more than 90% of the nutrients have been removed leaving primarily dietary fibre. The colon has two main functions: Firstly, to convert as much as possible of that dietary fibre into energy for the body. This is done by the intestinal microbiota fermenting the fibre, primarily into short chain fatty acids (SCFA). Secondly to remove most of the water from the chyme in order to minimise water loss from the body. The colonic fermentation of dietary fibre into SCFA releases some 40–50% of the energy in the fibre. Thus in the distant past, when humans were hunter gatherers and their diet comprised significantly higher levels of fibre, a significant amount of the energy supplied by the diet could come from the fermentation of fibre but in the modern western diet this is very much less (Cummings & Macfarlane, 1997).

The interactions between gut microbiota, diet, and the host have been the subject of extensive research over the last 10 years. It is not the intention of this chapter to cover this research other than to highlight some key points of relevance to gut function. Almost independently of the type of substrate the colonic fermentation produces SCFAs. Butyrate is the major energy source for colonocytes, propionate is largely taken up by the liver, and acetate enters the peripheral circulation to be metabolised by peripheral tissues such as muscle. Specific SCFA may reduce the risk of developing gastrointestinal disorders, cancer, and cardiovascular disease. Butyrate has been studied for its role in nourishing the colonic mucosa and in the prevention of colon cancer by promoting cell differentiation, cell cycle arrest, and apoptosis of transformed colonocytes. Therefore an increase in SCFA production and potentially greater delivery of SCFA, specifically butyrate, to the distal colon may result in a protective effect. As a result, butyrate irrigation has also been suggested in the treatment of colitis.

6 The Gastrointestinal Mucosa

The intestinal mucosa is the site of nutrient absorption and indeed the site where digested food and other components in chyme really come into close contact with the body. Thus, the intestinal mucosa has developed to optimise nutrient absorption while minimising pathogen infection. The intestine is lined with crypts (intestinal glands) and villi (finger-like protrusions). Intestinal epithelial cells are produced in

the base of the crypts and migrate to the villus tips over a period of 3–5 days. During this migration the cells are differentiated into a number of different cell types including:

- Enterocytes—the normal absorptive cells
- Goblet cells—responsible for secreting mucin
- Endocrine cells—responsible for the detection of a range of nutrients and the secretion of gastrointestinal hormones

Mucus is secreted by goblet cells throughout the GI tract. However, the composition of the mucus layer varies significantly depending on the location. In the stomach, the primary secreted mucin is MUC5AC but MUC6 is also present while in the intestine it is MUC2 (Phillipson et al., 2008/10). The enterocytes are also protected by the membrane bound mucin MUC1 that has a role to play in regulation of the secreted mucins but does not affect their adherence to the underlying epithelium. The architecture of the mucosa has a significant effect on the properties of the mucus layer. In the stomach the layer has been shown to be striated (Ho et al., 2004) and this has also been extensively shown in the colon where the mucus forms two distinct regions, the tightly and loosely adherent layers (Johansson, Larsson, & Hansson, 2011). The tightly adherent mucus layer in healthy individuals is thought to be an effective barrier to bacterial penetration while the loosely adherent layer can provide an environment that is more conducive to bacterial growth (Johansson et al., 2008). In the small intestine there is still evidence of a tightly adherent layer but it is significantly reduced in thickness (Bajka, Rigby, Cross, Macierzanka, & Mackie, 2015) and the loosely adherent layer is more heterogeneous. Because of these differences, there are a number of other factors that contribute to the permeability of the small intestinal mucus layer to both bacteria and nutrients. Although bacteria are more easily able to penetrate the mucus layer in the small intestine, a number of antimicrobial components are co-secreted by the mucosa.

The permeability of the mucus layer to particulates clearly depends on the size, charge, and surface properties of the particles but also on the pore size of the network through which it needs to diffuse. In general the pore size of the network has been shown to be of the order of 100 nm (Mackie, Round, Rigby, & Macierzanka, 2012). However, in addition the free diffusion of particulates depends on them carrying sufficient negative charge and in the small intestine this can be imparted by bile salts adsorbing to the particle surface. In studies following the diffusion of 500 nm latex beads and also lipid digestion products, it was found that in the absence of bile the particles/lipids were unable to penetrate the mucus layer (Macierzanka et al., 2011).

The properties of the mucus layer have also been shown to be affected by other endogenous and exogenous polymers. The first of these is DNA that is woven into the mucus layer as a result of cell shedding from the tips of the villi. This is part of the normal cellular turnover and is done in a very controlled way but it does mean that cell debris including DNA is regularly added to the mucus layer around the villus tips and this can significantly increase the local viscosity of the mucus layer (Mackie, Bajka, & Rigby, 2016). Soluble dietary fibre has been shown to have the

same affect. The soluble fibre sodium alginate was shown to freely diffuse into the mucus and to have minimal effect on the bulk rheology when added at concentrations below 0.1% (Mackie, Macierzanka, et al., 2016). Despite this lack of interaction between the mucin and alginate, the addition of alginate had a marked effect on the diffusion of 500 nm probe particles, which decreased as a function of increasing alginate concentration. It was also shown that diffusion of a fluorescently labelled digested protein stabilised emulsion was decreased by the addition of 0.1% alginate to porcine intestinal mucus. This reduction may be sufficient to reduce problems associated with high rates of lipid absorption such as hyperlipidaemia. In a study using β-glucan as the soluble fibre, a preliminary study investigated the effect of doubling the β-glucan content of a porcine diet for 3 days and focussed on the properties of the intestinal mucus layer (Mackie, Rigby, Harvey, & Bajka, 2016). In vitro digestion of the enhanced β-glucan and control meals showed that over 90% of the β-glucan was released from the enhanced β-glucan diet in the simulated proximal small intestine, although this did not alter rates of nutrient hydrolysis. Measurements of the permeability of the porcine intestinal mucus showed that the diet decreased permeability to 100 nm latex beads and more importantly reduced permeability to lipid from the digested diet. The dietary fibre, β-glucan, has been shown to lower cholesterol by reducing bile recycling. The authors suggest that reducing mass transfer of bile and lipid through the intestinal mucus layer may be one way in which this decrease in bile reabsorption is enabled and that postprandial lipid absorption is prolonged.

7 Feedback and Control

In some of the previous sections, references have already been made to the complexity of the systems that control digestion. In this section, some more details of that control will be described. The sensing of food by G-protein couple receptors (GPCRs) starts but is by no means limited to the mouth. Although it is clear that oral processing and taste can affect digestion this has already been discussed. There are a number of sensors in the stomach and small intestine that allow the body to respond to the food that has been consumed. Ghrelin is an acylated 28-amino acid peptide hormone produced primarily by the stomach and to a lesser extent the small intestine. Ghrelin increases appetite and gastrointestinal motility but decreases insulin secretion (Karhunen, Juvonen, Huotari, Purhonen, & Herzig, 2008). It also decreases the response of gastric mechanosensors making them less sensitive to gastric distension. Another GI hormone that has a significant effect on gastric behaviour is CCK, which is secreted by I-cells in the proximal small intestine. CCK affects digestion by slowing down gastric emptying, increasing gall bladder contraction and increasing gastric mechanosensor sensitivity. Another hormone that is secreted by endocrine cells in the proximal small intestine is glucose-dependent insulinotropic peptide (GIP), which shares the insulinotropic effect with

glucagon-like peptide 1 (GLP-1). GIP is secreted by K-cells, which like I-cells are in high density in the proximal small intestine but decrease in density more distally. GLP-1, oxyntomodulin and peptide YY (PYY) are secreted by L-cells that are in low density in the duodenum and increase in density towards the ileum and are most widespread in the colon. PYY mediates the so called ileal and colonic brakes, mechanisms that ultimately slow gastric emptying and promote digestive activities to increase nutrient absorption and enhance satiety. GLP-1 is thought to play an important part in the ileal brake a mechanism regulating the flow of nutrients from the stomach into the small intestine. In addition, GLP-1 is an incretin hormone and increases glucose-dependent insulin release, inhibits glucagon secretion, and increases pancreatic ß-cell growth. These gastrointestinal hormones along with others not discussed here are responsible for controlling the passage of nutrients, energy metabolism, and satiation.

8 Psychology Versus Physiology

This chapter has described the body's physiological responses to food. However, it is well known that there is also a significant psychological component to our response to food. A well-known example is the Nobel Prize winning work of Pavlov on conditioning associated with salivation in dogs (Pavlov, 1927). The same behaviour is seen in humans when we see or even think about certain foods; but does this affect responses such as appetite and satiation?

Satiation signals arise from multiple sites in the GI tract. Ingested food evokes satiation by two primary effects, namely gastric distension and release of peptides from enteroendocrine cells. The hindbrain is the principal central site receiving input from fast-acting satiation signals transmitted both neurally and hormonally. Although the perception of fullness clearly involves conscious awareness, perception of GI feedback signals is not required for satiation. Therefore, gut-hindbrain communication is sufficient for satiation, although this normally interacts with higher cognitive centres to regulate feeding (Cummings & Overduin, 2007). The article by Cummings and Overduin, gives an excellent overview of the physiological mechanisms involved in the regulation of food intake.

Because of concerns about the high levels of obesity in developed countries, there has been significant research on eating behaviour and appetite showing that in addition to the satiety signals, environment can have a significant effect on energy intake (Chambers, McCrickerd, & Yeomans, 2015; Rolls, Hetherington, & Burley, 1988). These articles concluded that food can be manipulated in terms of structure and composition to enhance the consumer's experience of satiety but that a combination of factors will ultimately determine the foods effect on appetite control. The authors suggested that taking this integrated approach to satiety will lead to the more optimised development of high satiety foods. The psychology of eating and feeling of satiation is discussed in more detail in Chap. 10.

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In vivo, In vitro, and In silico Studies of the GI Tract

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1 Introduction

Understanding the mechanisms of digestion is important in promoting the design of food formulations with increased health benefits by tailoring their digestive profiles. Such knowledge is also important for functional foods and pharmaceuticals. However, studying digestive processes is challenging due to reasons such as the complex processes occurring during digestion (see for example chapters "The Digestive Tract" and "Consumer Psychology and Eating Behaviour" for the physiology and psychology of eating, respectively); the complex nature of foods and meals (Bornhorst, Gouseti, Wickham, & Bakalis, 2016); the vast variability between individuals (Bratten & Jones, 2009), and the limitations of currently available techniques (Gidley, 2013). To date, knowledge of digestive processes typically comes from broadly three types of research methodologies. In vivo investigations involve human or animal studies, in vitro experiments study digestion outside the body, and in silico models simulate digestive processes using numerical and computational methods.

Important advantage of in vivo studies, in particular human studies, is the high relevance of the outcomes, as the subject of the study is also the targeted end user of the foods (Hur, Lim, Decker, & McClements, 2011; Minekus et al., 2014). On the other hand, in vitro or in silico methodologies may be preferred in studies aimed at

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gaining mechanistic understanding of digestion, as they offer the potential to operate at simpler, well-defined conditions. However, in vitro outcomes should be interpreted with caution to ensure physiological relevance.

A tiered approach has been suggested in studying bioaccessibility and/or bioavailability of nutrients from foods, in which in silico and in vitro models are used at a first step to provide evidence for the necessity of in vivo animal and human trials (Lefebvre et al., 2015). One of the aims of computational and experimental simulations may therefore be the reduction of the necessary in vivo studies, as the latter are generally expensive, time-consuming, laborious, and often ethically compromised.

The present chapter considers the three methodologies separately and briefly presents existing approaches and techniques used in each one. It is our intention to avoid replicating information provided elsewhere in this book, and in these cases the reader is referred to the relevant chapters.

2 In Vivo Methods

While traditionally linked with the medical/pharmaceutical sciences, in vivo methods provide a powerful tool for studying the link between food and health. For example, there are in vivo studies that aim to correlate a dietary exposure (e.g. saturated fat consumption) with a biomarker (e.g. serum cholesterol level) and ultimately with a health outcome (e.g. disease prevention). This type of study often lies on the border between digestion and nutritional studies, and typically involves epidemiological study designs (discussed in Sect. 2.1).

Another type of in vivo investigations focuses on gaining insight into the mechanisms of digestion. These include, but are not limited to, imaging techniques used to characterise flow of the material in the gut, and intubation techniques used to examine gut motility. Use of imaging techniques is extensively discussed in the chapter "Quantitative Characterisation of Digestion Processes" and will not be included here; intubation will be briefly introduced in Sect. 2.2. Section 2.3 briefly introduces animal studies.

2.1 Epidemiological Studies

The term "epidemic" was introduced by Hippocrates (ca. 460–377 BC) to describe conditions that occur during finite periods of time, for example an outbreak of a disease. On the other end, diseases that occur permanently within a population or region were termed "endemic", for example malaria in Africa is practically a permanent concern (Willett, 2013). In 1995, Last defined epidemiology as "the *study* of the distribution and determinants of health-related states or events (including disease), and the *application* of this study to the control of diseases and other health problems". This definition is largely applicable to date.

Epidemiological studies typically seek to investigate the link between an exposure and a health outcome. The three main common elements in all epidemiological studies involve (1) identification of an exposure (for example high fat diet) and how to measure it; (2) identification and evaluation of the associated health outcome (e.g. breast cancer); and (3) statistical analysis to assess potential correlation between the exposure and the outcome (Thiese, 2014). The overall aim of epidemiological studies is to either generate hypotheses or to provide evidence for existing hypotheses.

2.1.1 Epidemiological Study Designs

A number of epidemiological studies exists differing in the study design and/or the desired outcome. These will be briefly introduced in this section and the interested reader is encouraged to seek detailed information elsewhere [for example see Carneiro and Howard (2011), Grimes and Schulz (2002), Hajat (2011), Last and International Epidemiological Association (2001), Thiese (2014), and Timmreck (2002)].

Classification of the major epidemiological study designs is schematically shown in Fig. 1 (Grimes & Schulz, 2002). Depending on whether the investigator intervenes in the subjects' dietary habits or not, a study may be experimental (interventional) or observational, respectively. In observational designs the researcher studies the participants in their natural environments. The subjects' individual dietary habits are therefore determined by factors such as personal preferences, availability, doctors' prescriptions, fashion, and policy decisions (Carlson & Morrison, 2009). Further, in an observational study design, the investigator may study a single group alone (descriptive) or compare between two groups, one of which acts as the control (analytical). Individuals in the control group are expected to be unexposed to the predetermined exposure measure (or to the outcome, depending on the specific study design). Descriptive studies are often used to generate a hypothesis, while analytical studies may generate or support a hypothesis (Hajat, 2011).

Analytical observational studies further involve three main types of study design, depending on the relative time between exposure and health outcome. Cross-sectional studies consider exposure and associated outcome at a single point in time, and compare between the control and exposed groups. For example, between two groups of adults, one obese and the other not, the former shows higher rate of arthritis (Grimes & Schulz, 2002). This type of studies is usually inexpensive and straightforward in their design, implementation, and interpretation. However, they lack information about temporality. In the previous example, it is unclear whether the increased stress on the joints preceded arthritis or occurrence of arthritis resulted in reduced physical activity and increased body weight (Grimes & Schulz, 2002).

Cohort and case–control studies consider exposure and outcome in two reverse orders, as seen in Fig. 1. In cohort study design, the "active group" consists of individuals who are being/have been exposed to the identified risk factor (e.g. high-carbohydrate diet), while the other, the control, involves non-exposed participants.

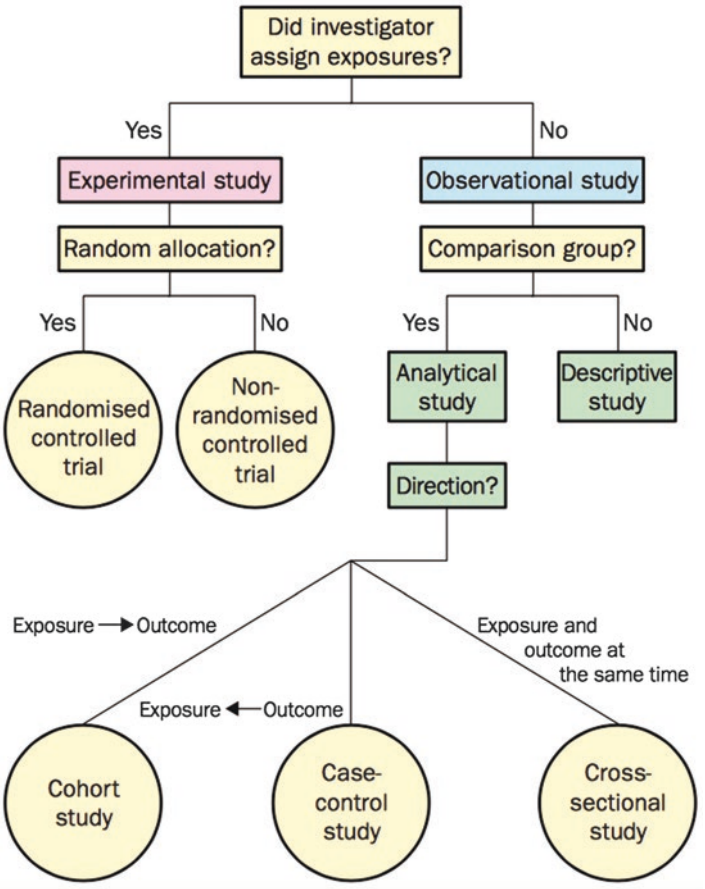

Fig. 1 Types of epidemiological studies (Grimes & Schulz, 2002)

The two groups are then monitored with time and the health outcome (e.g. occurrence of diabetes) is observed. Cohort studies demonstrate temporality, as the exposure precedes the outcome. However, they require time and they may be expensive. In addition, they are ineffective in the case of rare diseases as the probability of observing a health outcome is small (Thiese, 2014).

In case–control studies, selection of the two groups is based on their disease status. The "active" group is the one affected by the disease, whereas the control group(s) is disease-free. The researcher then investigates the degree of exposure of each group to a risk factor (Hajat, 2011). Using this method, it is possible, for example, to examine outbreaks of food-borne diseases. In a real case, the passengers of a ship that showed increased cases of vomiting and diarrhoea were divided into those who became ill and those who did not. Examination of their exposure to food identified a potato salad responsible for the outbreak of shigella (Grimes & Schulz, 2002).

Observational studies are popular among researchers. Using observational studies, for example, a link between high salt consumption and overweight/obesity (Boccia, 2015), or between high dairy consumption and metabolic syndrome in adults (Moosavian, Haghighatdoost, Surkan, & Azadbakht, 2017), or children's dietary habits and behaviour (Brown & Ogden, 2004) have been indicated. Furthermore, large-scale observational studies can provide vital information for generating and supporting generalised dietary advice and recommendations. For example, recommendations for increased consumption of vegetables or reduced consumption of salt are evidence-based on the outcomes of observational research (Gidley, 2013).

Two reported limitations of this study design involve (1) limitations on determining causality, as the evidence provided on the cause-and-effect relationship between the consumed food and the health outcome is weak, and (2) limitations on specificity, as due to the variability of human diet the effect of individual food components is unclear (Gidley, 2013).

In intervention studies, the researcher determines the degree of exposure of the "intervention group" to the exposure measure (e.g. the food under investigation) through the detailed experimental design. Intervention trials share similarities with the cohort study design in that exposure to a risk/treatment factor(s) differentiates the "intervention" from the "control" groups and participants are assessed over a period of time for health outcomes (Grimes & Schulz, 2002).

There are many elements that characterise intervention trials. Among these, the randomised, double-blind, placebo controlled study design has been reported as the optimal study design in clinical and nutritional studies (Misra, 2012; Slavin, 2013; Willett, 2013). Table 1 summarises important features and terminology of interventional studies.

Intervention studies are typically controlled trials. This is because they typically involve comparison between at least one "intervention" group that is exposed to the food under investigation and at least one "control" group that does not receive the investigated food. Control measures may vary. Placebo control refers to a measure that has the same form as the investigated treatment but it is free from the active component. As an example, the placebo sugary drink looks and tastes like the intervention sugary drink but without addition of the active component, which could be dietary fibre (Jenkins et al., 1978). The two groups may also be fed with alternative meals. For example, in a trial investigating the effect of structure and particle size on digestion, two groups were fed with an otherwise identical porridge meal prepared with either oat flakes or oat powder and the metabolic responses were measured (Mackie et al., 2017).

Another important element in intervention trials refers to how the individuals are allocated to the intervention or control group. By large, the preferred study design to assess a hypothesis is the randomised controlled study (RCT). In this method, participants with comparable baseline characteristics (e.g. age, weight, health conditions) are selected and they are randomly allocated to the intervention or control group. This process helps protecting the investigation from selection bias (Kahan, Rehal, & Cro, 2015). When RCT is considered complicated, expensive, or even not

Table 1 Glossary of terms used in intervention trials [for more information on terms for intervention study design see for example Deeks et al., 2003]

Controlled trial	An intervention experiment involving (at least) one "active" group that receive the treatment(s) under investigation and (at least) one group that does not receive the treatment and serves as the control group. Possible control measures may include placebo, no treatment, historical comparisons, etc.
Placebo Controlled	A controlled trial in which the control measure is a placebo treatment (e.g. a pill that does not contain the drug or a diet that does not include the component under investigation). It is the most common control measure in digestion studies [see for example Jenkins et al. (1978)]
Randomised	A controlled trial in which the individuals are randomly assigned a group (either the "active" or "control")
Quasi-randomised	A controlled trial in which the investigator allocates participants into the intervention or control group using a method that is not trully random (e.g. by age, height, etc.)
Non-randomised	A controlled trial in which the individuals are not randomly assigned a group (either the "active" or "control"). For example, the individuals may or may not choose to be exposed to a measure.
Single blinded	A controlled trial in which either the investigator(s) or the participant(s) is ignorant of which groups the subjects are assigned to
Double blinded	A controlled trial in which neither the investigator(s) nor the participant(s) is aware of which group the subjects are assigned to

feasible, for example for ethical reasons, other study designs are employed. In quasi-randomised and non-randomised designs, no effort is taken to account for any randomisation element. For example, the investigator may allocate subjects alphabetically, in order of age, etc. (Grimes & Schulz, 2002); or the participants may select their group allocation by volunteering to be exposed to an experimental treatment. Such methods are likely to introduce a selection bias in the study, which should be taken into account when interpreting the results.

Blinding of the intervention may further help reducing bias of the experimental outcomes. In single-blinded study design, either the investigator or the subjects (but not both) are aware of who is receiving which intervention diet. In double-blinded studies, neither the researcher nor the participants know which diet is linked with which subject.

2.1.2 Nutritional Epidemiology

Nutrutional epidemiology refers to the use of epidemiological principles for the study of nutrition and health, and it can be regarded as a subdivision of epidemiology. It has been recently introduced as a distinct field of study, although the practice is not new. For example, one of the first reported intervention trials was that of Lind, who in 1753 used controlled study design to study treatment of scurvy in the Salisbury. Lind split 12 crew members affected by the disease in groups of two. All participants had the same core diet, while each of the six groups additionally received cider, elixir of vitriol, vinegar, sea water, oranges and lemons, and a

purgative mixture, respectively. His findings enabled him to associate scurvy with orange and lemon consumption, which was later assigned to vitamin C (Sutton, 2003). This and many other examples gradually led to the introduction of nutritional epidemiology as a separate research field (Willett, 2013).

A distinctive aspect in nutritional epidemiological studies, when compared to medical epidemiology, is the nature of the exposure: the complexity and variability of what we eat, compared for example to the well defined nature of a medical pill (Willett, 1987; Wilson & Temple, 2001). Adding to this, the intra-human as well as interhuman variability of human metabolism, including the effect of non-dietary factors such as stress on digestion, poses further challenges to the nutritional epidemiologist and to those with interest in digestion studies. These factors are particularly challenging in the case of observational studies.

Indeed, one of the major acknowledged challenges for nutritional epidemiologists refers to the characterisation and practical measurement of dietary exposure (Willett, 1987; Wilson & Temple, 2001). Foods are inherently complex, multiphase systems that are kinetically trapped within a food structure. The way that the human digestive system acts on foods depends on food variability, including the exact food ingredients and structures, how we prepare the food (processing conditions), and the amounts and combinations that are consumed (Wilson & Temple, 2001). Even unprocessed, "simple" food ingredients, such as vegetables or fruits, can vary in their properties depending on the weather conditions, soil composition, ripening time, etc. (Wilson & Temple, 2001). In addition, it is known that the digestion of a food component may be affected by the digestion of other food components (Hur et al., 2011).

This uncertainty of determining and measuring dietary input has provoked a debate among researchers. For example, there are those who fully question the likelihood of acquiring useful dietary information of free-living individuals and therefore the usefulness of carrying out observational epidemiological studies at all. There are also those who regard diet within a country as too homogeneous to provide any useful correlations with health (Willett, 2013). In a more recent trend, some researchers take a different approach. They consider food groups or dietary patterns rather than individual dietary components and use statistical methods to link these food groups or dietary patterns with health (Hoffmann, Schulze, Schienkiewitz, Nöthlings, & Boeing, 2004; Hu, 2002; Wilson & Temple, 2001).

Compared to observational studies, quantification of diet is easier to determine in experimental trials. One reported limitation of intervention nutritional trials, however, refers to the fact that intervening to the subjects' food consumption habits renders the diet more artificial, and therefore any results should be treated with care (Gidley, 2013). As an example, while controlled metabolic studies have demonstrated that increased consumption of cholesterol or of saturated fats, and decreased consumption of polyunsaturated fats result in an increase in serum cholesterol levels, this has not been verified in a number of observational cross-sectional studies (Willett, 2013). A possible explanation for this observation is that the amount (and combinations) of lipids typically consumed as part of a diet have marginal effect on serum cholesterol and larger quantities, such as those offered in intervention stud-

ies, are needed to create any measurable effect (Willett, 2013). Another limitation of nutritional trials refers to the fact that the outcome is often a biomarker (for example serum cholesterol levels) and it is only indirectly related to the health condition (for example heart disease or stroke) (Gidley, 2013). The link between the biomarker and the health conditions needs to be separately verified.

2.2 Other In Vivo Studies

While epidemiology is a very popular technique for studying the link between nutrition, digestion, and health, there are a number of other methods (besides imaging, described in chapter "Quantitative Characterisation of Digestion Processes") that are used to obtain information about digestibility and bioavailability of nutrients. For example, intubation has long been used to provide insight on gut motility (Kong & Singh, 2008a). This is a highly invasive technique, which requires insertion of a measuring device into the subject's gut. The gastric barostat falls within this category and involves introduction of a balloon (max. volume 1.0–1.2 L) that is connected to a barostat into the subject's stomach. The intraballoon volume or pressure is measured under isobaric or isovolumic conditions, respectively, providing information on gastric response to consumption of a meal (Schwizer et al., 2002). Intraluminal manometry is another technique that determines gut motility by measuring pressure changes in the gut at fasting or during digestion (fed). It involves introduction of a catheter, typically through the nose down to the oesophagus, stomach, and small intestines, that has openings in predetermined positions to collect pressure information at different segments of the gut. In a more advanced version of this technique, the use of wireless capsules in the place of the traditional catheter that provide simultaneous information on pressure, temperature, and pH of the investigated segment has simplified the experimental set-up (Farmer, Scott, & Hobson, 2013). Other, indirect methods to assess digestibility include blood test, such as blood glucose level determination, and breath tests (Kong & Singh, 2008a).

2.3 Animal Studies

Animals are often used in nutritional studies as subjects in intervention trials. Compared to human trials, animal studies are typically cheaper and less laborious, while they may offer a degree of ethical flexibility that is prohibiting in humans (McClements, 2007). For example, one common technique to quantify digestion in animals involves animal sacrifice, where the subjects are slaughtered at a predetermined time after feeding and the contents of different sites of the gut are examined to determine progress of digestion and properties of the digested material (Bach Knudsen, Lærke, Steenfeldt, Hedemann, & Jørgensen, 2006; Bornhorst, Roman, Dreschler, & Singh, 2013). Another technique refers to the surgical introduction of

one or more permanent cannula(s) to the required site(s) of digestion (e.g. stomach, small intestine) that enables sample collection and characterisation at desired time intervals (cannulation) (Bach Knudsen et al., 2006). Or a catheter can be surgically introduced to the animal's portal vein and an artery and sampling is used to determine digestibility kinetics (Bach Knudsen et al., 2006).

An important limitation in the use of animals as subjects for studying human digestion reportedly refers to the differences between the animal and the human digestive and metabolic systems (McClements, 2007). This is often taken into account, together with other parameters such as cost and ease of handling, in the choice of animals for digestion studies (McClements, 2007). Example animals that are used in digestion experiments include rodents, pigs, cows, sheep, and horses, with the first two being the most commonly encountered (Darragh & Hodgkinson, 2000; Deglaire & Moughan, 2012; McClements, 2007). Animal selection depends on the targeted investigation, as well as on the targeted population that is studied. For example, use of 3-week-old piglet has been suggested as a model animal to study digestion in infants (Darragh & Moughan, 1995).

The use of animals in scientific studies has significantly progressed knowledge in areas such as digestion and health. In recent years there is a trend to reduce the number of in vivo tests [e.g. European Centre for the Validation of Alternative Methods (Le Ferrec et al., 2001)] and an overall tendency to provide animal-friendly scientific environments [e.g. the UK's 3Rs initiative with the aim to promote replacement of animals with non-animal alternatives when feasible, reduction of animal use to the minimum required for the targeted scientific advancements, and refinement of experimental designs to ensure minimal animal suffering during the trials (Home Office, Department for Business Innovation & Skills, Department of Health, 2014)].

3 In Vitro Methods

Similar to in vivo, studying digestion in vitro was probably first popularised within the pharmaceutical community, where tests assessing the disintegration of drugs were officially introduced in 1907 and were made compulsory in 1933 in Switzerland by the Pharmacopoeia Helvetica and later by other countries (Al-Gousous & Langguth, 2015). At present, a number of strictly regulated apparatuses is routinely used to assess drug dissolution in vitro (Al-Gousous & Langguth, 2015; McAllister, 2010).

Use of in vitro methods to study food digestion became largely popular in the 1990s. This has significantly boosted research in this area and has led in an inspiring exponential increase in the publications on the topic. It has also led to the introduction of terms such as nutraceuticals and nutrakinetics, which are the "food" analogues of pharmaceuticals and pharmacokinetics (McClements, Li, & Xiao, 2015; Motilva, Serra, & Rubió, 2015).

Simulating digestion outside the body is challenging, due to reasons such as the complexities of the digestive system as well as of the food materials. As an example, the length scales of foods as well as of digestive organs range between at least eight orders of magnitudes, from cm (e.g. first bite, small intestinal diameter) to mm (e.g. rice granules, villi organisations on the intestinal wall), to μm (e.g. starch granules, thickness of single villi layer), down to nm (e.g. plant cell walls, absorption sites in the intestinal wall), and angstroms (e.g. single molecules of sugar, water) (Aguilera, 2005; Bornhorst et al., 2016; Cozzini, 2015).

Adding to this, the digestive system is a complex, multicompartmental organisation that operates and controls digestion through a diverse and interconnected pool of processes and feedback mechanisms (see chapter "The Digestive Tract") (Cozzini, 2015). Variability in digestive responses between individuals may also be significant. Indicatively, in a study that compared duodenal pH of healthy individuals (control group) and patients with functional dyspepsia, "normal" pH values between four and seven were reported for the control group alone (Bratten & Jones, 2009). Digestive responses have further been associated with factors such as mood, time of the day, level of stress, consumed food, etc. (Bratten & Jones, 2009), further complicating the work of those wanting to replicate it in the laboratory.

Experimental challenges also exist. For example, some of the materials used, such as enzymes and mucins, may be biological and sensitive and/or expensive (Bongaerts, Rossetti, & Stokes, 2007). This may lead in experimental inconsistencies. Indicatively, an interlaboratory study of peanut protein gastric digestion using the same experimental protocol, reported digestion times varying from 0 to 60 min and interlaboratory agreement 77% (Thomas et al., 2004).

In vitro studies are, in principle, easy to carry out and reproducible, compared to in vivo. Ideally, they would also be cheap, high throughput and produce accurate, physiologically relevant results (Hur et al., 2011). Currently, in vitro experiments are often used for rapid screening of different food formulations (Hur et al., 2011) or to gain mechanistic understanding of digestion processes (Gidley, 2013). Besides their popularity in pharmacology, they are also widely used to study protein stability for allergenicity assessments (Dupont & Mackie, 2015; Wickham, Faulks, & Mills, 2009), and to estimate glycaemic index as well as starch fractions (i.e. rapidly, slowly, and non-digestible starch) in food materials (Englyst, Kingman, & Cummings, 1992).

3.1 In Vitro Digestion Models

In vitro models are typically application specific. For example, there are oral models that mimic biting (Meullenet & Gandhapuneni, 2006), mixing (de Wijk, Janssen, & Prinz, 2011), chewing (Salles et al., 2007), shearing (Lvova et al., 2012), tongue action (Benjamin et al., 2012), or compression (de Loubens et al., 2011; Mills, Spyropoulos, Norton, & Bakalis, 2011), and have been specifically developed to study processes such as taste and/or texture perception, or bolus formation. Model

selection therefore highly depends on the scientific question of interest and, of course, on the available resources.

There is a number of physiological conditions that a model may replicate. These include, but are not limited to (see also chapter "Influence of physical and structural aspects of food on starch digestion"), the temperature, pH and pH gradients, enzyme types and concentrations, composition and quantities of digestive secretions, residence times, flow and mixing, motility, diffusion and mass transfer, or absorption mechanisms. Usually, the temperature, pH, and enzymatic secretions are among the controlled variables, though the exact selected values may considerably vary depending on the experimental protocol and the specific application (Cozzini, 2015; Donaldson, Rush, Young, & Winger, 2014; Dupont & Mackie, 2015; Marze, 2017).

In vitro models may be monocompartmental, where digestion is simulated in a single container, or multicompartmental, which uses a number of containers to simulate different digestive processes or conditions. Depending on whether the model replicates time-related aspects of digestion (such as mechanical actions, flow, mixing, gut wall contractions, or dynamic pH changes) or not, in vitro models have been characterised as dynamic or static, respectively.

3.1.1 Static In Vitro Digestion Models

Static in vitro models typically offer a simple, fast, and flexible solution to digestion studies (see also chapter "Influence of Physical and Structural Aspects of Food on Starch Digestion"). They comprise a single or a series of batch containers that replicate the different stages of digestion. Often, there are three vessels that simulate oral, gastric, and intestinal digestion, respectively, with a fourth one replicating large intestinal digestion occasionally included (Marze, 2017). The experiment typically operates at 37 °C under mixing conditions that generate homogeneous mixing using devices such as magnetic or overhead stirrers, shaking incubators, or blood rotators [see for example Englyst, Veenstra, and Hudson (2007)]. The digestive fluids usually consist of water with electrolytes, enzymes, and possibly other compounds (mucins, bile salts, etc.), depending on the experimental protocol. The pH is typically adjusted at the beginning of each step to the desired, physiologically relevant value (Marze, 2017). The volume of the material analysed in static in vitro models may vary from µL of material [see for example the OCTOPUS (Maldonado-Valderrama, Terriza, Torcello-Gómez, & Cabrerizo-Vílchez, 2013)] to tens of mL of material [see for example the pH stat (McClements & Li, 2010)]. The pH stat is a popular model that was firstly introduced for lipid digestion studies, for which it has been extensively used (Ban, Jo, Lim, & Choi, 2018; Mun & McClements, 2017; Qin, Yang, Gao, Yao, & McClements, 2016; Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013).

Many static in vitro methods exist and it is often difficult to compare between their outcomes. This is partially due to the variability in the simulated physiological conditions used, such as pH or enzyme concentrations (Hur et al., 2011; Marze, 2017). For example, in a 2013 literature review on in vitro tests to study protein

allergenicity, protease concentrations in gastric digestion studies has been reported to vary between four orders of magnitude (Mills et al. 2013). Similarly, in a 2008 review on starch digestion, 36 protocols were reported (Woolnough, Bird, Monro, & Brennan, 2010). In an attempt to harmonise static in vitro methods, a network of scientists collectively working in the European (COST) action INFOGEST has published a suggested standardised protocol, which has shown good interlaboratory reproducibility (for more details in the INFOGEST protocol see chapter "Quantitative Characterisation of Digestion Processes") (Egger et al., 2016; Minekus et al., 2014). Applications of static models to study digestion of different components have been recently reviewed (Bohn et al., 2017; Mackie, Rigby, Macierzanka, & Bajka, 2015).

Static models have also been developed to study absorption of the digested material. These models often incorporate cell cultures (for example a monolayer of Caco2 cells or MDCK cells) (Marze, 2017). Absorption models, including cell culture models, as well as membrane models such as PAMPA and Ussing chambers, have been reviewed in relation to drug absorption studies (Deferme, Annaert, & Augustijns, 2008); however, the same principles pertain to nutrient absorption, including from functional foods (Motilva et al., 2015).

Studying starch hydrolysis is an example of simple static in vitro digestion assays and it has been used to quantify glucose release from carbohydrate food samples, such as rice (Chen et al., 2017; Dhital, Dabit, Zhang, Flanagan, & Shrestha, 2015; Hsu, Chen, Lu, & Chiang, 2015; Van Hung, Lam, Thi, & Phi, 2016), bread (Ronda, Rivero, Caballero, & Quilez, 2012), and oat (Brahma, Weier, & Rose, 2016). It can be used to estimate the glycaemic index of foods (Englyst, Vinoy, Englyst, & Lang, 2003; Goñi, Garcia-Alonso, & Saura-Calixto, 1997; Granfeldt, Bjorck, Drews, & Tovar, 1992) and to evaluate the fractions of starch that are rapidly digested (i.e. hydrolysed within 20 min), slowly digested (i.e. hydrolysed within 120 min) and not digested after the 120 min time (Englyst, Kingman, Hudson, & Cummings, 1996) (see also chapter "Influence of physical and structural aspects of food on starch digestion").

3.1.2 Dynamic In vitro Digestion Models

The importance of the dynamic nature of digestion has been indicated long before dynamic in vitro models gained popularity (Lea, 1890). Compared to static models, dynamic models offer the potential to replicate complex digestive actions, and they are therefore preferred in studying phenomena such as the effect of fluid dynamics on digestibility. They are, however, typically more laborious and time-consuming. Like static, they may reproduce one or more sections of the digestive process (for an introduction to dynamic digestion models see also Thuenemann, 2015). Examples of dynamic in vitro models are shown in Table 2 (with references).

Oral processing signals the beginning of digestion and it causes changes such as mechanical breakdown, lubrication through mixing with saliva, bolus formation, as well as initiation of enzymatic hydrolysis through the enzymes present in the saliva (see also chapter "Influence of physical and structural aspects of food on starch

Table 2 Example dynamic in vitro models

	Model	Characteristics	References
Mouth	In vitro mouth model	Replicates chewing by controlled motion of teeth, jaw, and tongue. Measures food breakdown and flavour release	Salles et al. (2007)
	Chewing device	Reproduces molar trajectory in realistic dimensions	Xu, Lewis, Bronlund, and Morgenstern (2008)
	Chewing machine (Bouche Artficielle)	Mimics mastication and measures food breakdown and volatile compound release	Mielle et al. (2010)
	Model mouth with artificial tongue	Reproduces tongue pressure patterns; provides online measurement of volatile compounds	Benjamin et al. (2012)
Stomach	Dynamic gastric model (DGM)	Mimics gut wall contractions by squeezing of the conical vessel	Wickham (2013), Mercuri, Lo Curto, Wickham, Craig, and Barker (2008), Lo Curto et al. (2011), Chessa et al. (2014)
	In vitro gastric model	Mimics fluid flow between a spherical probe and a cylindrical wall	Chen et al. (2011)
	Human gastric simulator (HGS)	Replicates mechanical forces by particle–particle abrasion	Kong and Singh (2008b)
	TIMagc (advanced gastric compartment)	Controls mixing/pressure profiles and flow rates, including gastric secretions and emptying	Bellmann, Lelieveld, Gorissen, Minekus, and Havenaar (2016)
	RD-IV-HSM A "near-real" in vitro human stomach model	Realistic morphology; mimics gut wall contractions	Chen et al. (2016)
Small intestine	Dynamic Duodenal Model	Mimics intestinal wall contractions and flow rates of digesta and intestinal secretions	Tharakan, Norton, Fryer, and Bakalis (2010), Gouseti et al. (2014)
	Human duodenum model	Replicates sigmoidal shape of duodenum and intestinal wall motility	Wright, Kong, Williams, and Fortner (2016)

(continued)

Table 2 (continued)

	Model	Characteristics	References
Large intestine	Simulator gastrointestinal (SIMGI)	Consists of a three-stage large intestinal fermentation model. Also contains gastric and small intestinal compartments	Barroso et al. (2015)
	Simulator of the human intestinal microbial ecosystem (SHIME)	Comprises three-stage large intestinal compartments. Also contains gastric and small intestinal compartments. Used to study microbial bioconversions in the colon	Van de Wiele et al. (2015)
	Dynamic colon model (DCM)	Non-microbial colon model that simulates gut wall motility and studies absorption in the large intestine	Stamatopoulos, Batchelor, and Simmons (2016)
Multicompartmental	Dynamic in vitro upper gastrointestinal model	Gastric and duodenal compartments. Controls flow rates and pH of the compartments. Used to study digestion of probiotics	Mainville, Arcand, and Farnworth (2005)
	Gastrointestinal digestion system (DIDGI)	Controls flow rates and pH of the compartments. Simulates pyloric sieve. Validated for infant formula	Ménard et al. (2014)
	TIM1 (gastric and small intestinal model); TIM2 (large intestinal model)	Controls gut wall contractions and flow rates of digesta and secretions. Commercially available	Minekus, Marteau, Havenaar, and Huis in't Veld (1995), Minekus et al. (1999), Marteau, Minekus, Havenaar, and Huis in't Veld (1997), Blanquet et al. (2001), Krul et al. (2000)

digestion"). Oral processing in vitro has been simulated using commercial meat mincer (Bornhorst & Singh, 2013), commercial/laboratory blender (An, Bae, Han, Lee, & Lee, 2016; Bordoloi, Singh, & Kaur, 2012; Dhital et al., 2015; Tamura, Okazaki, Kumagai, & Ogawa, 2017), or sophisticated mouth models (Benjamin et al., 2012; Mielle et al., 2010; Panouillé, Saint-Eve, Déléris, Le Bleis, & Souchon, 2014; Salles et al., 2007). For a review of dynamic oral processing models see Peyron and Woda (2016) and Morell, Hernando, and Fiszman (2014). In vitro dynamic oral processing models typically incorporate a mechanical element of oral

digestion and measure food breakdown and/or release of volatile compounds. As the mouth is also the organ where organoleptic characteristics of food are sensed, models have been developed to study texture and taste perception. For example, there are models that measure dynamic release of tastants such as salt (de Loubens et al., 2011; Mills et al., 2011), while addition of a microphone in the artificial mouth chamber has been reported, with the aim to gather acoustic information during eating. Analytical techniques, such as texture analysis or tribology, are further used to evaluate texture perception (van Aken, Vingerhoeds, & de Hoog, 2007; Vardhanabhuti, Cox, Norton, & Foegeding, 2011).

Gastric dynamic in vitro models typically incorporate a mechanical action (e.g. motility, mixing, and mechanical forces) by various techniques such as squeezing of the simulated gastric walls or relative motion between surfaces (see Table 2). They may or may not control flow rates of digesta and digestive secretions. These models typically study mechanical and/or enzymatic breakdown of the food bolus and have also been used to produce chyme that is then characterised using analytical techniques.

The chyme then passes to the small intestine, which is the site where most of the absorption occurs. Models that simulate intestinal wall motility (e.g. segmentation and peristaltic contractions) have been developed (examples shown in Table 2) and used to characterise chyme breakdown, bioaccessibility, and nutrient absorption rates. Absorption rates in these models are typically assessed by measuring the concentration of nutrients that pass through a semipermeable membrane simulating the intestinal walls. The semipermeable membrane acts as a sieve, which allows small molecules (products of digestion) to pass through the pores but retains large, undigested molecules in the luminal side. Like gastric, intestinal models may or may not incorporate fluid flow control.

In vitro models developed to study large intestinal digestion typically also consider the previous stages of digestion (oral, gastric, and small intestinal). Example models are the Spanish computer-controlled multicompartmental dynamic model of the gastrointestinal system (SIMGI) (Barroso, Cueva, Peláez, Martínez-Cuesta, & Requena, 2015) and the Belgian simulator of the human intestinal microbial ecosystem (SHIME) (Van de Wiele, Van den Abbeele, Ossieur, Possemiers, & Marzorati, 2015) used to study fermentation processes in the colon (see Table 2).

Multicompartmental digestive models to study combined digestive processes have been developed (examples shown in Table 2). The TNO's TIM1 (gastric and small intestinal digestion) and TIM2 (large intestinal digestion) are commercially available (for details see chapter "Influence of physical and structural aspects of food on starch digestion").

3.2 What Is Being Measured?

Typically, in vitro digestion models determine breakdown and digestibility of food materials. Measurements that determine mechanical breakdown, hydrolysis of macronutrients, release of compounds, and bioaccessibility of nutrients are often

selected to quantify digestion. However, other analytical methods have been combined with in vitro digestive systems, including in situ scattering techniques such as small-angle X-ray scattering (SAXS) or neutron scattering, nuclear magnetic resonance, mass spectrometry, and techniques studying the effect of interfacial features on digestion. These have been reviewed (Marze, 2017) and will not be extensively regarded in this chapter.

3.3 In Vitro Studies: An Application-Specific Methodology

It is important to keep in mind that in vitro models are application specific. Overall, the large number of in vitro models developed in the last decades indicate the challenges involved in replicating digestive processes outside the body. The continuing efforts to understand digestion in vitro are expected to increase in the forthcoming years, in line with the efforts to reduce the need of extensive in vivo studies. However, due to the complexity of the physiological processes that are involved in digestion, it is important to understand the limitations of each in vitro model. Model selection and implementation of acquired data should therefore be treated with care (Bidlack et al., 2009).

4 In Silico Methods

Simulating digestion using numerical/computational methods can provide insight to the processes involved and mechanistic understanding of the digestion steps. In silico models may further be used as predictive tools in digestion, for example to estimate digestibility or gastric emptying, by extrapolating existing data within the model's boundaries.

This section provides an overview of the current state of in silico modelling of the human digestive system. It will primarily focus on the gastric and small intestinal regions of the gastrointestinal tract. It will further focus on how the formulation of a meal and the body's physiological responses can influence the gastric emptying and ultimately the nutrient absorption profile of a consumed food.

4.1 The Stomach

The stomach serves a variety of purposes when a meal has been consumed, these can be broken into four categories (Barrett, 2005):

- Breakdown of solid food particles through the contractions of the gastric wall.
- Breakdown of food chemically via the action of enzymatic hydrolysis.

- Act as a reservoir to store food prior to further processing.
- Control the rate at which food is emptied to the duodenum through contractions of the pyloric sphincter.

4.1.1 Gastric Emptying

Most models for the gastric emptying of meals fit experimental data to empirical models to try and characterise the emptying, and express it in simple terms such as the half time ($t_{1/2}$), which is the time for half of the original meal content to have been emptied from the stomach. The simplest model for gastric emptying is the exponential emptying curve, expressed mathematically as the following ODE (Hellström, Grybäck, & Jacobsson, 2006):

$$\frac{\partial V}{\partial t} = -\gamma V \tag{1}$$

where V is the volume of meal remaining in the stomach, t is the time since consumption, and γ is the rate of gastric emptying, which can be expressed as the half time:

$$t_{1/2} = \frac{\ln(2)}{\gamma} \tag{2}$$

By setting the initial conditions, that is, volume consumed at time zero (V_0), Eq. (1) can be analytically solved:

$$V = V_0 \exp\left(-\frac{\ln(2)}{t_{1/2}} t\right) \tag{3}$$

The emptying curve produced by Eq. (1) is shown in Fig. 2 over a normalised time period. The emptying begins at a faster rate, but slows as time progresses and the overall volume of the meal remaining in the stomach is reduced. We can therefore link the gastric emptying to the volume of meal in the stomach, which has been viewed in vivo by a number of authors (Brener, Hendrix, & McHugh, 1983; McHugh, 1983; McHugh & Moran, 1979).

This type of emptying pattern is generally seen with liquid meals, but solid meals, which require breakdown prior to emptying, usually show a lag phase (a period with low rate of emptying before a faster rate initiates). For these cases, other empirical approaches have been taken. The first requires an additional factor k, which is defined as a shape factor (Kong & Singh, 2008a; Siegel et al., 1988):

$$V = V_0 - V_0\left(1 - \exp(-\gamma t)\right)^k \tag{4}$$

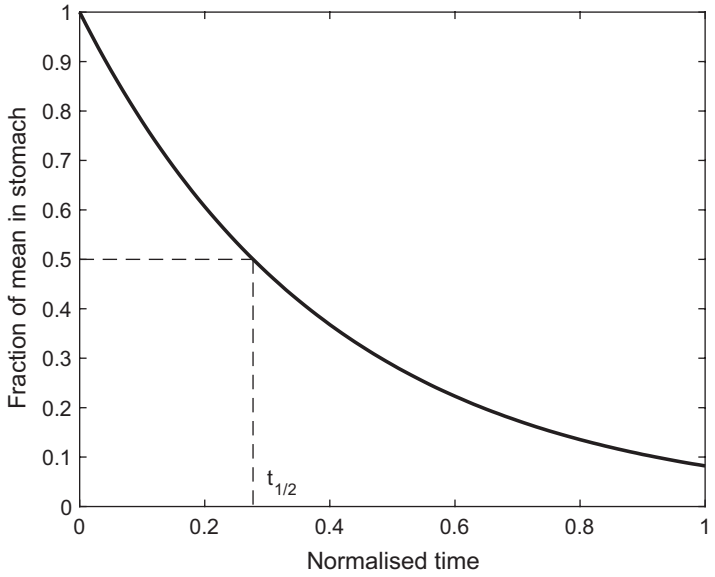

Fig. 2 Exponential emptying plot from the stomach, highlighting the time at which half the gastric content is emptied

Another alternative is the delayed sigmoidal model (Eq. 5) utilised to describe the emptying of solids (Kong & Singh, 2009), where k is used here to describe the lag phase. The curves for Eqs. (4) and (5) are shown in Fig. 3.

$$V = V_0\left(1 + k\gamma t\right)\exp\left(-\gamma t\right) \tag{5}$$

Equation (4) (Siegel model) shows an initial lag phase, common when looking at the emptying of the solid portion of a meal (Hellström et al., 2006; Kong & Singh, 2008a). The half time of emptying for Eq. (4) is expressed by Eq. (6) (Kong & Singh, 2008a).

$$t_{1/2} = \left(-\frac{1}{\gamma}\right)\ln\left(1 - 0.5^{1/k}\right) \tag{6}$$

The delayed sigmoidal model (Eq. 5) allows for the effect of secretions upon the stomach to be considered. Meals with high-viscosity liquids (Marciani et al., 2000) or high solid content (Kong & Singh, 2009), will stimulate higher gastric secretion rates. This may be due to increased stimulation of stretch receptors in the stomach (Marciani et al., 2000). Due to these secretions the curve initially increases, with extra volume in the stomach compared to the initial meal volume. After processing [disintegration of solid particles or reduction in viscosity of high-viscosity meals (Marciani et al., 2000)] the emptying begins in a similar pattern to other models,

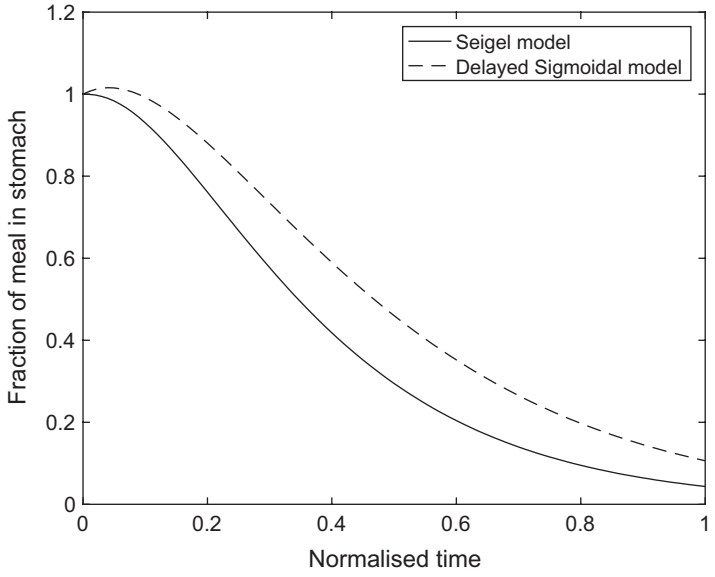

Fig. 3 Graph showing gastric content for two empirical gastric emptying models (Eqs. 4 and 5)

faster initially but slowing down as the fraction of meal remaining in the stomach is reduced.

The empirical models developed do not provide any predictive ability, but allow for in vivo data to be classified. However, one aim of producing mathematical models is to allow for predictions of how a meal will behave postprandially, and as such allow for more efficient design of meals that will have certain desirable traits.

Dalla Man, Camilleri, and Cobelli (2006) assumed that the stomach could be described as two compartments. The first accounts for the solid portion of the meal, containing mass M_{sto1}, the second accounts for the liquid portion of the meal of mass M_{sto2}, and this is the portion which can empty (Dalla Man et al., 2006).

$$\frac{\partial M_{sto1}}{\partial t} = -k_{12} M_{sto1} + D\delta(t) \tag{7}$$

$$\frac{\partial M_{sto1}}{\partial t} = -k_{empt} M_{sto2} + k_{12} M_{sto1} \tag{8}$$

$$M_{stom} = M_{sto1} + M_{sto2} \tag{9}$$

Equations (7)–(9) describe how the mass of both compartments changes with time (t), the input of initial meal is into the first compartment, with an initial mass D, where $\delta(t)$ is the dirac delta function (this will give an input at $t = 0$). The movement from compartment 1 to compartment 2 will be controlled by the rate constant k_{12}, which can be thought of as a grinding term from the solid portion of the meal.

The emptying from the second compartment will be at rate k_{empt}; this rate was linked to the remaining mass in the stomach and the initial mass (D) via the following equation:

$$k_{empt} = k_{min} + \frac{k_{max} - k_{min}}{2}[\tanh\left(\alpha\left(M_{stom} - bD\right)\right) - \tanh\left(\beta\left(M_{stom} - cD\right)\right) + 2] \quad (10)$$

Equation (10) introduces a number of parameters that require estimates. k_{min} and k_{max} are the minimum and maximum rates of emptying, respectively. Parameters b and c are fractions of a meal in the stomach, b being the fraction at which the rate is at $-(k_{max} - k_{min})/2$ and c when the rate is at $(k_{max} - k_{min})/2$. The additional parameters are defined as follows:

$$\alpha = \frac{5}{2D\left(1-b\right)} \quad (11)$$

$$\beta = \frac{5}{2Dc} \quad (12)$$

Equations (10)–(12) are used with the parameters defined in Dalla Man et al. (2006) to give the plots of the fraction of meal remaining in the stomach postprandially. Results are shown in Fig. 4a, b, where the gastric contents after consumption of an Oral Glucose Tolerance Test (OGTT, comprising a drink with 75 g of dissolved glucose, Fig. 4a) and a meal (containing 45% carbohydrates, 15% protein, and 40% fats in a predominately solid form, with a low-nutrient liquid portion also included, Fig. 4b) are plotted against time postprandially. Figure 4a shows that the OGTT meal empties initially quickly, followed by a slower linear emptying. This is expected for liquid meals containing nutrients, where initial rapid emptying is followed by a controlled rate of emptying due to the feedback mechanism observed in vivo (Brener et al., 1983; Calbet & MacLean, 1997; McHugh, 1983). However, Fig. 4b shows that the mixed meal behaves slightly differently. One would expect a long initial lag period before emptying begins, as the solid portion of the meal undergoes size reduction in the stomach, to ensure particles are below the 1–2 mm diameter threshold (Kong & Singh, 2008a) before they can pass through the pyloric sphincter and exit the stomach to the proximal small intestine. It appears that the plot of gastric content tracks both the liquid and solid fraction of the meal, with the liquid portion emptying independently of the solid portion (Marciani et al., 2012). As a result, there is an initial rapid emptying rate of the low-nutrient liquid. This is higher than the initial emptying rate of the high-nutrient OGTT liquid, reflecting the feedback mechanism that controls emptying nutrients to the small intestine. When a large portion of the liquid has emptied, and the solid has been reduced in particle size sufficiently to pass through the pylorus, a much slower emptying rate of the solid meal is observed. Figure 4c, d refer to the intestinal phase of digestion and will be discussed in Sect. 4.2.

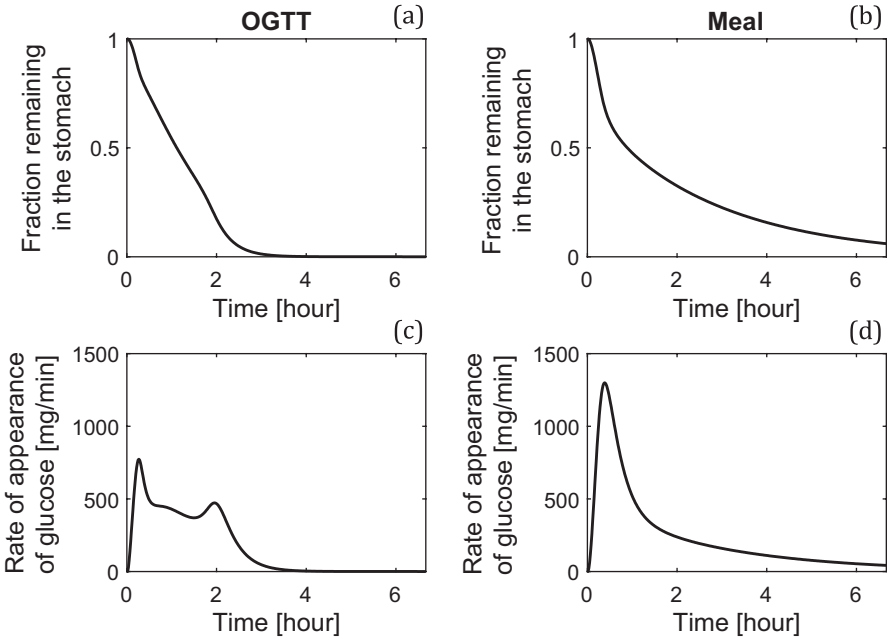

Fig. 4 Plot of Dalla Man et al. (2006) model (Dalla Man et al., 2006), the top two plots show the fraction of a meal remaining in the stomach postprandial for a Oral Glucose Tolerance Test (OGTT) and for a model meal, under these plots are the corresponding plots of rate of glucose appearance in the plasma [adapted from Dalla Man et al. (Marteau et al., 1997)]

4.1.2 Gastric Secretions

When modelling the gastric secretions one can either study the total gastric secretions in relation to the consumption of a meal (Marciani et al., 2001; Sauter et al., 2012), or take a more intricate approach and analyse the secretions on a cellular level (Joseph, Zavros, Merchant, & Kirschner, 2003; Marino, Ganguli, Joseph, & Kirschner, 2003). Only the first approach will be presented here. This generally involves linking in vivo measurements and empirical models developed to describe the change in volume in the stomach with the gastric emptying and secretions. Marciani et al. (2000, 2001) have studied in vivo the effect of gastric viscosity upon the secretion rate. Low and high-viscosity meals with low or high nutrient content were administered to volunteers and the postprandial gastric volume was measured (Marciani et al., 2001). A model that links the secretion rates to the volume of meal and a basal secretion rate, described by Eqs. (13) and (14), was compared with experimental data.

$$\frac{\partial V_m}{\partial t} = -pV_m \tag{13}$$

$$\frac{\partial V_s}{\partial t} = kV_m + S_0 - pV_s \tag{14}$$

where V is the volume at time t, of secretions (s) or meal (m), k is the secretion rate, p is the gastric emptying rate and S_0 the basal rate of secretions.

This model provided results similar to the experimental measurements when the parameters were fitted, but it does not take into account the effect of viscosity on the secretion and emptying nor other factors of the meal properties. The viscosity was shown to have a major influence upon the rate of secretions when the same group was fed with non-nutrient meals of varying viscosities (Marciani et al., 2000). The secretions, on the other end, reduced the gastric viscosity to manageable levels over short periods of time (a meal of viscosity 11 Pa s was reduced to 0.3 Pa s over 40 min).

Sauter et al. (2012) proposed two approaches to modelling. The first is similar to that of Marciani et al.; however, Sauter et al. assumed that the secretions were not affected by the meal and they were a function of the maximum secretion volume. The second model linked the meal and secretion volumes, giving two coupled ODEs (Eqs. 15 and 16):

$$\frac{\partial V_{\mathrm{m}}}{\partial t} = -kV_{\mathrm{m}} - k_{\mathrm{ms}}V_{\mathrm{m}} + k_{\mathrm{sm}}V_{\mathrm{s}} \tag{15}$$

$$\frac{\partial V_{\mathrm{s}}}{\partial t} = -k_{\mathrm{sm}}V_{\mathrm{s}} + k_{\mathrm{ms}}V_{\mathrm{m}} \tag{16}$$

where V is the volume of secretion (s) or meal (m), k is the rate of emptying, k_{ms} is a rate constant representing the effect that the meal has upon the secretion rate, and k_{sm} is a rate constant representing the effect that the secretions have upon delaying the gastric emptying. The authors further proposed a dimensionless term eff_{m} ($=k_{\mathrm{ms}}/(k+k_{\mathrm{ms}})$), which represents the efficiency of a meal at stimulating secretions, taking a number between 0 and 1.

This model was fitted to experimental data for a high-nutrient viscous meal (chocolate drink). It was found that the secreted volume over the 120 min of measurement was around 48–74% of the original meal volume, and that in this case the rate constant k_{ms} was around 2.3 times larger than the rate constant k_{sm}, indicating that the viscous meal influenced the secretion of gastric fluids to a greater extent than the gastric secretions inhibited the gastric emptying rate.

Moxon et al. (2017) linked the gastric secretion rate to the viscosity of the gastric chyme, taking into account that the secretions also had the effect of reducing the viscosity of the chyme. The secretion rate (K_{sec}) was defined as follows (Eq. 17):

$$K_{\mathrm{sec}} = \lambda_{\mathrm{s}}\mu^{\mathrm{b}} + S_{\mathrm{b}} \tag{17}$$

where μ is the viscosity of the gastric chyme, S_{b} is the basal secretion rate, and λ_{S} and b are constants linking the rate of secretion to the viscosity. The viscosity was

defined as a function of the concentration of a thickening agent; for the experimental data used in the paper Locust Bean Gum (LBG) was utilised as the thickening agent, and the following (Eq. 18) relationship was found:

$$\mu = 2C_{\mathrm{LBG}}^{4.21} \tag{18}$$

where C_{LBG} is the concentration of the LBG in the stomach.

Three ODEs were then defined (Eq. 19–21), one for the mass of nutrient in the stomach, one for the mass of non-nutrient liquid in the stomach, and one for the mass of thickener in the stomach:

$$\frac{\partial m_{\mathrm{N}}}{\partial t} = -\gamma m_{\mathrm{N}} \tag{19}$$

$$\frac{\partial m_{\mathrm{liq}}}{\partial t} = K_{\mathrm{sec}} - \gamma m_{\mathrm{liq}} \tag{20}$$

$$\frac{\partial m_{\mathrm{LBG}}}{\partial t} = -\gamma m_{\mathrm{LBG}} \tag{21}$$

Thus, the total mass in the stomach was defined as in Eq. (22):

$$m_{\mathrm{tot}} = m_{\mathrm{N}} + m_{\mathrm{liq}} + m_{\mathrm{LBG}} \tag{22}$$

The simulated results for a 11 Pa.s viscosity meal containing no nutrients are shown in Fig. 5. The simulated results were fitted to experimental data (Marciani et al., 2000), and highlight how large reduction in the chyme viscosity can be achieved through gastric secretions. It is anticipated that this will have a major impact on the mass transfer of nutrients in the intestine and influence the absorption rate of nutrients, which has been highlighted in-silico and during in vitro experimentation (Gouseti et al., 2014; Moxon et al., 2017; Moxon, Gouseti, & Bakalis, 2016).

4.2 The Small Intestine

Numerous authors have described the mass transfer and absorption of nutrients and/or drug compounds from the small intestine mathematically. The underlying assumption is that the mammalian digestive system can be described as a series of ideal reactor systems (Penry & Jumars, 1986; Penry & Jumars, 1987). Building upon this assumption, three types of systems are generally utilised to describe the small intestine: a single continuous stirred tank reactor (CSTR) (Dalla Man et al., 2006; Di Muria, Lamberti, & Titomanlio, 2010), multiple CSTRs in series (Bastianelli, Sauvant, & Rérat, 1996; Jumars, 2000; Yu & Amidon, 1999; Yu, Crison, & Amidon, 1996), or a plug flow reactor (PFR) (Logan, Joern, & Wolesensky, 2002;

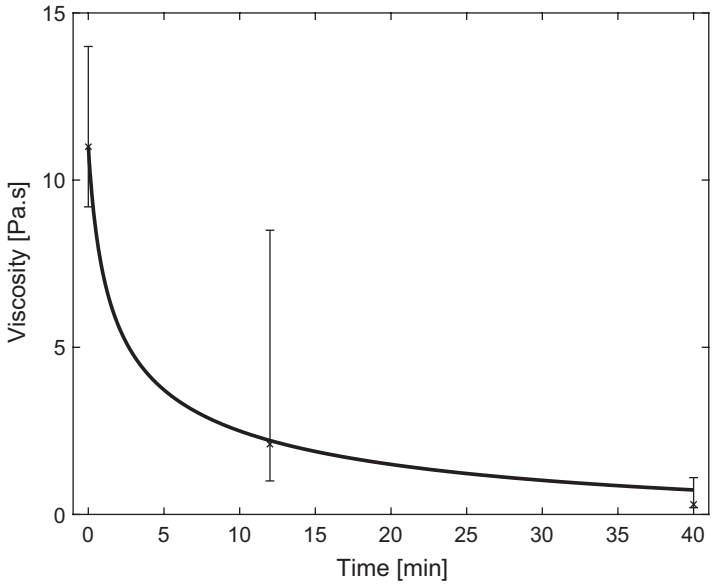

Fig. 5 Reduction in gastric chyme viscosity due to effect of secretions, modified from Moxon et al. (2017), experimental data from Marciani et al. (2000)

Ni, Ho, Fox, Leuenberger, & Higuchi, 1980; Stoll, Batycky, Leipold, Milstein, & Edwards, 2000). Figure 6 shows the different intestinal schematics for each set-up. Some of the models produced look only at the transit and absorption of nutrients in the small intestine (Logan et al., 2002), while others couple the model with gastric emptying (Dalla Man et al., 2006; Moxon et al., 2016), or with the nutrient/drug dynamics in the body after absorption (Dalla Man et al., 2006; Stoll et al., 2000; Yu & Amidon, 1999), or look at the whole digestive system from consumption to excretion (Bastianelli et al., 1996).

One of the simplest models for the small intestine was utilised by Dalla Man et al. (2006). It was assumed that the small intestine can be described as a single CSTR, with absorption being modelled as a first order reaction term, giving the following Eq. (23) (Dalla Man et al., 2006):

$$\frac{\partial m_{\mathrm{SI}}}{\partial t} = -k_{\mathrm{abs}} m_{\mathrm{SI}} + G_{\mathrm{empt}} \tag{23}$$

where m_{SI} is the mass of meal in the small intestine, k_{abs} is the absorption rate, and G_{empt} is the rate of gastric emptying [calculated from Eqs. (5)–(12)]. Rather than validating the intestinal model against blood glucose data, the authors considered a novel technique to quantify the rate of glucose appearance in the plasma near the gut, utilising multiple tracer compounds (Dalla Man et al., 2005). The rate of appearance of glucose in the plasma (Ra) was defined as the absorption rate multi-

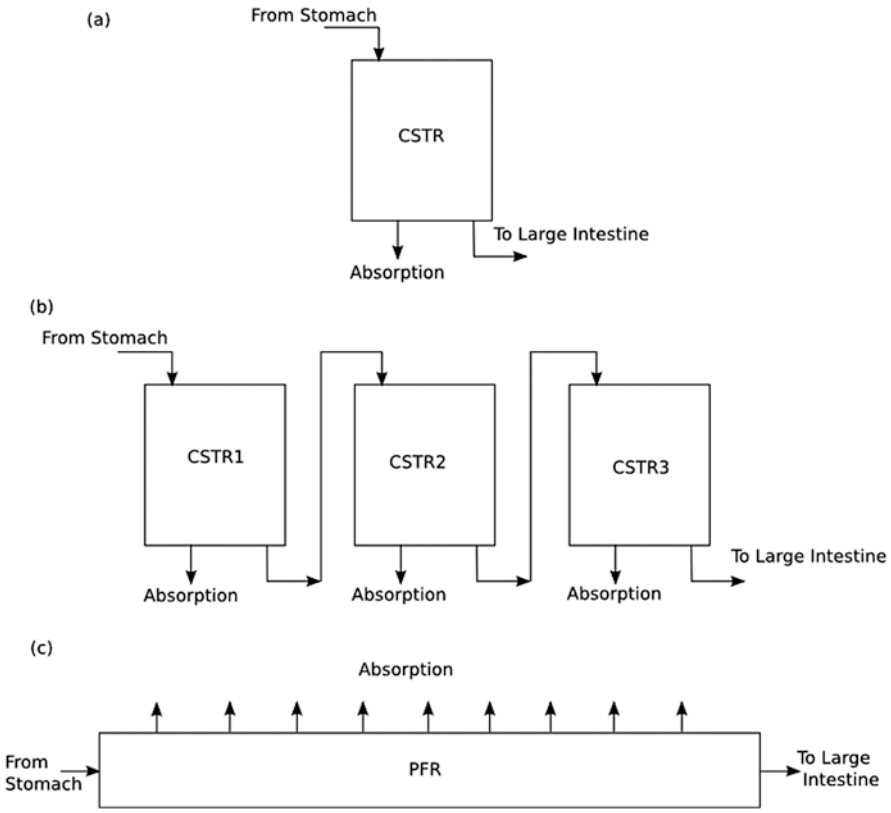

Fig. 6 Schematic of different reactor designs used to describe the small intestine. (**a**) is a single CSTR design, (**b**) multiple CSTRs in series, (**c**) is a PFR set-up

plied by a scaling factor, f, which is a fraction of the total mass of a meal nutrient that will be absorbed. This factor was set to 0.89 in the current model.

$$\mathrm{Ra} = fk_{\mathrm{abs}}m_{\mathrm{SI}} \tag{24}$$

The curves for two meals (oral glucose tolerance test, OGGT, and solid meal) are shown below the corresponding gastric content curves in Fig. 4. The rate of appearance of glucose after the consumption of a OGTT meal shows an initial high peak before dropping to a lower rate, which can be explained by the initial rapid emptying period of the model followed by a more linear, slower emptying. The mixed meal showed a different response. After an initial peak there is a major drop followed by a lower rate of absorption that is steadily dropping over a period of around 4 h. Overall, Dala Man et al.'s models gave a good fit to the averages of the rate of appearances and the measurements for each individual, though they require a large number of parameters to be estimated, and they are not able to describe the intesti-

nal transit time well due to the use of a single CSTR assumption for gastric digestion, as highlighted in the work by Yu et al. (1996).

Di Muria et al. (2010) chose to use one compartment to describe the small intestine in a whole body model for the absorption of zinc sulphate in rats. Along with the single intestinal compartment, six other compartments were defined to describe the distribution of the drug in the body: the stomach, large intestine, gastrointestinal circulatory system, liver, plasma, and tissue. In this model, the change in drug concentration in the small intestine was described as in Eq. (25):

$$\frac{\partial A_{\mathrm{SIL}}}{\partial t} = S\frac{\partial r}{\partial t} - J_{\mathrm{SIL}} - k_{\mathrm{ELSIL}} A_{\mathrm{SIL}} \tag{25}$$

where A_{SIL} is the mass of drug in the small intestine, the first term on the right handside, $S\frac{\partial r}{\partial t}$, describes the dissolution of the drug in the small intestine, J_{SIL} describes the mass transfer between the small intestine and the gastrointestinal circulatory system, and the final term describes the elimination of the drug from the small intestine. The mass transfer between the small intestine and the circulatory system was further defined using a first order mass transfer equation (Eq. 26) with a concentration driving force:

$$J_{\mathrm{SIL}} = k_{\mathrm{a}} V_{\mathrm{B}} \left(\frac{A_{\mathrm{SIL}}}{V_{\mathrm{B}}} - \frac{C_{\mathrm{GICS}}}{R_{\mathrm{GICS}}} \right) \tag{26}$$

where k_{a} is the rate of absorption, V_{B} is the volume of the bolus containing the drug in the intestinal tract, C_{GICS} is the concentration of the drug in the gastrointestinal circulatory system, and R_{GICS} is the drug partition coefficient. The formulation of equations for other compartments is not discussed here but can be found in Di Muria et al. (2010).

This model provides a good fit with experimental data, with only five parameters requiring estimation and an additional determination of the variable r (in Eq. 25) to describe the dissolution of the drug compound in the stomach, small intestine, and large intestine. Information to determine r in this model requires in vitro data [however, some methods for modelling the dissolution of drug compounds are discussed by Sugano (2009)]. Di Muria et al. (2010) applied their model to the drug Diltiazem from results on human oral consumption. Two parameters needed to be estimated from the in vivo data, the elimination of the drug from the plasma, and the distribution volume of the drug in the plasma. Three different formulations were utilised, these provided slow, medium, and high release rates for the diltiazem compound. The models described the general profile of drug concentration in the blood plasma, but underestimated the peak in both fast and slow release formulations, and overestimated the clearance from the circulatory system during the terminal phase of the profile.

Yu et al. (1996) analysed how the different types of reactor systems used to describe the small intestine affect the transit time, assuming that a drug does not

degrade or get absorbed. For continuous stirred tank reactors, the Eq. (27) was used to describe the percentage of drug in each compartment:

$$\frac{\partial Y_n}{\partial \tau} = Y_{n-1} - Y_n, \qquad n = 1,2,\ldots N \tag{27}$$

The amount of drug leaving the small intestine was described as in Eq. (28):

$$\frac{\partial Y_{\text{si}}}{\partial \tau} = Y_N \tag{28}$$

where $\tau = K_t t$, and K_t is the transit rate constant.

For the PFR assumption, a diffusion–convection equation (29) was further used:

$$\frac{\partial C}{\partial t} = \alpha \frac{\partial^2 C}{\partial z^2} - \nu \frac{\partial C}{\partial z} \tag{29}$$

where C is the concentration at a point z along the intestine and at time t, α is the diffusion coefficient, and ν is the axial velocity along the length of the small intestine.

These models were used to calculate the mean transit time along the small intestine, and compared to experimental data for the rate of appearance in the colon. The experimental data showed a mean small intestinal transit time of 199 min and a 95% confidence interval of 7 min, with a minimum transit time of 30 min and a maximum of 570 min, while the gathered data showed neither a Gaussian nor a lognormal distribution (Yu et al., 1996). The single compartment model showed a poor fit to the experimental distribution, with a SSE (sum of squared error) of 3542, much higher than the multiple compartment approach or the PFR approach.

For the multicompartment approach, the total number of compartments was varied to analyse the effect. Five, seven, and nine compartment models were simulated giving SSE of 79, 8, and 52, respectively. The seven compartment model gave the best fit, with the authors (Yu et al., 1996) rationalising this physiologically by stating that the first compartment represents the duodenum and proximal jejunum, the second and third compartment represent the mid jejunum and distal jejunum, respectively, and the rest of the compartments represent the ileum. Fitting the diffusion coefficient, the PFR was found to have a SSE of 20, giving a better fit to the experimental data than the single, five, and nine compartment models, but slightly worse than the seven-compartment model. The author chose the multicompartment model approach as the best when describing the intestinal transit, due to (1) the large reduction in SSE between the multicompartment and single compartment model, and (2) the fact that the multicompartment approach is simpler mathematically than the PFR model, though it should be noted that the work was published in 1996 and the complexity around solving the PDEs for the PFR model has since been reduced by the increased speed of modern computation.

According to the approach by Bastianelli et al. (1996) the small intestine is considered as two compartments [smaller than the recommended seven mentioned in the previous paragraph (Yu et al., 1996)] that are linked with one compartment for the stomach and another for the large intestine, giving a total of four compartments. All four compartments have an input of endogenous secretions, the three intestinal compartments have an additional output representing the absorption of nutrients, and the large intestinal compartment has a further additional output to represent faeces elimination. The model looks at all components of a meal given to a pig. The components in the initial feed are protein, starch, sugars, digestible cell wall, lipids, and minerals. These are hydrolysed to absorbable compounds in the different compartments (e.g. starch to sugars, and proteins to amino acids), and these absorbable compounds are absorbed via Michaelis–Menten kinetics.

Results of this model were compared to experimental data for the absorption rate of glucose, amino acids, and volatile fatty acids. The simulation underestimated the absorption rate of all three nutrients, and greater underestimation was seen with amino acids and fatty acids. The authors highlighted the need for advanced in vitro models to better understand the digestibility of a meal, so that parameters could be predicted more accurately (such as kinetics of the nutrients being absorbed). It is noted that since the publication a number of methodologies have been identified to study the in vitro digestion of food and elucidate some of these processes and parameter values, though the detailed understanding and in vitro representation of digestive processes is still not complete (see previous section). They also pointed out the added benefits of utilising a PFR style model for the intestine, which allows for the effects of viscosity and peristaltic propulsion and mixing to be included. There have been studies by later authors (Moxon et al., 2016; Taghipoor, Barles, Georgelin, Licois, & Lescoat, 2014; Taghipoor, Lescoat, Licois, Georgelin, & Barles, 2012) to look at the effect of viscosity or peristaltic waves upon absorption, but the effect of local mixing (due to segmentation waves) has not been included into models looking at absorption, though have been studied from a fluid mechanic perspective (Ferrua & Singh, 2011; Kozu et al., 2010; Love, Lentle, Asvarujanon, Hemar, & Stafford, 2012), to understand the mixing effect the gastric and intestinal wall contraction can have.

Yu and Amidon (1999) built on previous work from the group (Yu et al., 1996) by using the seven compartmental approach developed in the 1996 paper and adding two more compartments to represent the stomach and colon to look at the passage and absorption of drugs after oral consumption. It was assumed that the drug emptied exponentially from the stomach to the first intestinal compartment, and that the passage from one intestinal compartment to the next and the absorption from each compartment were both via first order kinetics (Yu & Amidon, 1999). Thus, the following Eqs. (30–32) were presented (compared to Eqs.19–21):

$$\frac{\partial m_s}{\partial t} = -k_s m_s \tag{30}$$

$$\frac{\partial m_n}{\partial t} = k_t m_{n-1} - k_t m_n - k_a m_n, n = 1,2,\ldots,7 \tag{31}$$

$$\frac{\partial m_c}{\partial t} = k_t m_7 \tag{32}$$

where m is the mass in the different compartments, and subscripts s is the stomach, c is the colon, and n is the small intestine; k_a is the absorption rate, k_t is the transfer rate between compartments, and k_s is the emptying rate from the stomach. The rate of absorption was taken as the effective permeability and data from literature was gathered for multiple drug compounds. To fit the simulated data to that of concentrations gathered from in vivo data, a model for the distribution of drug concentration in the plasma, which included one central compartment and two peripheral compartments, was taken from literature (Mason, Winer, Kochak, Cohen, & Bell, 1979).

The models gave good fit to experimental data, using an exponential as well as a biphasic emptying rate from the stomach. The biphasic emptying gave the best fit, though it resulted in around 77% of the drug fraction being emptied in the first phase of emptying, followed by a 2-h period in which no emptying occurred, before the rest of the drug emptied. As pointed out by the authors, this is something that it is unlikely to occur in vivo (Yu & Amidon, 1999).

A model developed by Moxon et al. (2016) linked the absorption rate to the viscosity of the intestinal chyme. The model assumed the intestine could be described as a plug flow reactor and used an advection–reaction equation to describe how the mass of nutrient varied along the length of the intestine (Eqs. 33):

$$\frac{\partial m_{SI}}{\partial t} = \begin{cases} \gamma m_{stom} - \bar{u}\frac{\partial m_{SI}}{\partial z} - \frac{2f}{r_m} K m_{SI}, & \text{if } z = l_0 \\ -\bar{u}\frac{\partial m_{SI}}{\partial z} - \frac{2f}{r_m} K m_{SI}, & \text{otherwise} \end{cases} \tag{33}$$

where m_{SI} is the mass in the small intestine, which varies with distance along the length z ($\in[0, L]$, where L is the total length of the intestine), and with time t. γm_{stom} represents the rate of emptying from the stomach into the intestine, which occurs at a distance l_0 along the intestine (l_0 is the radius of a bolus entering), and $\bar{u}$ is the velocity of the intestinal content along the length of the intestine. An exponential equation was assumed for the gastric content, as defined in Eq. (1). The final term ($\frac{2f}{r_m} K m_{SI}$) describes the absorption of the nutrients, where f is the increase in surface area due to protrusions (e.g. villi and microvilli), $2/r_m$ represents the surface area to volume ratio of the intestine, and K is the absorption rate constant. K was further linked to the convective mass transfer coefficient of the intestinal chyme, through the relationship between Sherwood, Schmidt, and Reynolds numbers, which gave the following definition (Eq. 34) of the mass transfer coefficient (Moxon et al., 2016):

$$K = 1.62\left(\frac{\bar{u}D^2}{Ld}\right) \tag{34}$$

where D is the diffusivity of the nutrient calculated from the Einstein–Stokes equation, and d is the diameter of the intestine.

Two dimensionless terms, the characteristic time of mass transfer, τ_{transfer}, and the characteristic time of gastric emptying, τ_{empty}, were defined (Eqs. 35 and 36) (Moxon et al., 2016):

$$\tau_{\text{transfer}} = \frac{2fK}{r_m}\frac{L}{\bar{u}} \tag{35}$$

$$\tau_{\text{empty}} = \gamma\frac{L}{\bar{u}} \tag{36}$$

In the Plot in Fig. 7, showing the relationship between the two dimensionless characteristic times, two regions are identified. The bottom region (below the black diagonal line) is controlled mainly by the emptying rate, where increasing the characteristic emptying time results in an increase in absorption of nutrients, but changes in the mass transfer rate do not have a major effect. In the top region (above the black diagonal line), the limiting factor is the mass transfer rate, and changes in gastric emptying rate do not seem to have a large effect.

In reality, the two factors are likely to be linked. Work by numerous authors (Brener et al., 1983; Calbet & MacLean, 1997; McHugh, 1983) has shown a nutri-

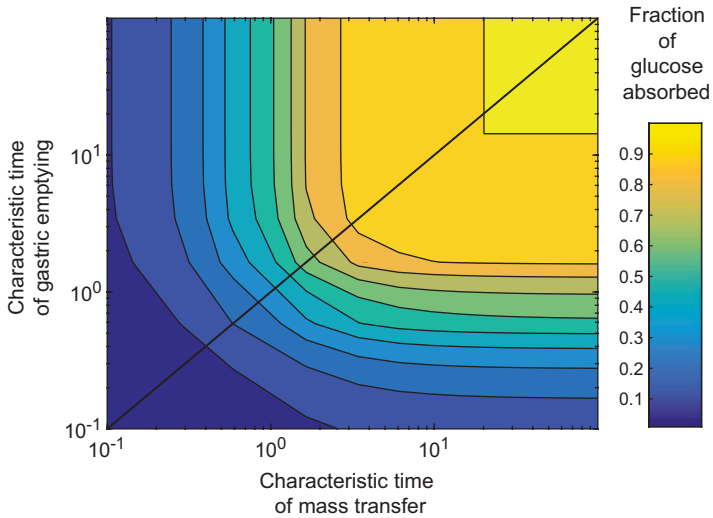

Fig. 7 Contour plot highlighting the effect of characteristic emptying time and characteristic mass transfer time upon the absorption of glucose over a 3-h period, adapted from Moxon et al. (2016)

ent based feedback mechanism that links the bioaccessibility of nutrients within the proximal small intestine to the gastric emptying rate. As such, high characteristic mass transfer rates would likely have the effect of slowing down the gastric emptying rate, thus reducing the amount of glucose in the intestine and maintaining a lower absorption rate of nutrients.

This was approached in Moxon et al. (2017), where the gastric emptying rate was linked to the bioaccessibility of the nutrients in the small intestine, through an on/off type feedback control system, as expressed in Eq. (37):

$$\gamma = \begin{cases} 0, & \text{if } A > A_{\max} \\ \gamma_0, & \text{otherwise} \end{cases} \tag{37}$$

where γ is the gastric emptying rate, γ_0 is the initial gastric emptying rate, and the rate is zero if the bioaccessibility is greater than a maximum value ($A_{\max}$). The bioaccessibility can be defined from Eq. (38), where it will be equal to the reactive term of the equations.

$$A = \frac{2f}{r_m} K m_{\mathrm{SI}} \tag{38}$$

This gave good fits to experimental data for both high- and low-viscosity liquid meals with high- and low-nutrient content (Fig. 8), when the parameters K, $A_{\max}$, and γ_0 were fit. It can be seen that for the low-nutrient meals there is little difference in

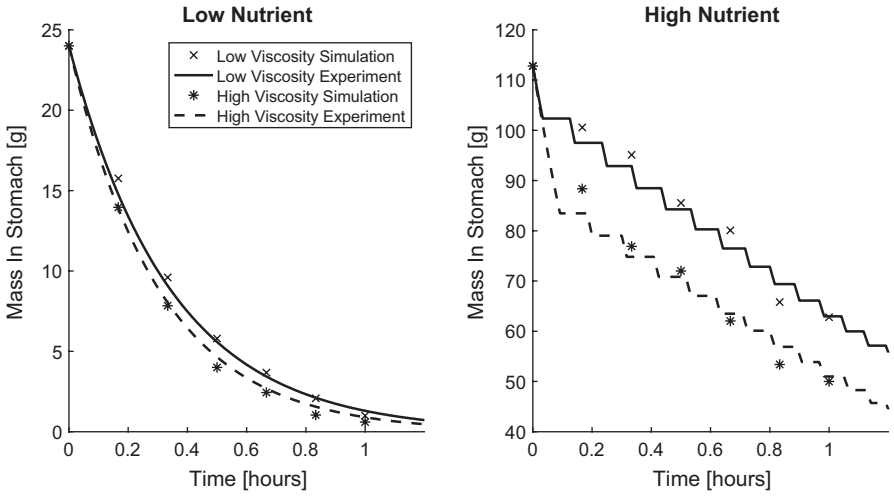

Fig. 8 Simulated (Moxon et al., 2017) and experimental data (Vist & Maughan, 1995) for the gastric retention profile of low-nutrient and high-nutrient meals at low and high viscosity. The simulated data highlight the presence of a nutrient-based feedback mechanism controlling the gastric emptying rate

the emptying rate between low- and high-viscosity meals, as confirmed by other in vivo experiments (Marciani et al., 2000). However, this is not the case of the high-nutrient meals. Here, there is a longer period of initial rapid emptying for the high-viscosity meal, compared to the low-viscosity one, prior to the initiation of the feedback mechanism. This can be attributed to the lower bioaccessibility of the nutrients in the high-viscosity meal, allowing for longer emptying times before any nutrients are detected in the small intestine signalling feedback for reduction in the gastric emptying rates.

This highlights the importance of the link between the mass transfer in the intestinal lumen and the gastric emptying rate. An additional complexity refers to the fact that the viscosity is not constant as previously demonstrated, and it will vary due to gastric and intestinal secretions. The mass transfer in the intestinal lumen will therefore vary over time and along the length of the intestine. This mass transfer will be influenced not only by the viscosity of the chyme, but also the intestinal wall contractions, something that has not been modelled so far when looking into the absorption of nutrients in silico. These contractions are expected to play a role by increasing the mass transfer rates, as demonstrated during in vitro experiments (Gouseti et al., 2014; Tharakan et al., 2010).

5 Conclusions

Studying digestive processes is challenging and research in this area is currently very active. The broad aspects of digestion, including the nature of the diet and the physiology of eating, pose fascinating questions that are yet to be understood before a complete, detailed understanding is achieved. Significant progress has been attained by studying digestion using in vivo, in vitro, and in silico approaches and this has led to improved dietary options and enhanced individual and public health. However, each methodology has its advantages and limitations that need to be taken into account when selecting an appropriate research approach and also when interpreting any acquired data. As this field of research is currently evolving, it is important to reflect on existing methodologies collectively and form the future of digestion studies according to the needs and gaps in the current practice.

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Part II
Considerations During Digestion Studies

Challenges in Quantifying Digestion

Robert Havenaar and Mans Minekus

1 Introduction

Eating habits are continuously changing, which is often related to new foods and food ingredients on the market. For example, due to the increasing demand for proteins, new sources of proteins are introduced on the market, such as proteins from insects, algae, and fish industry by-products. Another aspect that drives the production of new food products is new insights in nutritional quality in general or related to specific age groups, such as infants, the elderly, and people with disease conditions.

These changes give a continuous need for in vivo and in vitro studies to determine the quality of food products in terms of, among others, palatability, digestibility, and bioavailability of nutrients and/or functional compounds.

In this section we describe the challenges in qualifying digestion of food products and the bioavailability of nutrients and functional compounds in human and animal studies as well as in in vitro studies.

2 Challenges in Terminology

One of the first challenges is: do we speak the same language in food and nutrition research? It is important that the scientists in this field have the same perception and understanding of the terminology. So it is essential to use a standard type of "professional language" with uniform terminology and definitions. Different organizations have published guidelines on definitions and terminology. For example, the

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European Society for Clinical Nutrition and Metabolism (ESPEN; www.espen.org/education/espen-guidelines) appointed a Terminology Consensus Group in the field of clinical nutrition (Cederholm et al., 2017). Individual authors or institutes also publish research papers focused on stimulation of uniform terminology. In relation to digestion and bioavailability of food compounds it is important to have identical and consistent terminology, not only for terms as "in vivo bioavailability" versus "in vitro bioaccessibility" of nutrients (Fernandez-Garcia, Carvajal-Lerida, & Perez-Galvez, 2009), but also for definitions related to food compounds, such as "trans-fatty acids" (Wang & Proctor, 2013) and "dietary fiber" (Macagnan, Da Silva, & Hecktheuer, 2016; Miller Jones, 2014).

It is crucial to check these different guidelines on terminology and definitions for the specific food products and/or nutrients. We ought to use consequently the same terminology as far as possible or at least mention the origin of the definition used in the food digestion experiments.

3 Challenges in Analysis

Once we know which compounds are defined within a "definition" the next challenge is the standardization of the extraction and analytical method. Different organizations are involved in standardization of (bio)chemical analysis methods, such as the Food and Agriculture Organization (FAO) of the United Nations (www.fao.org/publications/en/) on food analysis in general and on food energy methods of analysis and conversion factors (FAO, 2003), the Association of Official Analytical Chemists (today: Communities; AOAC; www.aoac.org), and the European Commission for functional food ingredients (Buchgraber & Karaali, 2005). These organizations give information on (globally accepted) standardized analytical methods, including nutrients and active food ingredients. Approved methods related to specific food products are also available, such as those introduced by the American Association of Cereal Chemists (AACC[1] International; www.aaccnet.org). They offer descriptions of analytical methods for a broad variety of food compounds in cereal grains. The use of these approved methods in digestion studies will contribute to the standardization of experimental results.

To evaluate the quality of the analytical methods as used in your lab, it is possible to use reference materials for the calibration of your analysis instruments and to improve the reliability of the analytical results. Via the Institute for Reference Materials and Measurements (IRMM, ec.europa.eu/jrc/en/reference-materials) a catalogue with 800 different certified reference materials is freely available (IRMM, 2015).

[1] AACC also stands for American Association for Clinical Chemistry; a global scientific and medical professional organization dedicated to clinical laboratory science and its application to health care (www.aacc.org).

In case you want to validate your analytical method in a collaborative study, the AOAC International has guidelines available for the setup of these types of collaborative studies (AOAC International, 1995).

4 Challenges in Human Digestion Studies

Human clinical studies may be regarded as "the gold standard" for food digestion research. However, the performance of a human clinical study is a real challenge. For human intervention studies, to evaluate food digestion and quality, there are different general guidelines available, such as "scientific standards" for intervention trials and good clinical practice (GCP) by Woodside, Koletzko, Patterson, and Welch (2013) and Schmitt et al. (2012) or for evaluating health benefits of foods by Welch et al. (2011). These guidelines are mostly based on consensus by expert groups (e.g., ILSI Europe (Brussels, Belgium; ilsi.eu/task-forces/nutrition/)). Sometimes they are based on a review of methodologies, such as for analyzing the glycemic index in humans on the intake of carbohydrates by Brouns et al. (2005) and on energy metabolism in humans by Lam and Ravussin (2016).

A complicating factor in digestion experiments in humans is to follow exactly the digestion and bioavailability of a nutrient after oral intake. One of the techniques is the use of food compounds intrinsically labeled with stable isotopes, such as fatty acids (Ecker & Liebisch, 2014), proteins (Geboes et al., 2004), minerals (Abrams, 2003), or vitamins such as dietary carotenoids (Van Lieshout, West, & Van Breemen, 2003). Nevertheless, the collection of samples from human intervention studies is limited to, for example, blood, urine, and fecal samples. This may hamper the outcome of the studies.

The consequences of these intervention studies are that these studies are extremely expensive and time-consuming. Moreover, the pressure on ethical aspects is increasing, due to the rules for liability and corporate social responsibility. Based on the ethical principles for medical research involving human subjects (known as the Declaration of Helsinki) by the World Medical Association (www.wma.net/what-we-do/medical-ethics/), many (governmental) organizations have defined ethical rules for human intervention studies, such as the National Institute of Health (bioethics.nih.gov) and World Health Organization (who.int/ethics/research/en). These rules should at least be fulfilled for grant applications.

5 Challenges in Animal Digestion Studies

Animal studies are used already for a long time as model for human nutrition studies (Baker, 2008; Gallaher, 1992; Lovegrove, Hodson, Sharma, & Lanham-New, 2015), including neonatal nutrition (Puiman & Stoll, 2008), as animal models have species-specific possibilities and limitations. On the one hand, specific non-invasive

techniques such as the ^{13}C-labeled breath test (McCue & Welch, 2016) and invasive techniques such as fistulation (Swindle, Smith, & Goodrich, 1998), are available for animal studies, with legislative and ethical restrictions. On the other hand, there are challenges in the extrapolation of results to the human situation. For food digestion studies (e.g., protein quality assessment) pigs and rats are advised as animal models (FAO, 2013). However, it was found that the true ileal protein and amino acid digestibility in pigs was significantly lower than that in humans (Deglaire, Bos, Tomé, & Moughan, 2009; Rowan, Moughan, Wilson, Maher, & Tasman-Jones, 1994). The predictive quality of digestion experiment in rats showed a correlation coefficient of only 0.46 (Bodwell, Satterlee, & Hackler, 1980). The reason for discrepancy between results from human versus animal studies is the difference in gastrointestinal physiology. For example, the gastric pH and gastric emptying time can be drastically different between animal species and humans. This makes the selection of the animal species for digestion studies and the interpretation of results a real challenge (e.g., Fuller & Tomé, 2005).

Laboratory animals are also protected by legislation and guidelines, in Europe for example by Directive 2010/63/EU (http://ec.europa.eu/environment/chemicals/lab_animals/legislation_en.htm) and in USA by NIH guidelines (8th edition, 2010; https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals_prepub.pdf). In the UK they developed guidelines to improve the reporting of research using animals, aiming to maximize the published information and to minimize unnecessary animal studies (Animal Research: Report of In Vivo Experiments; ARRIVE; https://www.nc3rs.org.uk/arrive-guidelines).

6 Challenges in In Vitro Digestion Studies

In vitro digestion studies have been and still are performed in a broad range of digestion methods and models, from simple static beaker experiments (Babinszky, Van der Meer, Boer, & den Hartog, 1990) to highly sophisticated dynamic, computer-controlled gastrointestinal models (Bellmann, Lelieveld, Gorissen, Minekus, & Havenaar, 2016; Minekus, Marteau, Havenaar, & Huis in 't Veld, 1995).

Various review papers describe the differences between models and methods in relation to food digestion and measuring the availability for intestinal absorption of nutrients (bioaccessibility), such as for adults (Alminger et al., 2014; Guerra et al., 2012; Ting, Zhao, Xia, & Huang, 2015; Verhoeckx, 2015; Williams et al., 2015) and infants (Nguyen, Bhandari, Cichero, & Prakash, 2015). These differences in methods and models make the comparison between in vitro digestion experiments quite complex. Therefore, the EU project "InfoGest" tries to standardize the simulated in vitro conditions, first for the static digestion models for adults (Minekus et al., 2014) and later for dynamic in vitro models (Dupont et al., 2017). These standardizations should result in more comparable in vitro data. Regardless of the attempt to standardize static digestion methods, there was consensus about the limited predic-

tive quality of static methods due to lack of the simulation of realistic kinetic gastrointestinal conditions (Minekus et al., 2014).

To simulate the realistic conditions in the stomach and small intestine for digestion experiments, the (average) physiological kinetic conditions in the lumen of the stomach and small intestine should be "translated" to dynamic in vitro models. Many review articles describe the gastrointestinal physiology after intake of different types of meals for adults (e.g., Barros, Retamal, Torres, Zúñiga, & Troncoso, 2016; Culen, Rezacova, Jampilek, & Dohnal, 2013; Varum, Hatton, & Basit, 2013) as well as for infants (e.g., Bourlieu et al., 2014; Kamstrup, Berthelsen, Sasene, Selen, & Müllertz, 2017).

This "translation" to dynamic in vitro gastrointestinal models also has several challenges (Guerra et al., 2012). One of these challenges is the interpretation of enzyme activities, especially the pancreatic digestive enzymes (DiMagno & Layer, 1993). Different definitions of digestive enzyme activities and enzyme assays have been published, including the use of coenzymes, different substrates and pH values for digestion, such as those described for infant digestion by Abrahamse et al. (2012). The next challenge is the availability of appropriate, purified digestive enzymes. Specifically, human gastric and pancreatic enzymes as well as brush border enzymes (Picariello, Ferranti, & Addeo, 2016) are not commercially available. So alternative enzymes such as pancreatic enzymes from pigs are used based on the knowledge that the pig is the best available animal model for human digestion (Guilloteau, Zabielski, Hammon, & Metges, 2010). As alternative for gastric lipase and purified proteases, commercial enzymes of animal or microbial origin are available. They need to be selected on their physicochemical characteristics, such as activity and stability under site-specific human gastrointestinal conditions (Minekus et al., 2014). Likewise, bile is an important secretion compound for food digestion (Maldonado-Valderrama, Wilde, Macierzanka, & Mackie, 2011), facing the same challenges for in vitro models as digestive enzymes in relation to the secreted amount during the digestion process, composition of bile salts, and availability of human bile. Commercially available porcine or bovine bile is often used as an alternative to human bile (Minekus et al., 2014).

After the optimal in vitro model (hardware), settings (software), and composition of secretion fluids have been set up, the next important challenge is the validation of the in vitro digestion model. First, an operational quality (OQ) validation is necessary: does the dynamic model simulate in a controlled and reproducible way the in vivo physiological conditions? An example of such an OQ validation has been described by Bellmann et al. (2016) for the simulated conditions in the stomach in comparison to human physiological data. Second, a performance quality (PQ) validation should take place: are the in vitro results predictive for human clinical digestion studies? The challenge is how to compare in vitro bioaccessibility data with human bioavailability data, in the light of the abovementioned challenges of human clinical studies. The optimal way of PQ validation is the use of in vitro vs. in vivo studies specifically dedicated to the in vitro–in vivo comparison, such as those described by Verwei, Freidig, Havenaar, and Groten (2006) for folate and Bellmann, Minekus, Sanders, Bosgra, and Havenaar (2017) for carbohydrate digestion. In these

studies, the in vitro gastrointestinal models were used in combination with in silico modeling for optimal prediction and comparison with human bioavailability data.

In most cases this optimal way of validation is not possible. In those cases, relevant clinical human data must be found for reproducing in vitro studies. Examples of this type of validation or evaluation are protein and fat digestibility studies under infant, adult, and elderly digestive conditions (Denis et al., 2016; Fondaco et al., 2015; Gervais et al., 2009; Havenaar et al., 2016; Maathuis, Havenaar, He, & Bellmann, 2017) as well as in vitro bioaccessibility studies for minerals and vitamins (Déat et al., 2009; van Loo-Bouwman et al., 2014; Verwei et al., 2003, 2006). These evaluation studies demonstrate that digestion experiments in dynamic in vitro gastrointestinal models may have a high predictive quality for the human situation. Validated in vitro digestive models contribute to the replacement of animal studies and the cost-efficient development of new food products.

7 Conflicts of Interest

Irrespective of the type of study, the setup and performance of the experiments, the descriptions and interpretation of the results and the final conclusions should be based on scientifically sound arguments (e.g., based on a broad literature survey). It may not in any way be biased by (vested) interests that could inappropriately influence the work. Various guidelines are available to learn more about financial or personal conflicts of interest, such as that by scientific organizations (e.g., NIH; https://www.ncbi.nlm.nih.gov/ pubmed/ 21872119) and publishers (e.g., https://www.elsevier.com/conflictsofinterest; https://publishing.aip.org/authors/conflict-of-interest).

It is advised to read one of these guidelines, in fact before starting a project, but especially before writing a scientific publication. A conflicts of interest form can be downloaded from the International Committee of Medical Journal Editors (ICMJE; http://www.icmje.org/conflicts-of-interest/). This form can be filled out, saved on your computer, and be attached to the submitted manuscript.

8 Conclusion

The continuous need of results from reliable food digestion and nutritional quality studies creates the challenge to find the most optimal way of a cost-efficient and time-efficient, ethically liable experimental setup with optimal predictive quality. Although human clinical studies seem to be the gold standard, these studies are complex, expensive and have ethical constraints and therefore only applicable for single specific studies and not for routine digestion experiments. Animal models, on the other hand, may have a low extrapolative quality due to physiological differences in comparison to humans. Thus, a conscientious selection of the animal

model in relation to the aim of the study and the ethical constraints is necessary. The latest generation of dynamic in vitro gastrointestinal models makes it possible to accurately simulate the human digestive conditions, even in relation to age (infants, adults, and the elderly). The results from food digestion studies with these dynamic models show a high predictive quality for the human situation.

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Exploring and Exploiting the Role of Food Structure in Digestion

Matt Golding

1 Introduction

It is well understood that our health and physical well-being is intimately related to what we eat. However, dietary practice at an individual level is a complex issue, being determined by a number of aspects such as personal preference, choice, cultural definition, food availability and affordability, amongst others. To maintain an acceptable quality of life, our daily food intake must comprise an appropriate balance of macronutrients and micronutrients. Whilst the range of food available to us should readily supply such nutritional needs, it needs recognising that our nutritional requirements are actually ultimately met (or not met, as the case may be), based as much on the foods that we would prefer to eat, or perhaps can afford to eat.

It is also increasingly becoming apparent that nutritional content of a food does not necessarily reflect the nutritional *value* of that same food once consumed. Nutritional labelling on products gives a relative indication as the content of a food in basic terms of protein, carbohydrate and fat, as well as some indication of vitamins and minerals. However, we now have greater understanding that there can be considerable variation in the nutritional value of different protein types, for example in regard to the relative abundance of essential amino acids. Likewise, the nutritional value of lipids can vary according to compositional variation between different fat and oil types, notably in the degree of saturation or unsaturation, and acknowledging that the greatest nutritional value may come from raw materials abundant in polyunsaturated triglycerides. Carbohydrates provide equal complexity in their nutritional representation, in that the term carbohydrate can encompass simple monosaccharide or disaccharide sugars through to oligosaccharides and long

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chain biopolymers such as starches and polysaccharides. Needless to say that within each of these particular classes of carbohydrates, there can be considerable diversity of materials.

A similar picture emerges when we further consider the intake of micronutrients. Whilst the biological role of vitamins, minerals and other bioactive components for our health and well-being is now well understood (along with the consequences of omitting these from our dietary food intake), it is increasingly apparent that availability and efficacy of such materials goes beyond simply consuming an appropriate amount on a daily basis. This has been elegantly demonstrated by a short communication produced by Jeanes and co-authors (Jeanes, Hall, Ellard, Lee, & Lodge, 2004), who demonstrated how the extent of vitamin E uptake was influenced by the manner in which the vitamin was consumed. Jeanes' findings showed that uptake of vitamin E was compromised when consumed in the absence of lipid (i.e. when consumed with water or skimmed milk). However, the introduction of various lipid components in combination with the consumption of the vitamin, was able to greatly enhance uptake, reporting increased levels of plasma tocopherol when the supplement was taken with either buttered toast or cereal with whole milk (Fig. 1).

Similar findings were observed regarding the bioavailability of other lipophilic vitamins and micronutrients (carotenoids) through work undertaken by Agustiana, Zhou, Flendrig, and White (2010), which demonstrated negligible uptake of these nutrients when consumed in a salad based meal in the absence of fat. Inclusion of fat in the form of a salad dressing showed improved uptake, with increasing absorption observed as fat levels from the dressing were increased. These studies provide pertinent examples of how the nutritional value of food can be greatly influenced by even small changes to composition and the manner in which it is consumed.

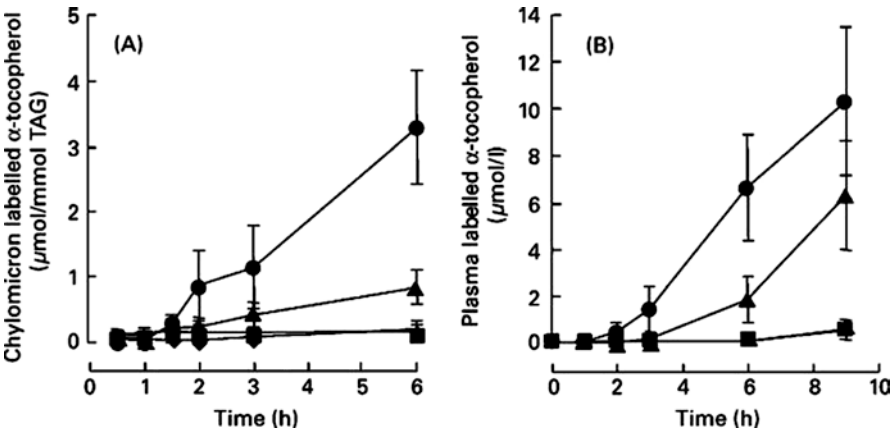

Fig. 1 H-labelled a-tocopherol concentration in (**a**) chylomicrons and (**b**) plasma following ingestion of a capsule containing 150 mg 2 H-labelled RRR-a-tocopheryl acetate with various test meals. Values are means for eight subjects: Toast with butter; (–O–), cereal with full-fat milk; (–V–), cereal with semi-skimmed milk; (–B–), water; TAG, triacylglycerol. Reproduced with permission from Jeanes et al. (2004)

Such studies invariably lead to the question of the broader role of food structure in nutrient digestion and uptake. To a degree this is a relatively recent consideration, possibly due to the fact that whilst our diet comprises a highly diverse array of structured food materials, our digestive system demonstrates a remarkable ability to (almost entirely) utilise nutritional value regardless of the structure of the food in question, and thus its contribution to the digestive process has tended to be ignored. However, it is increasingly being recognised that the structural assembly of nutrients, and the nature of their interactions within a food system—ranging from molecular to material—can be highly impactful on the manner in which those nutrients are digested, and potentially provides a lever by which the health and wellness value of a food, whether wholefood or manufactured, can be optimised. This chapter aims to provide some context regarding the effects of food microstructure on the primary nutrient systems present in foods.

2 Defining Food Structure

The role of the digestion process can be grossly simplified as a mechanism by which ingested nutrients can be rendered into a state amenable for assimilation and utilisation by the body. This simple statement belies the complex biological pathway that has evolved to achieve this effect and which, as indicated, provides humans with the ability to consume, and gain nutritional value, from a vast array of different foodstuffs, and which have equally large diversity in their structural complexity. The study and characterisation of food structure as a scientific discipline has become increasingly prominent over the past few decades, not only in developing a greater understanding as to the relationship between food structure and digestion (Lundin & Golding, 2009; Norton, Wallis, Spyropoulos, Lillford, & Norton, 2014) but also in relation to other attributes of food materials, such as their sensory properties and shelf-life (as determined by physical, chemical and microbiological stability).

Accordingly, attempts have been made to specifically define the concept of food structure. Aguilera summarises two particular definitions, one from 1993, which states that "food microstructure can be defined as the spatial arrangements of elements in a food and their interactions" and a second and similar definition from 1980, determining that structure is "…the organisation of a number of similar of dissimilar elements, their binding into a unit, and the interrelationship between the individual elements and their groups". This common theme of spatial arrangement and interactions can be applied over multiple length scales from molecular through to microscopic (e.g. colloidal particles, cellular organelles), mesoscopic (e.g. colloidal aggregates, gel structure, cellular assemblies) and material, and with the integration of these length scales defining the overall properties of food.

In this regard, all raw materials contributing to our diet will possess an inherent nature-defined structure which is constructed over time during the farming, harvesting and post-harvest treatment of that material. For some foodstuffs, mainly wholefoods such as fruit and vegetables, it is the naturally assembled food structure that

will be extant at point of consumption (whilst acknowledging that gradual changes to structure will inevitably occur post harvesting). For wholefoods commonly consumed raw, this endogenous structure will be determinant on the sensory characteristics of the food, as well as having the potential to influence the subsequent digestion behaviour of the nutrients present within that food. For other wholefoods, such as meat and fish and vegetables, additional processing (usually in the form of heating) is commonly applied prior to consumption. Whilst the primary purpose of such processing has been to ensure that the food is safe for eating, we also recognise the role of heating in imparting desirable sensory properties such as the development of flavours (e.g. through the Maillard reaction) and textural attributes (such as those arising from the denaturation of proteins, breakdown of cellular structures, the melting of fat or gelatinisation of starch). The development of sensory characteristics during what has effectively become the cooking process, is a consequence of changes taking place to the native structure of the food material in response to the applied conditions, noting that such changes will also invariably impact on the digestive properties and nutritional value of the food.

Extending this further, as part of modern food manufacturing, it is also the case that we can use processing pathways to effectively disassemble the native structure of particular raw materials for subsequent use as ingredients in the production of processed foods. The degree of structural modification or disruption can be altered across the various length scales dependent on the requirements of the product. Notably, the production of ingredients with highly defined functional or sensory properties, for example fats and oils derived from plant materials, requires processes capable of complete disruption of the spatial arrangement and interactions of the various structural components within the raw material, thereby enabling the separation, extraction and concentration of the particular molecular component(s) of value. It is important to note that the processing required to achieve separation can potentially impact on the molecular structure of the ingredient being isolated. For relatively non-labile materials that can be separated, refined and concentrated using relatively mild processing conditions (such usually the case for simple sugars, fats and oils), molecular structure is not necessarily altered during the production of the ingredient. However, for more labile components the native molecular structure may be modified by processing, such as for proteins for which denaturation may occur as a consequence of thermal treatments applied during production. Any such modifications at a molecular level will invariably influence the functionality of those ingredients when used in manufacture of a processed food, as well as potentially impacting on the manner in which those materials can be digested.

The definitions of food structure as provided by Aguilera can equally be applied to the production of manufactured or processed foods. In this case, the structural elements are determined by the formulation of the product. Interactions between elements will be dependent on the functionality of the raw materials comprising a formulation (noting that not all ingredients may play a functional role in creation of a food structure, as well as the potential use of additive systems capable of providing highly specific functional roles in food products). However, the enabling of interactions and creation of an appropriate spatial organisation of structural ele-

ments is equally achieved through the processing operations utilised in the manufacture of the food (such as thermal treatment, homogenisation and shear). This assembly of structure through formulation and process design determines the material and product properties, enabling manufacturers to produce foods with well-defined and highly reproducible attributes.

The assembly of food structures (whether wholefood or manufactured) towards the point of consumption is invariably a dynamic process (Fundo, Quintas, & Silva, 2015). For manufactured foods, processing during production constitutes the main dynamic pathway for constructing a particular food structure. However, it is important to acknowledge that food structures can continue to be modified through physical, biochemical or chemical means during distribution, storage and utilisation. Likewise, the consumption and digestion of food is a dynamic process, in which food structures are broken down, initially in the mouth and subsequently during gastrointestinal transit.

The deconstruction of the material and structural properties of a food under conditions of oral processing (i.e. the combined role of in mouth shear, combination with saliva, temperature and time) serves to define the sensory characteristics of a food, creating a temporal profile of texture, flavour and taste perception (Foster et al., 2011). Oral processing also serves to render foods in an appropriate structural state for both swallowing and the onset of digestion in the stomach. In this regard, the structural state of liquid foods, such as milk, may be relatively unaltered during the short oral residence time for consumption. In contrast, the structures of solid, or semi-solid foods may be considerably broken up and mixed with saliva in the formation of a swallowable bolus. Accordingly, the digestive behaviours of a bolus formed through oral processing may represent a largely modified structure relative to that of the food system prior to consumption (Bornhorst & Singh, 2012; Wang & Chen, 2017). The following sections will explore the role of hierarchical assembly and food structure on the digestion of the main macronutrient components of protein, fat and carbohydrate.

3 The Influence of Food Structure on Protein Digestion

Mapping of the human proteome has determined that the human body comprises approximately 100,000 different proteins, providing a plethora of highly specific physiological functions. Maintenance and replenishment of the human proteome is supported through consumption and digestion of various sources of dietary protein, which can be broken down initially into oligopeptides before further hydrolysis to free amino acids that are transportable across the small intestinal epithelium. Without expanding into detail regarding the entire physiological processes of protein digestion and uptake, the reduction of dietary protein to smaller peptide fragments occurs through exposure to gastric and pancreatic endopeptidases (pepsin, trypsin and chymotrypsin) with subsequent breakdown into amino acids being achieved through interaction of oligopeptide fragments with digestive

exopeptidases. The biochemical hydrolysis of proteins is facilitated by the variable pH conditions in the stomach and small intestine. Thus, the gastric protease pepsin, has optimum activity in the pH range 1.5–2.2 (Schlamowitz & Peterson, 1959), whilst the pancreatic lipases have optimal activity in the range 7.5–8.2.

3.1 Influence of Protein Molecular Structure on Digestion Behaviour

The rate and efficacy by which proteins can be digested can be influenced across all length scales representative of food structure. At the shortest length scale, the interaction between the digestive endopeptidases and the protein substrate can be affected by the molecular conformation of the protein. In the specific case of pepsin or chymotrypsin, which exhibit preferential hydrolysis at peptide bonds comprising hydrophobic or aromatic amino acids (such as phenylalanine or tyrosine), the internalisation of these residues within the native, folded structure of a globular protein can render the protein more resistant to digestion. This has been observed for the milk protein β-lactoglobulin, which has been shown under in vitro gastric conditions as undergoing only limited hydrolysis by pepsin or chymotrypsin whilst in the native state (Loveday, Peram, Singh, Ye, & Jameson, 2014; Mullally, Mehra, & FitzGerald, 1998; Schmidt & Vanmarkwijk, 1993). In contrast, the more disordered molecular structure of the casein protein fraction in milk provides greater exposure of preferential sites for proteolysis and thus (without considering any structural contribution) the protein tends to be far more readily hydrolysed (Guo, Fox, Flynn, & Kindstedt, 1995).

Prior studies on β-lactoglobulin molecular structure (Iametti, DeGregori, Vecchio, & Bonomi, 1996; Townend, Herskovits, & Timasheff, 1969) have determined that significant proportion of amino acids residues susceptible to hydrolysis to be either hidden through quaternary interactions or buried within the protein secondary and tertiary structures, creating a hydrophobic cavity that is not readily accessible by the enzyme. Perhaps understandably, unfolding of the native protein structure and exposure of these buried hydrophobic/aromatic domains has been seen to greatly increase the susceptibility of the protein to not only peptic hydrolysis, but also when exposed to trypsin and chymotrypsin (Iametti et al., 1996). This demonstrates one potential consequence of food processing on protein digestion, in which the denaturation of the protein through various mechanisms leads to an enhancement in the extent of proteolysis such through the application of thermal processing (Kitabatake & Kinekawa, 1998; Mullally et al., 1998). The application of static high pressure represents another processing pathway allowing for enhancement of peptic digestion. This is exemplified in a study by Zeece and co-authors (Zeece, Huppertz, & Kelly, 2008), who demonstrated that pressures in excess of 600 MPa for at least 10 min were able to greatly enhance the digestion of 1 wt% β-lactoglobulin solutions under simulated gastric conditions, observing the complete disappearance of the primary structure occurred after only 1 min of in vitro

incubation after pressure treatment. Proteolysis has also been shown to be improved by other mechanisms capable of exposing suitable hydrolysis sites. This includes protein denaturation through addition of urea (Guo et al., 1995), and even the unfolding of the protein as a consequence of adsorption at the oil–water interface when used for emulsification (Macierzanka, Sancho, Mills, Rigby, & Mackie, 2009; Nik, Wright, & Corredig, 2010).

Modification to molecular structure through these various mechanisms can also impact digestion as a consequence of additional intermolecular interaction and self-assembly. Again, this can occur across increasing length scales from mesoscopic to macroscopic, creating structures ranging from oligomeric to colloidal through to percolating gel structures. The extent and nature of aggregation is dependent on a number of variables, including protein concentration, solvent conditions (pH and ionic environment), processing conditions, and their influence on the specific bonding mechanisms responsible for association (i.e. hydrogen bonds, electrostatic interactions, covalent linkages and hydrophobic effects). It can also be noted that association can occur between like molecules, either directly or via an appropriate bridging mechanism, or between different moieties. Whilst the formation of aggregated structures can impact on digestion (again, primarily considering this from the perspective of enzymatic hydrolysis) due to the alterations to the accessibility of peptide linkages susceptible to hydrolysis, other factors can influence digestibility.

3.2 Influence of Intermolecular Interactions on Protein Digestion

Cross-linking mechanisms between proteins and other molecular species has been demonstrated as impacting on the bioavailability of particular amino acids groups that are involved in cross-linking. This is exemplified by the Maillard reaction, which can occur between proteins and reducing sugars, in which the bioavailability of the essential amino acid lysine (along with other reactive amino acids such as arginine, methionine, tryptophan, and histidine) can be compromised as a consequence of cross-linking mechanisms between the amino acid and the sugar (Obrien & Morrissey, 1989). Lysine is reported as supporting a variety of physiological functions including the production of carnitine, lowering cholesterol levels, as well as assisting in the absorption and conservation of calcium. The Maillard reaction not only negates the metabolic availability of lysine, but can also influence the ability of peptidases to hydrolyse particular sequences on the protein chain and leading to the formation of different polypeptide fragments (Schumacher & Kroh, 1996). This can reduce the nutritional value of the reacted protein, and can be problematic for processes and formulations that facilitate the Maillard reaction, such as those occurring during the manufacture and storage of milk powders and sterilized milks, in which the protein component of the milk can form cross-links with lactose during thermalisation (Guyomarc'h, Warin, Muir, & Leaver, 2000; Mehta & Deeth, 2016). For infant nutrition this can be a particular issue, noting that the manufacture, and

especially the extended storage, of both UHT and powder based formulations can result in a reduction to biologically available lysine, which is considered a critical amino acid for supporting early growth and development (Ferrer et al., 2003).

The specific loss of amino acid bioavailability as a consequence of Maillard complexation is, however, not necessarily exhibited in other forms of intermolecular cross-linking. For example, biochemical protein polymerisation can be mediated through the use of the enzyme transglutaminase, which catalyses the formation of intermolecular covalent bridges between lysine and glutamine amino acids. Studies on the digestion of transglutaminase cross-linked proteins have demonstrated that exopeptidase digestion is able to cleave the lysine-glutamate isopeptide, liberating free lysine (Romeih & Walker, 2017). That said, the polymerisation of proteins through transglutaminase can influence their susceptibility to hydrolysis via endopeptidase action. For example, in vitro studies on the enzymatic digestion of soy protein isolate carried out by Tang and co-authors (Tang, Li, & Yang, 2006) determined that the rate and extent of protein hydrolysis (as measured by % nitrogen release) was inhibited as a consequence of transglutaminase polymerisation of the protein. This was observed for both proteolysis by pepsin and subsequent digestion by trypsin. Interestingly, Tang observed that whilst heating of the soy protein enhanced peptic digestion by exposing hidden hydrolysis sites through unfolding of the native structure, denaturation was also able to provide greater access to lysine and glutamine sites for transglutaminase polymerisation. Accordingly, the more extensive cross-linking of the heat-treated soy protein was found to result in a marked decrease in peptic digestion (although this was less pronounced for trypsin hydrolysis).

A separate study by Monogioudi and co-authors (Monogioudi et al., 2011) investigated the effects of transglutaminase polymerisation of the milk protein β-casein in relation to its proteolysis by pepsin. They observed a retardation in the rate and reduction in the extent of hydrolysis that increased with extent of protein cross-linking. In vitro studies revealed that after extended incubation, the degree of proteolysis of the non-cross-linked casein was 50% higher than that of the fully cross-linked protein. Interestingly, whilst there was a clear inhibition of proteolysis, the peptides that were generated during digestion were essentially the same, regardless of whether the protein had been cross-linked or not.

Curiously, a prior study undertaken by Roos and co-authors (Roos, Lorenzen, Sick, Schrezenmeir, & Schlimme, 2003) exploring the effect of transglutaminase cross-linking on the gastric and small intestinal hydrolysis of sodium caseinate, the authors determined that the extent and pathway of proteolysis was unaffected by the transglutaminase cross-linking of the protein. Their initial in vitro findings were following up with a mini-pig in vivo study, which demonstrated no significant differences in either the kinetics of digesta flow, or in the total protein digestibility between the cross-linked and untreated protein samples.

Other process-induced intermolecular interactions can have significant consequences for the digestibility of foods without necessarily impacting on the material or sensory properties of those systems. An example of this has been presented by Ye and co-authors (Ye, Cui, Dalgleish, & Singh, 2016b) who studied the effects of heat

treatment on the in vitro coagulation and gastric digestion of milk. Their findings showed significant differences in the structure of clot formation, with a milk sample heated at 90 °C for 20 min displaying a much looser clot structure compared to that of an unheated milk. The denser protein network of the unheated milk under gastric pH was found to be more resistant to peptic hydrolysis (as observed for both casein and whey protein digestion), due to limited structural breakdown during gastric incubation, and restricted diffusivity of the enzyme into the interior of the structure. These effects were attributed to the cross-linking of whey proteins to the surface of the casein micelle structure during heating, which significantly altered the interactions and structuring of the casein micelles on lowering of pH towards the isoelectric point. Interestingly, a repeat of the study using whole milk indicated that heat treatment also affected the location and distribution of the fat droplets within the gastric environment, noting that fat droplets were more readily liberated from the heated milk coagulum compared to that of the unheated sample (Ye, Cui, Dalgleish, & Singh, 2016a).

3.3 Influence of Protein Polymers and Self-assemblies on Protein Digestion

The self-assembly of protein molecule into moieties of varying size and structure can be generated through other mechanisms, leading to the formation of a range of structural assemblies such as fibrillar, stranded, branched and random aggregate. These have been termed giant supramolecules, and whilst the assembly of these may extend into colloidal dimensions, the structural dimensions are not yet sufficient to generate fully percolating networks characteristic of protein gels [although these structures may cause weak gelation through entanglement and interaction at sufficiently high volume fractions (Veerman, Sagis, & van der Linden, 2003)]. Formation of these aggregated supramolecules can be achieved through a number of predominantly globular proteins, with whey, egg and soy proteins providing examples of edible proteins known to exhibit this behaviour (Akkermans et al., 2007; Veerman et al., 2003). The mechanisms of assembly of these structures, and the consequences of these on material and functional properties has been an area of considerable research interest (Zhao, Pan, & Lu, 2008), with the digestive properties of these assemblies coming under particular scrutiny in recent years (Moayedzadeh, Madadlou, & Asl, 2015).

The formation of aggregates and the type of structures generated is highly dependent on processing considerations, such as the application of heat and shear, as well as the properties of the solvent as influenced by pH and ionic strength. These treatments can promote the establishment of a number of attractive interactions (electrovalent, hydrophobic, hydrogen and covalent bonding and van der Waals), that lead to the bonding of protein molecules into discrete aggregates. A widely studied example of these effects has been the fibrillar assembly of the whey protein β-lactoglobulin, that has been shown to be highly sensitive to processing conditions

in producing a variety of different structures (Loveday, Su, Rao, Anema, & Singh, 2012). As a general rule of thumb, fibrils can be formed at elevated temperatures close to the denaturation point of the protein, at pH conditions removed from the isoelectric point (IP) of the protein and at low ionic strength (Venema, Minekus, & Havenaar, 2004). Fibril formation also tends to require extended heating periods, in the order of hours. Adjustment of pH conditions towards that of the protein IP and increasing ionic strength can be used to change structure from essentially fibrillar entities with high aspect ratio to progressively branched structures and denser random aggregates. For example, in the particular case of β-lactoglobulin, fine stranded fibrils could be formed at a pH of 2, ionic strength of 0.03 M coupled with heating at 80 °C for 10 h (Veerman et al., 2003). Studies of the digestion of β-lactoglobulin fibrils under in vitro gastric conditions showed that pepsin was able to readily hydrolyse fibrillar structures over very short times (Bateman, Ye, & Singh, 2010). The susceptibility to gastric digestion was understood to be enhanced by the unfolding of the native structures during extended heat treatment and extreme pH conditions. An interesting observation from the digestion process was the characterisation of the peptide fragments generated during hydrolysis, that suggested that the fibril structures themselves were comprised of peptide sequences that had previously been hydrolysed during the treatment for fibril formation. However, a more recent in vitro study on amyloid type fibrils assembled from a broader range of proteins, including whey, soy, egg white and kidney bean indicated certain fibrillar structures showing resistance to not only gastric proteolysis but also after treatment with pancreatin (Fig. 2) (Lasse et al., 2016).

Findings from this study have indicated that, whilst there is potential value in the technical functionality of these supramolecular structures, further work is required to ensure that any variance in digestion behaviours observed for such structures does not pose any health risk arising from their consumption.

3.4 Influence of Protein Gels and Protein-Structured Foods on Protein Digestion

Much of the discussion thus far has centred on how protein digestion is influenced through alterations to molecular conformation, either at an individual level or within complexes, self-assembled and polymerised structures. By and large, the material properties of these systems are not considered as providing meaningful contribution to their digestive behaviours, with investigations carried out with the protein essentially present in a fluid state in solution (and usually under relatively dilute conditions). However, aggregation of protein structures (whether in isolation, or in combination with other components) can be extended such that percolating networks are formed, resulting in the transition of material state from liquid to solid and the formation of gelled structures. In addition to changes to molecular conformation caused by the creation of the gelled state that may influence susceptibility to digestion, the change in material state is likely to influence the diffusivity and accessibility of the digestive enzyme in relation to the protein substrate.

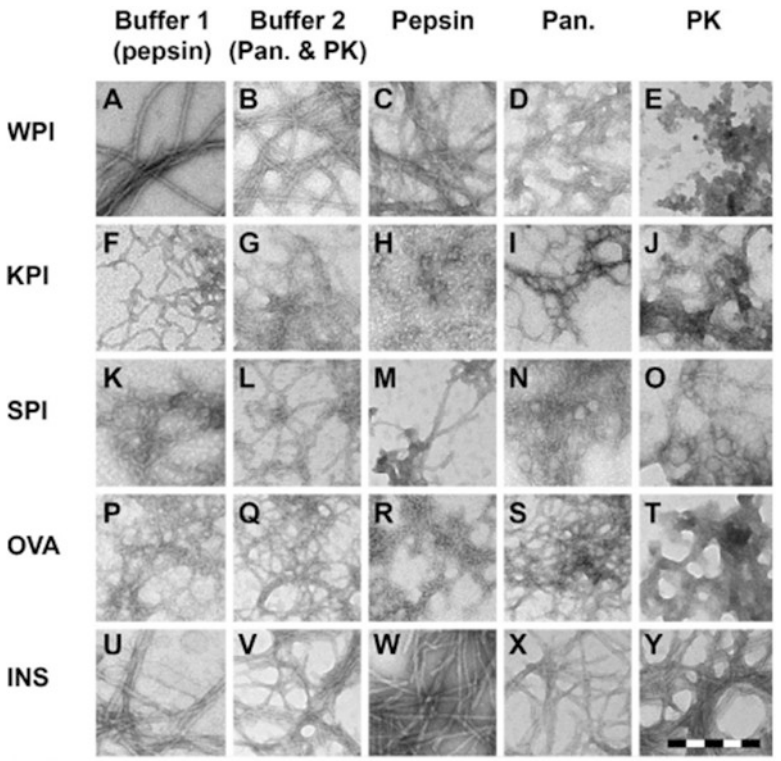

Fig. 2 TEM of fibrils after 3 h of incubation in buffer (column 1, pepsin buffer; column 2, pancreatin (Pan.) and Proteinase K (PK) buffer), and after 3 h of proteolysis by pepsin (column 4), pancreatin (column 4) or Proteinase K (column 5). Panels are organised in rows depending on protein source. From top to bottom: WPI, KPI, SPI, OVA, INS. The scale bar is 200 nm. (Reproduced with permission from Lasse et al. (2016)

A commonly cited example is the difference in digestion of raw and cooked egg protein. From a molecular perspective, the native structure of the globular proteins comprising egg white represent a more resistant conformation for enzymatic hydrolysis, as discussed in Sect. 3.1. This is in spite of the fact that the physical state of raw egg is liquid, and therefore should be expected to provide a more effective medium for enzymatic diffusion into the ingested material relative to the gelled cooked egg (acknowledging that the structure of the cooked egg would have been comminuted to varying degrees through mastication). In contrast, the denaturation and unfolding of the egg protein as consequence of heating would be expected to provide a more amenable substrate for proteolysis. This hypothesis was explored in a human study undertaken by Evenepoel and co-authors (Evenepoel et al., 1998), who used a stable isotope method to determine both the assimilation and gastric emptying time of raw and cooked egg. Raw egg was found to be significantly less digestible over the entire course of digestion compared to the cooked egg. In fact, the total ileal digestibility of the raw egg was determined as 51.3% (±9.8) compared to 90.9% (±0.8) for the cooked egg. In this regard, the molecular structure of the

native, liquid protein was seen to be the greater barrier to digestion compared to the material properties of the cooked, denatured egg. It was also noticeable that the different material states of the cooked and raw egg contributed to significant differences in gastric emptying time. In the case of the raw egg, the gastric half emptying time was determined as 25 min (±9), whilst the cooked egg resulted in a pronounced increase in half empting time at 68 min (±6). Whilst it can be reasonably argued that the faster rate of emptying of the raw egg was due to its liquid state, it may also be the case that the poorer extent of digestion may result in reduced hormonal cholecystokinin (CCK) feedback signalling as a controlling factor in the rate of emptying when compared to the more readily digested cooked egg (Liddle, 1995).

A similar study, but under in vitro conditions was carried out by Luo and coauthors (Luo, Boom, & Janssen, 2015; Luo, Borst, Westphal, Boom, & Janssen, 2017) studying the peptic digestion of egg and whey protein solutions and comparing their behaviours to gelled systems comprising the same concentration of protein. Interestingly, they observed that the material properties of the gelled systems provided greater resistance to hydrolysis compared to the protein solutions. For the protein solutions, after 3 h gastric incubation, the extent of hydrolysis was determined as 11% and 15% for the egg and whey respectively. In contrast the extent of hydrolysis of the gels was found to be 2.5% and 7.9% for the egg and whey protein respectively. The reduced degree of hydrolysis for the gel systems was considered a consequence of limited diffusivity of the enzyme into the interior of the gel structure, and accordingly hydrolysis progressed primarily at the surface of the gel structures (Luo et al., 2017). The difference in observed effects when compared to the Evenepoel study (Evenepoel et al., 1998) may be due to the preparation of gel particles themselves, noting that the in vitro study was based on gel particles of cylindrical particles with dimensions approximately 5 × 5 mm. In contrast, the gelled egg in the human study would have undergone mastication during the eating process, and whilst the size of the particles was not reported, it might be reasonably assumed that these particles were both smaller and more structurally damaged as a consequence of the eating process, thereby enabling greater diffusivity of the proteases during digestion.

The role of food gel microstructure and assembly on subsequent digestive behaviours has been studied for other food proteins, indicating that the nature and strength of interactions can influence the manner in which the protein component is digested. This is exemplified in an in vitro study carried out by Rui et al. (2016) investigating the effect of different coagulants on the rate and extent of gastrointestinal proteolysis of tofu. Findings showed that under gastric conditions digestion was proceeded most rapidly for the acid coagulated gel, which displayed a higher overall extent of proteolysis. Digestion rate and extent was least for the tofu samples prepared through covalent cross-linking using transglutaminase. An interesting aspect of the research was the lack of correlation of digestion with material properties, noting that the acid tofus were significantly firmer than those prepared using divalent cations as a cross-linker. Indeed, it was observed that increasing the concentration of GDL used to coagulate the acid tofu was found not only to increase the firmness of the tofu, but also lead to a greater extent of proteolysis. One argument for the

observed differences might be that acid tofu was at a lower initial pH, and therefore more favourable for pepsin activity compared to the other samples; however, the in vitro model used standardised the pH conditions at pH = 2 for all samples prior to addition of the pepsin. In the case of the samples comprising transglutaminase, the reduced level of proteolysis is consistent with observations made in Sect. 3.2 indicating that intramolecular and intermolecular cross-linking may limit the availability of hydrolysis sites for enzymatic binding.

Differences in protein digestion pathways can also be observed for other structured protein assemblies, such as demonstrated by Barbé and co-authors (Barbe et al., 2014). Their study investigated the digestion of two dairy gel systems of equivalent protein concentration but formed via different structural pathways, namely acid gelation and rennet gelation. Employing an in vivo pig model, they demonstrated that the acid milk gel was more readily proteolysed than the rennet gel. Additionally, significant differences in plasma peptides were observed, with the acid gel displaying elevated levels relative to the rennet get. These behaviours also manifested other variations in physiological biomarkers, for example in the release of ghrelin and CCK (although there was apparently no consequence of these variations on rate of outflow from the stomach). A number of reasons for these differences were postulated, noting that variations in sample pH (and the possible contribution to buffering effects in the stomach) might lead to differences in pepsin activity, with the acid gel enabling higher relative activities, at least during the early stages of digestion. Additionally, the two gels showed varying structural behaviour in the stomach, with the rennet gel undergoing extensive syneresis and consequential compaction of the protein network. This structural densification was considered inhibitory for diffusion of protease enzymes into the interior of the gel structure.

This particular hypothesis was explored further in considering how pore structure in protein particle networks might impact on pepsin enzymatic diffusivity (Thevenot, Cauty, Legland, Dupont, & Floury, 2017). Findings demonstrated that increasing the tortuosity and decreasing the pore size of protein gels through increasing protein concentrations did indeed reduce the ability of the enzyme to diffuse within the gel structure. It is important to note that such observations are also dependent on the overall structure dynamics of the gel system in response to the pH, temperature and shear conditions in the gut, and thus multiple dependencies are likely to determine the overall manner in which the protein is digestion.

In this regard, research to date is now starting to progress towards the complexities of protein digestion in multicomponent food systems. Findings are increasingly corroborating the hypothesis that the digestive breakdown pathway of any given protein component in a food will be determined by an integration of interactions across all length scales, from molecular to material, that are known to be influential in the breakdown of protein molecules during gastric and small intestinal residence. Arguably, and as observed with other macronutrients, human digestion and utilisation of dietary protein is remarkably efficient regardless of the type of protein or its structure. However, it is clear that there are variations in the digestive pathways of proteins based on their type, interaction within a formulation, structure and material properties and the response of these to the conditions in the GI-tract. Such variations

may impart particular physiological outcomes, such as the management of satiety, or potentially have consequences for the manner in which other macronutrients and micronutrients are digested. Accordingly, there remains considerable research interest in exploring such relationships.

4 The Influence of Food Structure on Lipid Digestion

The primary source of dietary lipids comes in the form of triglycerides, which are naturally present in a variety of raw materials, of both plant (e.g. nuts, seeds and certain legumes) and animal origin, as well as being formulated into many processed foods. The apolar nature of triglycerides and their poor solubility in aqueous media presents a compositional state in food that is not immediately utilisable by the human body. Thus, the digestive process for lipids focusses on the conversion of dietary triglycerides into fatty acids and monoglycerides via enzymatic hydrolysis in the stomach and small intestine. These component products of lipolysis have a higher degree of polarity, and in combination with secreted bile salts and cholesterol, can be associated into nano-structured assemblies (the so-called mixed micelles), that are small enough to interact with, and diffuse across the epithelial membrane, a process that takes place in the small intestine. The nutritional value of lipids is not simply in the provision of fatty acids (acknowledging that whilst the body can synthesize most of the fats it needs from a range of dietary lipid sources, two essential fatty acids, linoleic and alpha-linolenic, cannot be synthesized in the body and must be directly obtained from foods in which they are naturally present) but also in acting as delivery vehicle for a range of lipophilic micronutrients. As indicated in the introduction, the role of fat is not just to act as a medium for ingestion of such micronutrients but also provides a critical role in their uptake and assimilation by the body during digestion (Agustiana et al., 2010; Jeanes et al., 2004).

4.1 The Role of Fat Structure in Food Systems

Lipid composition can vary widely according to source material; however, lipids tend to be generically classified in accordance with their physical properties at ambient temperature, that is, fats, such as butter, palm and coconut being solids under ambient conditions; and oils, such as sunflower, olive and canola, being liquids. Fats and oils play a significant role the hedonic aspects of foods, contributing to the temporal dynamics of lipophilic flavour perception (Arancibia, Jublot, Costell, & Bayarri, 2011; de Roos, 2006), arguably recognised as a tastant (Kindleysides et al., 2017; Running & Mattes, 2016; Stewart et al., 2010), and eliciting a broad array of textural descriptors, such as oiliness, greasiness and creaminess, amongst others (Scholten, 2017). This gamut of sensory characteristics is achieved through a remarkable diversity in the structural assembly of lipids within both natural and manufactured foods.

Within the context of lipid structuring in foods, we can broadly classify lipid structures as either non-colloidal, colloidal continuous or colloidal dispersed. Non-colloidal foods encompass a range of (generally low moisture) food materials for which the fat or oil is in a relatively free state, such as the presence of visceral fat on meat, or the use of oil for preparation of fried foods. Colloidal fat continuous foods are typically characterised by products such as butter and margarine (for which the dispersed phase is water), and in chocolate (for which the dispersed phase is solid particles of sugar and other non-fat cocoa solids). Colloidal fat or oil dispersed foods are probably the most predominant class of fatty food products, occurring naturally, as part of the cellular structure within many plant materials (such as oil bodies, within nuts, grains and seeds), or as emulsion droplets within mammalian milks. Colloidal fat dispersed structures are also widely encountered in manufactured liquid or soft solid food products, such as ice cream, mayonnaise and cheese. These emulsified systems are usually produced through the use of homogenisation to form typically micron-sized droplets, and through interfacial stabilisation of these droplets through adsorption of a surface active component within the formulation, either an ingredient (e.g. milk proteins in the manufacture of ice cream) or an additive, specifically added to the formulation for the purposes of emulsification (such as the use of gum Arabic in the stabilisation of flavour emulsions).

Arguably, the main purpose of emulsification is to provide a means for enabling a kinetically stable structure for dispersing lipids in an aqueous environment. This is exemplified in a number of foods, such as UHT milks, cream liqueurs, and liquid infant formulae, for which the formation of small stable droplets is able to maintain a homogeneously dispersed emulsion state for shelf lives in excess of a year. However, it should also be noted that controlled destabilisation of food emulsions has been used extensively in many products. This is particularly evident in soft solid (weak gel) and solid (strong gel) emulsions, where the material properties of the food are able to provide kinetic trapping of emulsion structures, thereby inhibiting phase separation over the product lifetime. Manipulation of droplet interactions into various states of aggregation, such as flocculated or partially coalesced is able to assist in the creation of soft solid or solid material states, and as well as assisting in the physical stabilisation of the system, can provide a significant contribution to the material, technical and sensory properties of the food.

Examples of this include the formation of protein-fat flocculated networks in the manufacture of yogurt and cream cheese, which provides a mechanism by which the firmness of the product can be enhanced, and the structuring of fat in ice cream through partial coalescence, which has been shown to markedly reduce the melting rate of the product. A similar partial coalescence mechanism that occurs in the manufacture of cheese, provides an important contribution to both the material properties of the cheese and associated characteristics, such as oiling off during melting. The ability to understand and control the structure of food emulsions has become an integral part of food design and development for many emulsified foods, with the ability to manipulate colloidal interactions and their spatial assembly within food structures enabling a degree of predictable control over product properties.

4.2 Motivations for Manipulating Fat Digestion

Naturally a question as arises as to whether our ability to digest and fully utilise dietary lipids is impacted by the structural diversity of fats and oils. In this regard, the human physiological pathway demonstrates a remarkable ability to process ingested fats and oils with equal efficacy regardless of their material or structural properties. This appears to apply irrespective of whether the lipids structures are present within natural food materials, or as part of manufactured products (noting that the consumption of processed emulsified foods forms a relatively recent component of our diet in terms of human evolution). Indeed, for healthy adults, the digestive efficiency of food lipids is typically in the order of ~95% for, when consumed as part of what can be considered normal dietary intake of fats and oils.

In recent years there has been significant intellectual investment directed to understanding how the lipid structures present in food impact on subsequent lipid digestion and digestibility (Mao & Miao, 2015). Such understanding has led to the wider consideration as to whether the processes of fat digestion can be manipulated to achieve a particular physiological outcome. Much of this research has been undertaken from a perspective of developing strategies for mitigating the current obesity crisis affecting significant populations across the planet. This includes the exploration of approaches capable of inhibiting or negating lipid uptake during digestion, and thus reduce the caloric intake associated derived from the lipid component of a food. Notable approaches to this effect include the utilisation of lipid-like materials which are able to impart equivalent technical and sensory properties relative to fats and oils, but which possess molecular structures impervious to digestion. Arguably, the most recognised of these materials are the sucrose polyesters (also known by the trade name Olestra) (Bimal & Zhang, 2006), which are essentially sucrose molecules with fatty acid chains linked through ester bonds at available hydroxyl sites. Liberation of these grafted fatty acids is not achievable through digestive lipolysis, and as the molecular structure of olestra is cannot be metabolised, it remains undigested across the entirety of the GI tract.

A slightly different approach involves the blocking of the digestive enzymes responsible for conversion of triglycerides to fatty acids, such as through the use of tetrahydrolipstatin (also known as orlistat) (Heck, Yanovski, & Calis, 2000). Orlistat acts by binding covalently to the serine residue of the active site of gastric and pancreatic lipases, disabling the ability of the enzymes to interact with the substrate (Guerciolini, 1997). As there is no mechanism by which non-hydrolysed triglycerides can be transported across the small intestinal epithelium, inhibition of hydrolysis means that varying amounts of ingested fats and oils can remain undigested. Both approaches provide mechanisms by which dietary fat intake can be lowered, and whilst at first glance it might be considered that such effects would be of benefit in developing strategies for mitigating the ongoing obesity pandemic, there remains an issue that the role of food is to provide nutrients for utilisation body. As such, for fats and oils for which digestive efficiency is particularly high, any mechanisms that lead to malabsorption to can cause physiological side effects (Harp, 1998). This

includes concerns regarding inhibition in lipophilic micronutrient uptake (Borel, Caillaud, & Cano, 2015), potentially leading to deficiencies over extended periods, as well as issues relating to the excretion of non-digested (or non-digestible) lipids, leading to abdominal discomfort and steatorrhea (loose stool formation and anal leakage) (Kelly et al., 1998). Somewhat paradoxically, such strategies may actually promote greater food intake, with some studies indicating that any inhibition of fat digestion can result in increased appetite (Ellrichmann et al., 2008).

The complete inhibition of fatty acid uptake as detailed above represents something of an extreme approach towards manipulating lipid digestion. However, extensive research has shown that the dynamics of fat structure during digestion can be more subtly manipulated to achieve particular physiological responses. These include the ability to influence satiety signalling through particular pathways, such as controlling the rate of gastric emptying (Marciani et al., 2009), or by triggering the so-called ileal break mechanism (Madadlou, Rakhshi, & Abbaspourrad, 2016). Other potential outcomes include the ability to influence the oxidative stresses associated with fat digestion (Michalski, Vors, Lecomte, & Laugerette, 2017), enhancing the efficacy of lipophilic micronutrient bioavailability and lowering cholesterol levels (Chen, McClements, & Decker, 2013). In this regard, whilst such approaches generally conclude that it is actually quite difficult to manipulate the overall extent of lipid uptake associated with consumption of fats or oils, it is possible to exert some control over the rate and location of digestion. It should be noted that there is now extensive coverage in this field, and that collated findings are well represented in a number of excellent review articles (Golding & Wooster, 2010; Mao & Miao, 2015; McClements, Decker, & Park, 2009; Singh, Ye, & Horne, 2009; van Aken, 2010).

4.3 Principles of Gastric Fat Digestion, and Structural Pathways for Controlling This

The digestion pathway of lipids can again be influenced across varying length scales, from molecular through to material, and the dynamic response of these various length scales to the conditions encountered from point of ingestion and subsequent transit through the GI tract. However, the colloidal state is arguably of greatest consequence in ensuring effective lipid digestion. The poor miscibility of fats and oils in aqueous media requires the hydrolysis of triglycerides to be mediated through the adsorption of lipases at the oil–water interface. This facilitates the formation of nano-structured mixed micelles comprising a number of amphiphilic moieties including the hydrolysed fatty acids and monoglycerides, as well as bile salts, phospholipids and cholesterol. Interaction of these micelles and the brush border of small intestinal enterocytes enables diffusion of these structures across the membrane and into the epithelial cells (Lentle & Janssen, 2011).

Ignoring the debatable role of lingual lipase, lipid hydrolysis primarily occurs in the stomach via an acid stable gastric lipase, and in the small intestine via a bile salt dependent lipase–co-lipase complex. Gastric lipase is an acid stable enzyme that is

secreted by the gastric chief cells in the fundic mucosa in the stomach. It has an optimum pH range of 3–6 and its amphiphilic structure allows for effective adsorption and positioning of the active site at the oil–water interface. Gastric lipase partially hydrolyses triglycerides to one fatty acid and one diglyceride, and is estimated as providing around 10–30% lipolysis in adults (whilst accounting for up to 50% total lipolysis in neonates, due to immaturity of the pancreas). Lipid digestion is continued in the small intestine via action of pancreatic lipase. Pancreatic lipase is the primary lipolytic enzyme during digestion, hydrolysing triglycerides at the 1,3 position to liberate two molecules of fatty acid and an Sn-2 monolgyceride. It is less effective at adsorbing at the oil–water interface than gastric lipase, and activity is greatest in the presence of co-lipase and where the adsorption of surface active bile salts to the oil–water interfacial layer has taken place (it can be argued that a key role of gastric lipolysis is to promote the occupancy of fatty acids at the oil–water interface, thereby facilitating the initial adsorption of the bile–lipase–co-lipase complex). The activity of pancreatic lipase can vary according to the presence of bile, but under physiological conditions is optimum at slightly alkaline pH. Furthermore, the adsorption of bile salts at the oil–water interface appears not only to enable pancreatic lipase adsorption, but is considered critical in the assembly of the mixed micelle moiety.

Lipolysis during both gastric and small intestinal transit represents an integral aspect of lipid digestion, and one that provides the most likely opportunity for manipulation. Indeed, much of the research focus in recent years has been the investigation of mechanisms by which the rate and extent of lipolysis can potentially be influenced. The previous section has indicated one such approach, in the use of drugs such as orlistat that specifically bind to the enzymes, effectively disabling their catalytic functionality to hydrolyse triglycerides. Whilst the side effects of enzyme blocking have been reported, it is noteworthy that this approach can be effective regardless of the structural or compositional state of the ingested lipid. In contrast, other approaches capable of influencing lipolysis are more reliant on manipulation of the colloidal state to achieve an effect.

The first of these is the creation of interfacial layers capable of acting as barriers to lipase adsorption. This concept tends to be most readily applicable to processed foods, such as milks, yogurts and ice cream, which incorporate oil-in-water emulsions, and for which the interfacial layer can be designed and manipulated through appropriate formulation and process control. The stabilisation of commercially produced food emulsions is generally achieved through two main classes of emulsifier: high molecular weight biopolymeric species or low molecular weight surfactants (a third option, that is, Pickering stabilisation via particulate adsorption has received considerable attention in the scientific literature but is not currently an established approach in product manufacture).

High molecular weight biopolymeric materials typically encompass a broad range of proteins, most commonly milk proteins (and for which there are multiple ingredient options provided by suppliers), but also increasingly from non-dairy sources such as soy, wheat, egg, pea and rice, amongst others. In addition, a number of additive systems capable of stabilising emulsions (generally derived from poly-

saccharides) are commercially available. These include gum Arabic, acetylated pectins and a number of chemically modified materials, such as OSA starch, propylene glycol alginates and hydrophobised cellulose derivatives (e.g. hydroxypropyl cellulose).

Low molecular weight surfactant species are based specifically on edible lipids, and tend to have well defined amphiphilic characteristics in which the hydrophobic domain is based on a fatty acid chain (which can vary in length and degree of saturation) and for which the acid group is esterified with a polar or charged moiety which provides the hydrophilic aspect of the molecule. These materials are generally classed as additives, and are more commonly termed as emulsifiers, which can be misleading as their technical function in food extends beyond just the stabilisation of droplets. Phospholipids represent the most naturally derived class of low molecular weight surfactants, and can be structurally summarised as two fatty acid chains linked to a phosphate head group. Collectively termed as lecithins, phospholipids can be obtained from a number of dairy and plant sources, including milk, eggs and seeds (e.g. sunflower and canola). Depending on source material, phospholipids can show broad structural variation in both fatty acid composition and in regard to the side-groups attached to the phosphate head group, which in turn influences their functionality as used in food products. Commercially produced lecithins typically comprise a mix of phospholipids reflective of the raw material used in their production. Chemically synthesised polar lipids are also widely utilised within the food industry, in which fatty acids (usually based on palmitic, stearic or oleic fatty acids chains) are chemically esterified with a head group containing at least one reactive hydroxyl group. Monoglycerides, which are produced through reaction of triglycerides or fatty acids with glycerol, are the largest class of chemically manufactured class of emulsifiers. Further variations in head-group type are able to produce an extended range of edible surfactant systems, which are broadly classified according to the relative contribution of hydrophobic and hydrophilic domains within the molecular structure (the so-called hydrophilic–lipophilic balance, or HLB). The specific properties of emulsifiers can vary considerably according to the choice of fatty acid and head group, and thus this particular class of food additives is able to provide very broad, yet specific, technical function in food production, including the stabilisation of oil-in-water emulsions.

Both biopolymeric and low molecular weight surfactants are extensively used in the stabilisation and structuring of food emulsions, noting that the particular choice of emulsifier, its behaviour during processing, and its interactions with other components within a formulation provide a determining effect on the stability, structure and properties of the emulsion. As such, there is considerable interfacial and structural variety across the range of emulsified foods that we consume. The particular interfacial composition of food emulsions can influence the pathway by which these materials are processed during gastrointestinal transit; however, as indicated earlier it appears that our physiology is well equipped to manage different interfacial states and structure, given the almost complete ability to uptake dietary lipids when integrated across the entirety of the stomach and small intestine.

The digestion of food emulsions and the particular role of interfacial composition have been widely explored, from model systems to actual foods and from in vitro characterisation through to human trials. In considering the gastric stage of digestion, notable differences are observed depending on whether emulsified lipids are stabilised with proteins, polysaccharides or surfactants. If we first consider the stabilisation of emulsion systems with protein (which is arguably the most widely utilised approach for formulating soft solid and solid emulsion-based foods), it becomes apparent that such systems can be particularly sensitive to the pH dynamics in the under gastric conditions. In the fasting state, the pH of the stomach is typically 1.9–2.0. However, this can rise significantly on ingestion of food due to the buffering effects of the food system, and this is particularly evident for protein-based compositions. In the case of protein-stabilised emulsions, the initial elevation of gastric pH is followed by gradual lowering back towards fasting levels, which can effect a transition through the isoelectric point for some protein systems, such as the caseins (van Aken, Bomhof, Zoet, Verbeek, & Oosterveld, 2011) and whey proteins, and which can lead to rapid flocculation of emulsion droplets in the early stages of gastric incubation (Bellesi, Martinez, Ruiz-Henestrosa, & Pilosof, 2016). The stability of protein stabilised emulsions can be further compromised during residence in the stomach as a consequence of proteolysis of the interfacial layer by pepsin.

The consequences of peptic hydrolysis on protein coated interfacial layers and emulsion properties have been explored in a number of studies. At the planar oil–water interface, Maldonado-Valderrama and co-authors (Maldonado-Valderrama, Gunning, Wilde, & Morris, 2010) showed how proteolysis under gastric conditions affected the structural and mechanical properties of a β-lactoglobulin adsorbed layer, demonstrating that whilst partial hydrolysis of the interfacial protein layer took place leading to a reduction in surface tension, complementary atomic force microscopy showed that, to a degree, the interconnected interfacial network remained intact. Accordingly, the dilational elasticity of the interface was only partly lowered as a consequence of enzymatic digestion. The retention of the interfacial network was speculated to be due to strong hydrophobic interactions, not only between protein and the interface but between neighbouring peptide fragments.

In the case of a model β-lactoglobulin-stabilised emulsion, Sarkar et al. (2010a) showed that in vitro exposure of the emulsion systems to gastric fluid at pH 1.2 and containing pepsin resulted in a time-dependent reduction in zeta potential from +50 to +17.6 mV during a 2-h incubation. This reduction in zeta potential was attributed to the detachment of charged domains of the protein layer from the interface. SDS-PAGE measurements were additionally used to show that after 2 h of digestion only ~20% of the interfacial protein membrane remained intact.

Proteolytic removal of the electrostatic and steric stabilising layer can also lead to flocculation of protein-stabilised emulsions (Hur et al., 2009; Golding et al., 2011), promoting droplet association through increased hydrophobic interactions (as observed, this may occur in the later stages of gastric incubation, compared to flocculation as a consequence of pH effects). A further consequence of the interfacial proteolysis is a reduction in the mechanical strength of the interface (Maldonado-Valderrama et al., 2009). Thus, for flocculated, weakly stabilised emulsion droplets,

even mild shear conditions can be sufficient to induce coalescence. For liquid-oil-stabilised emulsions this has been shown using microscopy in a number of separate studies (Hur et al., 2009), which has shown coalescence susceptibility as a consequence of prolonged exposure to the simulated gastric environment. Both flocculation and coalescence of may lead to a phase separation of the emulsion during gastric digestion.

It should also be noted that the initial interfacial proteolysis of protein stabilised emulsions may facilitate the adsorption of gastric lipase at the oil–water interface by creating hydrophobic domains that provide preferential adsorption sites for the lipase enzyme to bind to (Lueamsaisuk, Lentle, MacGibbon, Matia-Merino, & Golding, 2014, 2015). As indicated earlier, gastric lipolysis only accounts for approximately 10–30% of total synthesis of fatty acids. However, this initial lipolytic step can itself result in dynamic changes to the composition of the interfacial layer during gastric incubation, due to the accumulation of surface active fatty acids at the oil–water interface (Pafumi et al., 2002b). The build-up of fatty acids at the interface ultimately becomes inhibitory to lipase accessibility to the interface (such that lipase action effectively ceases at surface fatty acid concentrations of between 110 and 120 μmol m^{-2}).

Thus, the extent of gastric lipolysis is essentially surface limited, and accounts for the relatively low degree of overall conversion. An accompanying effect of gastric lipolysis is that the presence of surface polar lipids can render droplets sticky leading to aggregation (Fig. 3) and propensity towards phase separation (although it has been noted that coalescence arising from droplet association via this mechanism does appear limited) (Golding et al., 2011; Pafumi et al., 2002a). In considering the overall mechanisms of lipid digestion, an argument can be made that a key purpose of gastric lipolysis is to create a favourable interfacial environment for subsequent small intestinal lipolysis, on the basis that a fatty acid coated layer would most likely provide interactive sites for bile salt association, thus commencing the formation of mixed micelles and facilitating the adsorption of the lipase–co-lipase complex.

Whilst there are a number of non-protein biopolymeric emulsifiers capable of stabilising food emulsions, these are less widely encountered in actual manufactured food products. The use of gum Arabic as an emulsifier for the production of encapsulated bioactive materials or in the stabilisation of lipophilic flavour emulsions provides specific examples, and indicates some of the benefits in using such materials compared to proteins, such as maintenance of physical stability over a wide range of pH, ionic and thermal conditions. For these same reasons, non-protein biopolymer stabilised emulsions can display distinctly different behaviours during gastric incubation when compared to protein stabilised emulsions. Notably, adsorption of particular polysaccharide layers can provide effective steric stabilisation under the acidic conditions encountered in the stomach, and therefore do not undergo pH mediated flocculation. This is exemplified by research undertaken by Bellesi and co-authors (Bellesi et al., 2016), who demonstrated that emulsions stabilised with hydroxpropylmethyl cellulose, a non-ionic cellulose derivative, did not undergo significant destabilisation during gastric in vitro treatment when compared to similar emulsions stabilised with either soy protein or whey protein isolate. In

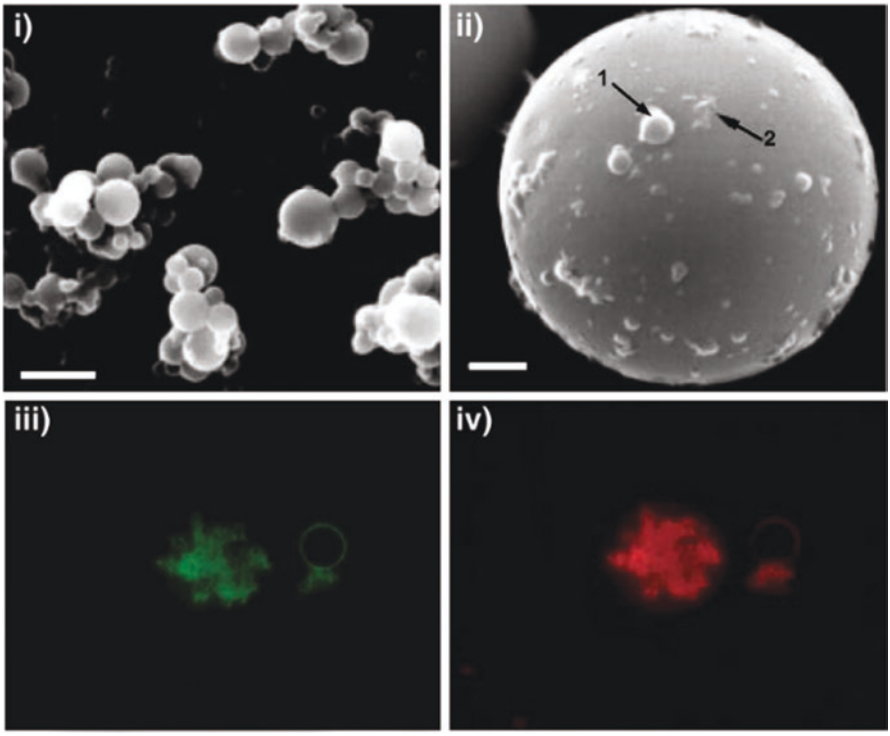

Fig. 3 Evolution of protonated fatty acids during digestion inhibits gastric lipase: During the digestion of triolein emulsions (**a**) bumps on the surface of the emulsions are seen to appear over time (**b**). Labelling of gastric lipase with fluorescein isothiocyanate FITC (green) and the fatty acids with copper (red) revealed that they were co-localised within the surface bumps. This finding explains why gastric lipolysis action ceases when surface fatty acids concentrations reach 110–120 μmol m^{-2}. Reproduced with permission from Pafumi et al. (2002b)

addition to resistance to flocculation under gastric pH, it was also observed that polysaccharide stabilised interfaces were impervious to peptic hydrolysis, which provide an additional mechanism for maintaining physical stability. Accordingly, consumption of such emulsions may lead to a more homogeneous distribution of droplets within the stomach during gastric residence which, as will be discussed, can have implications on gastric emptying rate. However, it is less clear, at least under in vitro conditions, as to whether such interfacial layers are in any way inhibitory to gastric lipase adsorption. This is primarily due to the current limited availability of human gastric lipase (or appropriate mammalian alternative) for incorporation into in vitro models. The use of various fungal lipases has been employed in some models, but these can lack physiological equivalence, with variations in active site location, specificity and pKa.

Whilst the above examples give consideration to the use of various amphiphilic gums as emulsifiers, it should be noted that the majority of food grade polysaccharides are hydrophilic and thus not surface active, and cannot therefore be used directly

for the stabilisation of emulsions. However, it is possible to attach polysaccharides to protein coated droplet through various binding mechanisms, most commonly electrovalent or covalent cross-linking or combinations of the two. Interfacial layers comprising protein-polysaccharide conjugates have also been shown to impart effective droplet stabilisation when exposed to pH conditions representative of the stomach (McClements & Li, 2010; Tokle, Lesmes, Decker, & McClements, 2012).

4.4 Fat Partitioning in the Stomach and Gastric Emptying

Variations in interfacial composition show that the physical stability and extent of lipolysis of food emulsions within the stomach can differ considerably according to formulation and thus be manipulated. Whilst such variations tend not to have significant consequence in altering the total lipid uptake from a food or meal, there can be some interesting differences relating to the kinetics of digestion, notably the specific influence of fat partitioning within the stomach on gastric motility and the rate at which lipids are released into the small intestine. The relationship between fat distribution in the stomach and gastric emptying has been established using model beverage emulsions in a number of separate in vivo studies. This includes research by Marciani and co-workers (Marciani et al., 2009; Marciani, Wickham, Bush, et al., 2006; Marciani, Wickham, Singh, et al., 2006; Marciani et al., 2007), who demonstrated how the partitioning of the fat phase in the stomach affected emptying. Consumption of 500 ml beverage emulsion systems that were designed to either separate or remain stable in the stomach were used to explore how fat separation influenced rate of emptying. The distribution of fat in the stomach was visualised using magnetic resonance imaging (MRI), which was able to clearly distinguish between the gastric-stable and gastric-separating compositions. For these model emulsion formulations, gastric emptying was observed to be initially more rapid for consumption of gastric-unstable emulsions, due to release of the oil-depleted aqueous phase into the intestine, with the emptying rate significantly decreasing on release of the oil-separated portion into the intestine. In comparison, the gastric-stable emulsion displayed a slower and more uniform rate of emptying, due to consistent release of fat from the stomach during the emptying period. Differences in emptying behaviour showed correlations with CCK response, such that the gastric unstable emulsion induced significantly lower measured plasma CCK in comparison to the gastric-stable emulsion, for up to 6 h after initial consumption of the emulsion. Data from satiety assessment also indicated that the acid-stable emulsion was more effective at suppressing hunger.

Further research comparing separated and stable emulsion systems across a range of fat and macronutrient concentrations showed this to be a reproducible and controllable phenomenon (Foltz et al., 2009; Keogh et al., 2011).The specific emptying behaviour of the layered emulsion is attributed to a two-stage mechanism with rapid emptying of the oil-depleted aqueous portion of the meal. With little or no delivery and detection of fat in the intestine, there is minimal secretion of CCK, and

accordingly emptying progresses rapidly. Once the fat layer leaves the stomach and enters the intestine there is a corresponding increase in CCK which slows down the rate of emptying. In the case of the emulsified beverage, commencement of gastric emptying results in immediate delivery of lipid into the intestine. The corresponding stimulation of CCK then regulates the rate at which meal contents is released from the stomach and this feedback mechanism proceeds according to the continuing detection of fat on emptying. In comparison to the layered emulsion, this results in a more uniform emptying rate, with markedly slower emptying in the early stages of digestion.

Arguably, much of the research conducted on lipid digestion, including the examples provided thus far, applies primarily to stable, non-interacting emulsions prior to exposure to gastric conditions. Thus, in terms of food formats, the model systems presented within these studies would mostly be considered representative of beverage formulations, such as milks, infant formulae, liquid dietary supplements and even ice cream (which can be considered liquid post-consumption).

In contrast to many of the systems discussed above, non-colloidal fatty foods and for fat continuous emulsion systems do not typically enter the stomach in the form of finely emulsified droplets. The markedly low surface area of such foods might be expected to reduce the rate and extent of gastric lipolysis, as this will greatly reduce the availability of binding sites for lipase adsorption compared to finely dispersed oil–water emulsion systems for which the surface area may be orders of magnitude higher. The surface area of unstructured fatty foods can be increased through oral processing, noting that the mechanical action in mouth coupled with mixing with saliva can enable the crude emulsification of fats into large droplets (Adams, Singleton, Juskaitis, & Wilson, 2007). Likewise, the eating process of fat continuous foods such as chocolate or butter can lead to the phase inversion of the initial structure during oral residence, resulting in the formation of oil-in-water type structures, albeit with large droplet size distribution (Rodrigues et al., 2017). Even crude homogenisation can greatly increase surface area compared to a fully phase separated oil layer, which can assist with the onset of lipolysis. It should be noted however that the large size of such droplet will still most likely result in some partitioning of the lipid phase towards the stomach, which as indicated may lead to differences in rate of emptying compared to finely dispersed gastric stable compositions.

For semi-solid or solid protein stabilised emulsion foods (such as cream cheese, Greek yogurt or processed cheese), the composition and structural state of the food causes greater complexity in the way in which these systems behave during digestion. A complicating factor is that whilst the structure of liquid emulsions tends to be minimally impacted during consumption, the oral processing of soft solid or solid food can lead to a considerable alteration in structure. Most examples of such foods are in the form of oil-in-water emulsions; however, there can be considerable variations in droplet size, surface composition and interactions with the surrounding continuous phase. For example, many soft solid emulsions comprise an appreciable amount of protein, which can contribute to the structure as well through formation of the type of network structures discussed in Sect. 3.4. Given that the kinetics of lipolysis are determined by the ability of the lipase to access the droplet

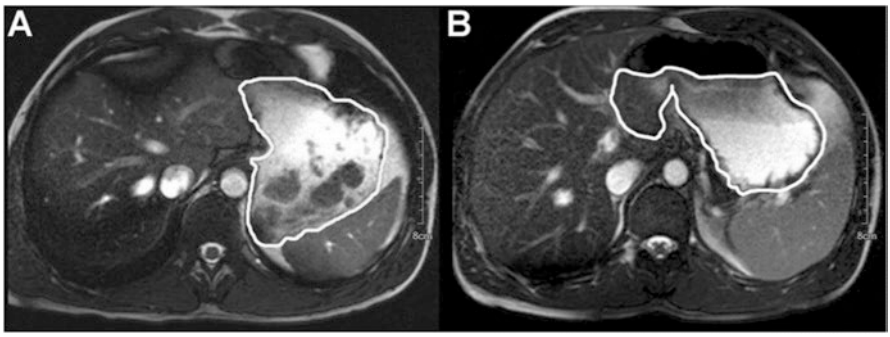

Fig. 4 MRI images of the semi-solid meal in the stomach (outlined) 5 min after consumption (**a**) and the liquid meal in the stomach (outlined) 25 min after consumption (**b**). Reproduced with permission from Mackie, Rafiee, Malcolm, Salt, & van Aken (2013)

surface, such structures may initially be inhibitory to lipase adsorption. The mechanical action in the stomach and the concerted action of pepsin will invariably lead to a deconstruction of the ingested food structure, but the onset and rate of lipolysis may be slower than that of non-structured emulsions (Wooster et al., 2014).

The behaviour of these more complex food structures during gastric digestion may additionally influence the location and distribution of fat. This is exemplified by studies undertaken by Mulet-Cabero and co-authors (Mulet-Cabero, Rigby, Brodkorb, & Mackie, 2017) and by Mackie and co-authors (Mackie et al., 2013), who demonstrated differences in gastric emptying rates for two isocaloric meals, one of which was in the form of a liquid emulsion and the other as a semi-solid comprising a mixture of yogurt and cheese. In the case of the semi-solid meal, gastric sedimentation was observed, whilst the liquid composition resulted in creaming of the lipid phase (Fig. 4). The effect of these differing structural dynamics was reflected in modification to a number of associated physiological responses. Notably, gastric emptying rate was initially slowed for the sedimenting meal, leading to relative higher retained gastric volume over a 180-min period when compared to the creaming meal. This is attributed to the sedimenting meal providing an initial release of nutrients to the small intestine, thereby regulating the subsequent rate of release from the stomach of the remainder of the meal.

4.5 Lipid Digestion in the Small Intestine

Lipolysis in the small intestine continues to completion through adsorption of colipase-dependent pancreatic lipase, which hydrolyses triglycerides at the sn-1 and sn-3 positions to liberate two fatty acids and an sn-2 monoglyceride. Pancreatic lipase is active under the neutral to mildly alkaline conditions found in the small intestine, with optimal activity at ~6.5. Whilst able to catalyse lipolysis through direct adsorption to an interface, its adsorption is more readily inhibited by the

presence of any surface-active components already occupying the interface. Accordingly, lipolysis efficiency is greatly enhanced by the secretion of co-lipase and bile salts (Blackberg et al., 1979). Co-lipase is a non-enzymatic protein co-factor which is more amphiphilic than pancreatic lipase, and is able to form complexes with the C-terminal region of the enzyme. This complexation provides a more favourable environment for adsorption at the oil–water interface (Lowe, 1997). However, lipase–co-lipase adsorption may still be inhibited by highly surface-active molecules adsorbed to emulsion droplets on entry to the small intestine. For example, stabilisation of an emulsion system with the small molecule surfactant Tween 80 was shown to render the interface impervious to both pancreatic lipase and complexed lipase–co-lipase (Gargouri, Julien, Bois, Verger, & Sarda, 1983).

Bile salts and phospholipid secretion by the liver can facilitate intestinal lipolysis of fats and oils (Maldonado-Valderrama et al., 2011). Whilst bile salts are amphiphilic, their structure is atypical of the usual head group–tail group molecular make-up of small-molecule surfactants. The basic structure of bile salts can be described as a rigid steroid backbone comprising hydrophobic and hydrophilic faces which is attached to a flexible region. Bile salts can be expected to possess greater surface activity than most edible amphiphiles used for interfacial stabilisation of fats and oils and will tend to displace these through a mechanism of orogenic displacement (Bellesi, Ruiz-Henestrosa, & Pilosof, 2014; Maldonado-Valderrama et al., 2008).

However, for polar lipid surfactants with greater relative surface activity than bile salts orogenic displacement is less likely to occur. Instead, it has been shown that for phospholipid monolayers, a synergistic interaction between the bile salts and the polar lipid interface allows for bile salts to be co-adsorbed at the surface, as evidenced by a lowering of interfacial tension beyond that of the surfactant system alone (Gallier, Shaw, et al., 2014).

The adsorption of bile salts at the interface is an important step in the digestive process, as it provides binding sites for the co-lipase–lipase complex at the interface, allowing lipolysis to proceed. This has been exemplified through investigation of the digestion of model emulsions stabilised by the non-ionic surfactant Tween 80, where it was observed that in the absence of bile salts, the lipolysis of the Tween stabilised emulsions was inhibited, whereas the inclusion of bile salts enabled the adsorption of the lipase–co-lipase complex, allowing lipolysis to proceed (Gargouri et al., 1983).

With this in mind, any interfacial mechanism that is able to inhibit adsorption of bile salts to the oil–water interface is likely to limit lipolysis. This has been demonstrated for oil droplets stabilised with non-ionic galactolipids. In comparison to charged phospholipids, galactolipids had reduced interaction with bile salts whilst imparting a more densely packed interface, thus restricting bile salt accessibility to the oil surface, and with a corresponding reduction in susceptibility to lipolysis (Chu et al., 2009). The formation of multilayered interfacial layers has also been shown to be effective against bile-salt adsorption, as exemplified by the formation of charge complexed bilayers comprising anionic lecithin and cationic chitosan, which was shown to have a reduced rate of lipolysis under in vitro intestinal conditions when compared to a control emulsion solely stabilised by lecithin (Mun,

Decker, Park, Weiss, & McClements, 2006). However, whilst the effects of interfacial complexation clearly resulted in a modified lipolysis rate under in vitro conditions, replication of the study design in vivo (using a mouse study), did not appear to have any influence on key digestive biomarkers relating to fat uptake when compared to an unmodified control (Park et al., 2007).

In this regard, it should be noted that whilst (under in vitro conditions and potentially via direct small intestinal intubation) variations in interfacial composition as presented by these example can influence small intestinal lipolysis, it is less likely that for the majority of consumed foods, the interfacial composition is likely to be inhibitory to bile salt adsorption, and indeed it may be the case that the original interfacial composition of any ingested food colloid may have already been displaced by fatty acids as part of gastric lipolysis.

However, lipase accessibility to an interface is not the only mechanism by which emulsion lipolysis in the small intestine can be influenced. As stated, lipolysis is an interfacially mediated process, and thus the efficiency of enzymatic hydrolysis should be determined by the available surface area of the substrate, that is, the oil–water interface. On this premise, decreasing the size of emulsion droplets entering the small intestine should, in principle, lead to increased rate and extent of lipolysis due to the increased availability of lipase binding with increasing surface area. In vitro studies on model emulsion systems designed to undergo gastric destabilisation destabilisation do appear to show that the rate and extent of pancreatic lipolysis is suppressed as surface area is reduced (Golding et al., 2011), with emulsions displaying coalescence exhibiting lower levels of released fatty acids in comparison to stable emulsions retaining high surface area. Additional in vitro studies, in which the droplet size of the emulsions is controlled prior to exposure to simulated intestinal fluid, also show that lipolysis is correlated to surface area (Li, Hu, & McClements, 2011).

However, these findings do not necessarily correlate when applied in vivo. Certainly, where gastric digestion is bypassed and emulsions are delivered directly into the small intestine through intubation, a relationship between droplet size and lipid digestion efficiency does appear to exist. This was demonstrated by Seimon and co-authors (Seimon et al., 2009) who investigated the small intestinal digestion of model emulsions with droplet size ranging from 0.26 to 170 μm. Their results indicated that the highest surface area emulsions generated statistically significant elevated levels of CCK and PYY, as well as higher levels of plasma triglycerides. The finer emulsions also had a marked effect on intestinal motility, reducing intestinal transit rates to ensure full digestion and uptake of fat. A further study, also looking at the effects of direct intubation showed that increasing surface area influenced not only digestive biomarkers, but also relative food intake.

In determining a correlation between surface area and intestinal digestibility of fat, these studies intentionally ignored the role of the oral and gastric environments on the emulsion structure and digestion dynamics prior to entry in the small intestine. A notable in vivo study by Armand and co-authors (Armand et al., 1999) took a more integrated approach, employing intubation of two model emulsions of differing size (10 and 0.7 μm) into the stomach and monitoring changes to size distribu-

tion, gastric and pancreatic lipase activities and fat digestion. Lipolysis under gastric conditions was seen to be greater for the fine emulsion, consistent with the argument that higher surface area facilitates lipolysis. However, lipolysis was accompanied by an increase in droplet size during gastric incubation. A difference in lipolysis between the two emulsions was also observed in the duodenum; again, somewhat greater for the fine emulsions. However, the overall plasma triglyceride counts were not significantly different between the two emulsions, indicating that the overall lipid uptake was unaffected by the initial droplet size. One other point of consideration from this study was the fact that the peak point for plasma triglyceride was significantly delayed in the case of the fine emulsion, indicating that differences in fat distribution during gastric digestion may have affected the rate of stomach emptying of the two emulsions. This apparent normalisation of emulsion structure during digestion is perhaps understandable, given the biological requirement to achieve effective lipid nutrient uptake. A number of studies suggest that bile salt/phospholipid adsorption appears to be an important factor in regulating the colloidal state during the intestinal stage of digestion (Golding et al., 2011; Nik, Wright, & Corredig, 2011; Sarkar, Horne, & Singh, 2010b), all indicating that for fine emulsions (droplet size typically <1 μm), significant coalescence can take place during incubation in intestinal fluid containing bile salts (irrespective of surface composition).

4.6 Fat Digestion: Mechanical Factors Altering Digestion

Thus far it appears that human physiology is well equipped to ensure effective lipid digestion across most natural or processed foods that comprise the human diet. The combined pH, enzymatic and physical conditions across the mouth, stomach and small intestine are able to accommodate a broad array of colloidal structure with remarkable variations in size, surface area, interfacial and lipid composition, enabling full hydrolysis of triglycerides from these diverse lipid sources, and complete utilisation of the rendered fatty acids. The above sections indicate that lipid and emulsion based interfaces and structures can be designed that extend beyond normal digestive mechanics, leading to variations in digestive outcome, these are not widely encountered as part of food design. However, it should be noted that lipid digestion can be influenced by the mechanical properties of the food, and where any ingested food system is consumed such that its material properties are resistant to digestion, this can lead to variations in ability of the body to full process the nutrients present within those structures. This has been observed for both natural and constructed food materials.

The most notable natural food system studied that exemplifies this approach is the digestion of almonds (and for which the findings have relevance in a number of nut, seed and grain based food materials). Almonds provide a rich source of protein, lipids and micronutrients. Indeed, the oil content of almonds can range from between 44 and 61% (Grundy, Lapsley, & Ellis, 2016). The lipids fraction of almonds is

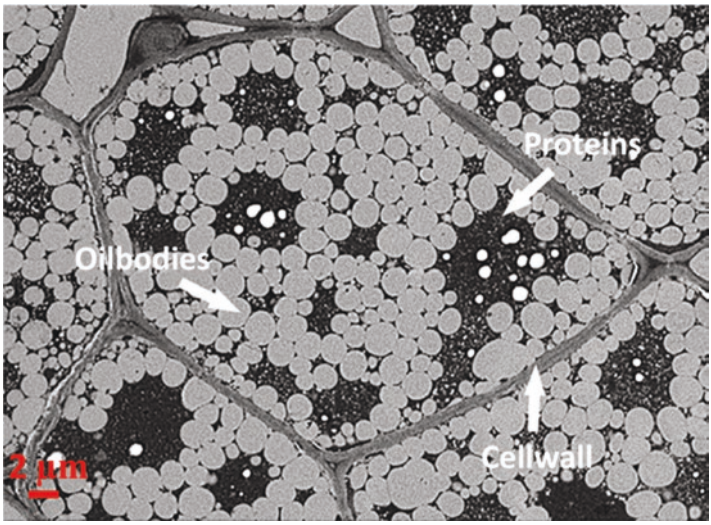

Fig. 5 Transmission electron micrograph image of almond kernel showing oil bodies (white inclusions), protein bodies (black inclusion) and the cell walls. Scale bar = 2 µm (Reproduced with permission from Grundy et al., 2016)

structured as discrete oil bodies contained within rounded parenchymal cells along with separate protein domains (Fig. 5). The size of the cells is ~35 µm and these are surrounded by a cell wall of 0.1–0.3 µm in thickness. The oil bodies themselves are stabilised by a phospholipid monolayer along with co-adsorbed oleosin protein and are of the order or 1–5 µm in size. In principle, the particle size distribution of the oil bodies within almonds should present an appreciable surface area for adsorption of digestive lipase and thus be readily digested. However, lipids have been detected in faecal samples obtained as part of a human study exploring the digestion of almonds, indicting incomplete uptake of lipids during digestion (Ellis et al., 2004).

Findings from this study and elsewhere (Grundy et al., 2015) imply that the oral processing of almonds is insufficient to fully disrupt the cellular structure, thus liberating the encapsulated oil bodies for digestion. Indeed, it is believed to be primarily the outer layer of the seed that is sufficiently masticated to enable access for lipase enzymes during gastric and small intestinal digestion, and that where disruption has taken place oil body coalescence can occur resulting in formation of liberated droplets in the order of 10–40 µm that are readily accessible for enzymatic adsorption (Ellis et al., 2004). Where cellular disruption has not occurred, the intact cell wall appears remarkably resistant to decomposition in either the stomach or small intestine, and thus oil bodies retained in these structures are not able to be hydrolysed and remain unavailable for transport across the epithelium. It has been postulated that some lipid leeching can occur during gastrointestinal transit, and that swelling of cellular structures may ultimately allow diffusion of digestive enzymes and bile salts into the interior of the cell at sufficiently long digestion times. It has also been observed that pectic fermentation by colonic microflora can eventually

start to breakdown cell structures along with fermentation of the lipid component. However, it is clear that a considerable portion of the lipid component is not available as metabolisable energy and thus the utilisable energy content of almond seeds is lower than the total energy content (Novotny, Gebauer, & Baer, 2012). A further consideration is that the inhibited uptake of the oil content present in almonds extends to a reduction in uptake of lipophilic micronutrients (such as tocopherols or phenolics) that are associated with the lipid phase.

Any modification of the native structure through processing might be reasonably expected to impact on the digestibility of the material; accordingly, the impact of thermal processing (roasting) and mechanical disruption have been investigated in relation to the bioaccessibility of the lipid fraction of the almonds. A number of studies show that roasting appears to actually have limited impact on the availability of the lipid component when the almonds are consumed whole kernels (Bornhorst et al., 2013; Mandalari et al., 2014). Likewise, limited comminution of the intact seeds into a ground state has only a marginal effect of availability. In contrast, processing approaches that fully disrupt the cellular structure, thereby liberating the entrapped oil bodies, such as in the production of almond milk, have been shown to greatly enhance the viability of the droplets towards lipolysis, and thus greater uptake is observed (Gallier, Rutherfurd, Moughan, & Singh, 2014).

Increasing the mechanical structural resistance of lipids to digestion has also been observed in manufactured emulsion systems. The particular approach demonstrated by Golding and co-authors (Golding et al., 2011) explored the hypothesis that lipid digestion rate and extent could be decreased in accordance with a decrease in surface area of fat during the digestion process. To this end, the study design focussed on synthesising a number of model emulsion systems that could be dynamically structured under gastric condition, from stable to flocculated to coalesced, allowing control over surface area of the emulsion during digestion. Whilst in vitro measurements showed that rate of lipolysis and liberation of free fatty acids could be affected by changes in surface area, a corresponding human trial showed that plasma triglyceride concentrations were not significantly affected as a consequence of any structural changes occurring during digestion (although it was observed that the emulsion predicted to undergo gastric coalescence did demonstrate a delay in onset of plasma triglycerides, consistent with previous studies in which lipid separation in the stomach delayed the entry of lipids into the small intestine) (Golding et al., 2011; Keogh et al., 2011).

These findings applied when the emulsions comprised oils that were fully molten at in body temperatures. When reformulated using a fat blend that had a solid fat content of 25% at 37 °C, the digestion properties were more noticeably affected. This was particularly true of an emulsion system designed to undergo partial coalescence (i.e. droplet agglomeration) in the stomach, leading to the formation of visible lumps of aggregated fat. Under in vitro conditions the partially coalesced emulsion was shown to have a markedly reduced rate and extent of fatty acid liberation arising from lipolysis. When extended to the human study, a similar pronounced suppression of plasma triglyceride was observed. The interpretation of these findings suggested that whilst the surface area reduction of liquid oil droplets was ultimately

limited by gastrointestinal emulsification, ensuring that a colloidal state could be maintained throughout the digestion process, the biomechanics of the GI-tract were insufficient to break up the partially coalesced aggregates of fat, and thus the low surface area was constant during digestion.

Interestingly, an additional study not only provided further evidence of this effect, but also determined that the interactions leading to partial coalescence during gastric digestion could be influenced by the continuous phase composition of the emulsion, leading to variation in rate and extent of fat digestion dependent on whether partial coalescence was able to take place in the stomach (Wooster et al., 2014). Whilst the findings demonstrated that this approach could modulate the rate of lipid digestion, the translation of this effect into food systems is still limited, since most dietary fats or oils (whether consumed as part of natural or processed food materials) tend to be fully molten at in body temperatures. A particular challenge of utilising this effect in a food product (acknowledging that a slower rate of lipid digestion may have beneficial effects in reducing lipaemic inflammation) is the potential alteration of textural properties associated when utilising a fat source that remains solid at in-body temperatures.

5 Effect of Structure and Composition on the Digestion of Carbohydrates

5.1 Digestion of Glycaemic Carbohydrates

5.1.1 Monosaccharides and Disaccharides

As with protein and lipids, the purpose of carbohydrate digestion is to render the carbohydrate component of food materials into a molecular state utilisable by the human body: in the case of carbohydrates, primarily as a source of metabolisable energy. Similarly, this process is predicated through biochemical translation of carbohydrate molecules through a suite of different carbohydrase enzymes, producing component monosaccharides capable of diffusing across the small intestinal epithelium. It is worth noting that certain dietary monosaccharides, namely glucose, galactose and fructose, are in a form already able to be transported across the epithelium, noting that whilst glucose immediately utilisable for metabolism by all tissues, the majority of fructose and galactose is metabolised in the liver (Englyst, Liu, & Englyst, 2007). Monosaccharide carbohydrates are highly water soluble, and in aqueous media (such as beverage formats, particularly those designed for sports nutrition) can be rapidly absorbed due to fast transit of liquids through stomach and small intestine, along with the lack of need for hydrolysis.

For foods with a higher level of structural complexity, the bioavailability and rate of uptake of the becomes more dependent on the liberation of the carbohydrate from the food matrix during digestion. This can apply to both natural food materials. For wholefoods, such as fruit and vegetables, the carbohydrate is solubilised within the

cell structure of the material. For manufactured foods, the structural state of the carbohydrate can vary considerably, being present in liquid form, in products such as ice cream and yogurt, through to incorporation as a solid in low moisture compositions, such as confectionary, biscuits and cereal foods. Liberation of monosaccharides from these carbohydrate containing foods commences in the mouth, where oral processing is able to breakdown structures thereby exposing the carbohydrate to the oral cavity. Additionally, mixing with saliva is able to commence the solubilisation of solid state monosaccharides leading to dissolution of structures. These processes are invariably linked to temporal hedonic perception of sweetness (Arancibia, Costell, & Bayarri, 2013; Kohyama, Hayakawa, Kazami, & Nishinari, 2016).

It should be noted that the extent of release of soluble material will be partly dependent on the bolus forming properties of the consumed food. For example, food structures formulated predominantly with soluble carbohydrate components, such as confectionary products can be mostly disintegrated during the eating process, leading to extensive liberation of any monosaccharide component on entry to the stomach. Conversely, solid foods containing other macronutrient components may be broken down in the mouth, releasing a certain amount of carbohydrate prior to bolus formation. Subsequent bolus formation may, in turn, cause some reassembly of the components in the food, thereby reducing the extent of oral solubilisation. Likewise, for carbohydrates contained in plant tissue in fruits and vegetable, cellular breakdown during oral processing may release some of the encapsulated sugars; however, depending on the material properties of the food in question (which in turn may be governed by factors such as ripeness), the mechanics of eating may not complete breakdown all the cell structures leading to limited retention on entry into the stomach (Harker, Amos, Echeverria, & Gunson, 2006). On entry to the stomach, exposure to gastric fluids and mixing causes further release of carbohydrates from structured food systems—a process which can be aided by the hydrolysis of protein structures. For multicomposite natural or manufactured food material, the kinetics of food structure breakdown in the stomach can influence the rate of small intestinal uptake of monosaccharides (Southgate, 1995). This can be due to either the rate and extent of liberation of the carbohydrate component from the digesta during gastric incubation, or alternatively due to a variation in rate of gastric emptying arising from the breakdown and digestion of food structures and specific effects such as CCK regulation of emptying rate as a consequence of fat digestion (Rayner, Samsom, Jones, & Horowitz, 2001).

As indicated, for the monosaccharide carbohydrates glucose, fructose and galactose, digestive hydrolysis is not required to render these molecules in a transportable state across the epithelium. For other simple, soluble carbohydrate components, such as the range of disaccharides that can comprise our diet (most notably sucrose), a similar consideration in terms of release, and depending on structure, solubilisation of these materials during the eating and digestion process can influence their rate of uptake. However, these sugars require additional digestive hydrolysis to convert them into component monosaccharides that are compatible with epithelia diffusion. This hydrolysis step is located in the domain region of the small intestinal enterocytes that are correspondingly located proximally to the transporters which will carry the

hydrolysed sugars into the epithelial cells. The enzymes responsible for this part of the digestion process (isomaltase, sucrase and lactase inter alia) are not unbound in the intestinal lumen, but anchored to the membrane proteins in the plasma membrane of the enterocyte. The apical plasma membrane housing these so-called brush border enzymes comprises microvilli, which protrude from the cell and constitute the brush border region (Hooton, Lentle, Monro, Wickham, & Simpson, 2015). Arguably the combined action of oral, gastric and small intestinal digestion ensures that most disaccharide carbohydrate is in a bioavailable state (i.e. fully solubilised and not trapped within digesta material) for hydrolysis by the brush border enzymes.

5.1.2 Starch

The third glycaemic carbohydrate fraction that is assimilated in the small intestine is starch, which forms a significant compositional and structural component of many natural and manufactured foods. Starch is a polymeric form of glucose, and is produced by many plant materials as an energy store, with staple crops such as corn, potato and wheat being notably starch-rich. The molecular composition of starch comprises two main types, the linear, unbranched amylose fraction and the branched amylopectin fraction. Generally, amylopectin is the more abundant of the two, typically comprising 70–80% by weight of most plant materials; amylopectins are also of higher molecular weight relative to the amylose fraction. The large size of starch molecules prevents direct transport across the epithelium and thus enzymatic hydrolysis yielding free glucose is the mechanism by which both amylase and amylopectin can be digested. This is achieved at two specific locations during digestion.

Starch digestion is initiated in the mouth by secretion of an α-amylase (which is also termed ptyalin) present in saliva (Butterworth, Warren, & Ellis, 2011). This enzyme has optimum activity under in-body conditions, that is, at a pH of 7, and temperature of 37 °C. Whilst salivary amylose can hydrolyse starch into the disaccharide maltose, it is not common for this to happen during eating (as evidenced by the fact that starchy foods do not generally tend to increase in sweetness during oral residence). Oral processing can be sufficient for initial conversion of starch into oligosaccharide fraction as well as enhancing the solubilisation of insoluble starch materials. Whilst salivary amylase is inactivated in the acidic conditions in the stomach, in reality, inactivation can be delayed as a consequence of any pH buffering effects generated by an ingested food capable of retaining gastric pH levels elevated above that required for amylase inactivation (noting that this can be particularly evident in foods comprising high levels of protein). Additionally, bolus formation may lead to entrapment of amylase within the bolus structure. Slow diffusion of gastric juices into the bolus may reduce the rate of inactivation of the entrapped enzyme (Mennah-Govela, Bornhorst, & Singh, 2015), allowing hydrolysis to be continued within the stomach.

Starch hydrolysis is continued in the small intestine through action of pancreatic amylase which randomly cleaves the glycosidic bond to progressively reduce starch to oligosaccharide fragments and ultimately disaccharide units of maltose. The

maltose is then further acted upon by the brush border enzymes maltase and isomaltase, yielding free glucose that can in turn be transported across the epithelium.

The small intestinal digestion of starch is strongly dependent on the structure of the starch during the digestion process. The most digestible form of starch is typically found in manufactured foods where starch has been used for texturisation, noting that the use of starch as thickening system has been used as part of food preparation for hundreds of years. The mechanism of action arises as a consequence of heating in the presences of water which essentially melts and hydrates the crystalline starch structures leading to the swelling and rupture of starch granules along with the release of the amylose component, thereby causing a thickening effect. This expanded state provides a ready environment for diffusion and hydrolysis by both salivary and pancreatic amylases (Colonna, Leloup, & Buleon, 1992). Starch hydrolysis of cooked starches is further facilitated for food structures that are readily diluted or disintegrated during oral processing or gastric digestion. This is particularly evident in soft solid foods, such as custards (Zhou, Topping, Morell, & Bird, 2010). In contrast, more structurally resistant foods (during both eating and gastric digestion) or those able to form compact dense boluses during oral processing may serve to inhibit amylase diffusion and access to the starch component of foods, thus slowing the rate of hydrolysis (Fig. 6).

This has been observed with digestion of pastas, in which the gelatinised starch-protein network forms large fragments during oral processing that are relatively inhibitory to amylase diffusion during gastrointestinal transit (Thomsen et al., 1994). This is also noted to occur in cooked beans (e.g. baked beans), in which the starch component is present within the cellular structure of the bean. Cellular structures damaged during mastication enable accessibility for the amylases, however, where cell structures are undamaged, the encapsulated starch can be retained in an unswollen state due to spatial limitations, and accordingly remains impervious to digestion (Zhou et al., 2010).

The structural or compositional nature of a food can sufficiently restrict the amylolysis of the starch component of that food material. Where small intestinal uptake of hydrolysed starch does not occur, then digestion can still take place through fermentation in the colon. This is said to occur for non-glycaemic carbohydrates, which includes the so-called “resistant” starch as well as soluble polysaccharide components (such as pectin), and insoluble fibres (e.g. cell wall materials including cellulose and hemi-celluloses. The digestion of these materials will be discussed in the next section.

5.2 Digestion of Non-glycaemic Carbohydrates

5.2.1 Resistant Starch

Native starch in plant materials exists in the form of granules in which the starch is arranged in the semi-crystalline state. Granule size and shape can vary depending on the source material ranging from approximately 1–100 μm in size. The amylose

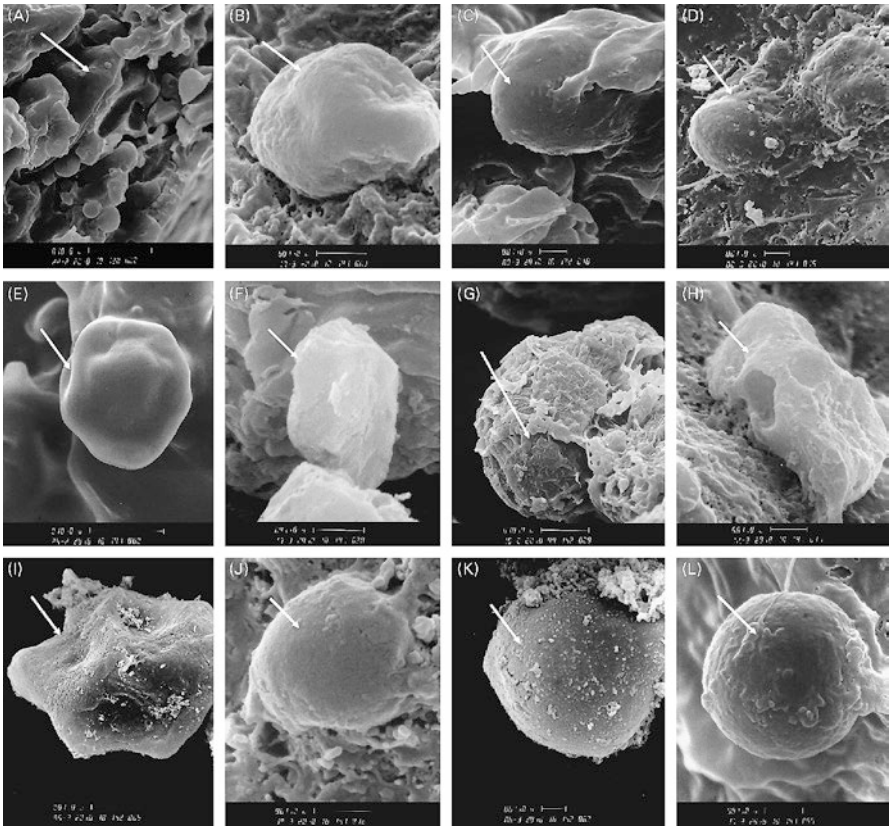

Fig. 6 Scanning electron micrographs of starches present in test foods and corresponding digesta. (**a**) and (**b**), muesli food and its digesta; (**c**) and (**d**), bread food and its digesta; (**e**) and (**f**), fried food and its digesta; (**g**) and (**h**), bean food and its digesta; (**i**) and (**j**), custard containing conventional maize starch and its digesta; (**k**) and (**l**), custard containing high-amylose maize starch and its digesta. Starch particle. Scale bar = 1.0 μm. (Reproduced with permission from Zhou et al., 2010)

fraction of starch tends to form single helical structures that are able to align into a double stranded crystallite arrangement whilst amylopectin is considered as forming double helix arrangements which can undergo further self-assembly into radially expanding domains of crystalline and amorphous structures. The crystalline structures, as present in the native plant source are effectively resistant to enzymatic hydrolysis (providing the terminology "resistant starch"), with amylose considered to be the more resistant of the two fractions (Sajilata, Singhal, & Kulkarni, 2006).

As indicated, heating in the presence of water leads to the formation of glycaemic starch due to expansion and hydration of the amylose and amylopectin structures. This can occur during the cooking of raw materials, such as rice and potato, as well as during the thermal processing of manufactured foods for which derivatised starch is used as an ingredient. Amylose tends to form stronger crystalline

assemblies that are surprisingly hydrophobic, and thus requires higher temperatures to undergo gelatinisation (Gallant, Bouchet, Buleon, & Perez, 1992). Accordingly, high amylose starches such as maize (or starch compositions with amylose added) can show resistant behaviours when the thermal processing is insufficient to disrupt the native crystalline state. This can also occur when starches are heated in the absence of water, which can be inhibitory to pasting. Under such circumstances, the starch component of low moisture baked or extruded foods can display delayed digestive behaviours. It should be noted that whilst maintaining the crystalline state of the native starch structure during processing is able to impart resistant behaviours during digestion, this can invariably lead to a loss of technical functionality, as the starch will not undergo pasting and gelatinisation. In this regard, product manufacturers need to have clarity about the rationale for starch inclusion as part of formulation design.

Another mechanism by which starch can demonstrate resistant properties is through retrogradation, which is a time-dependent recrystallization phenomenon that can occur after gelation has taken place—notably for high amylose starch compositions. This is the effect that causes the staling of bread, and can result in formation of increasing amounts of retrograded resistant starch (noting that for bread, such an effect is invariably considered a loss of product quality). Retrogradation can also lead to increased levels of resistant starch in cooked rice, in which cooled storage of the rice after cooking can cause recrystallization. This is exemplified in research undertaken by Nakayoshi and co-workers (Nakayoshi et al., 2015) determined that levels (% dry weight basis) of resistant starch increased from 2.5% for cooked rice to 7% when the rice was stored overnight at 4 °C. In the case of rice flour, the resistant starch content increased from 1.5% (after cooking) to 5% after chilled storage. These effects were most apparent in high amylose rice cultivars, noting that low amylose cultivars did not demonstrate any significant increase in resistant starch levels on storage (Hu, Zhao, Duan, Zhang, & Wu, 2004). Manufactured foods comprising starches used for the purposes of texturisation tend to be formulated with modified starches. Chemical reaction enables the attachment of various side groups (e.g. phosphorylation and hydroxylation) to the starch chains, which can provide a broader range of material and function properties. One particular consequence of the introduction of these side groups is that modified starches display a greater resistance to retrogradation than native starches, noting that such modifications do not appear to greatly influence the susceptibility of starch molecules towards amylolysis.

The relative balance of glycaemic and non-glycaemic starch has consequences for the levels of blood glucose arising from digestion. Purely glycaemic starch when consumed in foods that readily undergo rapid digestive breakdown, can lead to elevated postprandial blood sugar levels, are fall under the definition of high glycaemic index (GI) foods. The GI of glycaemic starches can be reduced by the matrix properties of the food, in which slower disintegration or more densely structured digesta can reduce the rate of starch hydrolysis and uptake (often termed as slowly digestible starch). In the case of resistant starch, the greater imperviousness to hydrolysis leads to a marked decrease in blood sugar levels and accordingly, foods comprising higher concentrations of resistant starch tend to be categorised as

being of low GI. The lower GI values associated with resistant starch are considered as beneficial in relation to a number of physiological biomarkers. The suppression of postprandial glucose is understood to improve insulin response as well as promoting lipid oxidation. Health benefits are reported to support mitigating obesity and reducing the propensity towards type 2 diabetes (Ashwar, Gani, Shah, Wani, & Masoodi, 2016).

Whilst resistant starch is less effectively digested in the small intestine, it is able to be broken down into utilisable by-products in the large intestine. The colonic microflora is able to ferment resistant starch fractions producing a number of short chain fatty acids. Short chain fatty acid profiling has indicated high levels of butyrate and somewhat lower levels of acetate being produced in comparison with other forms of non-glycaemic edible fibres. Short chain fatty acids provide an effective energy source for the colonic cells as well as broader utilisation by the body. Human studies have indicated that between 30 and 70% of resistant starch is metalisable, with the remainder excreted in faeces (Cummings, Beatty, Kingman, Bingham, & Englyst, 1996; Ranhotra, Gelroth, & Glaser, 1996). Variations in levels of malabsorbed resistant starch tend to arise as a consequence of the amount of starch consumed in the study design.

5.2.2 Non-starch Soluble and Insoluble Dietary Fibres

In addition to resistant starch a number of non-glycaemic carbohydrate material are consumed as part of regular dietary food intake (Dhingra, Michael, Rajput, & Patil, 2012). The generic terminology of these is fibre and this term can be further segmented into soluble and insoluble fibre. Both types are defined as being resistant to digestion and absorption in the small intestine, but capable of undergoing partial or complete fermentation in the large intestine. Both soluble and insoluble fibres are regularly encountered as part of consumption of fruit and vegetable and cereals. Insoluble fibre tends to be derived from the main structural elements in plant systems, notably as components in cell walls, such as cellulose and hemicellulose, or as a reinforcing component between cell wall structures as in the case of lignin. Whilst cellulose is a hydrophilic biopolymer possessing a linear primary structure comprising multiple glucose monosaccharides. In the absence of any branching or secondary or tertiary structure, its rigid, rod-like conformation allow it to undergo extensive intermolecular hydrogen bonding leading to the formation of fibrillary crystallites that are insoluble and, unlike starch, does not or hydrate on heating in aqueous media. In humans, it is also completely resistant to hydrolysis by any digestive enzymes within the gastrointestinal tract. Lignin is equally insoluble in water, but is markedly more hydrophobic than cellulose, possessing a highly branched phenolic structure. As with cellulose, it is impervious to gastrointestinal hydrolysis or digestion.

Soluble fibres are also inherently present in dietary fruit and vegetables and are generally classed as polysaccharides. Chief amongst these are the pectins, but also other soluble biopolymers such as the fructans (a class of polysaccharide that

includes inulin and which comprises fructose units), and the glucans. Pectin itself also contributes to cell wall mechanics, notably in supporting cell wall extension during plant growth. Whilst pectins can show some variation in molecular structure and composition, they share a common linear polymer backbone based on interspersed galacturonic acid and galacturonic acid methyl ester units in an interrupted repeat arrangement. Compositional variations are due to side chain attachment by various saccharide units. The functional properties of pectins vary accordingly with composition, demonstrating the ability to provide viscosification and gelling behaviours depending on conditions. All forms of pectin are resistant to small intestinal digestion but can undergo varying degrees of fermentation in the large intestine.

Ingestion of natural insoluble and soluble fibre is recognised as having a number of dietary and health related impacts (Potty, 1996; Slavin, 2013). The release of short chain fatty acids during colonic fermentation provide an energy source for the microbiota, as well as undergoing absorption in the colon. Dietary fibre may additionally assist in the regulation of blood sugar levels, as well as reducing total and LDL cholesterol levels. The effect of water binding and viscosification of both soluble and insoluble fibre may also to reduce rate of gastric emptying thereby enhancing satiety signalling, as well facilitating gastrointestinal transit and aiding in faecal bulking.

Consumption of fruit and vegetable rich foods provide one pathway for incorporation of fibre as part of dietary intake. However, manufactured foods may contain varying degrees of both soluble and insoluble fibres as part of product formulation. These fibre components are isolated from diverse raw materials and (particularly in the case of soluble polysaccharides) provide a broader spectrum of dietary polysaccharides than would normally be present in wholefoods. Thus, in addition to pectin (commercially derived various fruit sources such as citrus peel and apple pomace), other polysaccharides such as the alginates and carrageenans (from seaweed), guar and locust bean gum (from seeds), gum arabic and gum tragacanth (exudate gums) and xanthan (microbial expression) are now widely used in food production. These various polysaccharides are extracted from source materials for which their native function ranges from structural support through to acting as an energy source, or (as in the case of the exudate gums) providing a wound healing mechanism against structural damage.

The main soluble fibres materials used in food manufacturing share some common attributes, namely being high molecular weight biopolymer species assembled as extended chains of monosaccharide units. Variation in primary structure occurs through the presence and location of the different monosaccharide units comprising the polymer chain, whether these are non-ionic or ionic (noting that water structuring capacity of ionic polysaccharides can be sensitive to variable pH and ionic conditions), and whether the chains are linear or branched. The conformation of the primary structure can influence intramolecular interactions (predominantly hydrogen bonding) within the chain, leading to varying degrees of folding. The nature of polysaccharide secondary and tertiary structure can in turn impact on the ability of polysaccharides to undergo intermolecular interaction, leading to the formation of quaternary network assemblies, and which is characteristic of gelling polysaccha-

rides. In some cases, such as for the alginates and cellulose derivatives, additional chemical or biochemical treatments are able to alter the native molecular structure as a means of further tailoring physicochemical properties. Accordingly, their function in foods is primarily technical, enabling control over material properties through viscosification and/or gelation as well as other abilities such as emulsification, inhibition of ice recrystallization, film forming and foaming. For high moisture products such as ice creams, dressings and sauces, inclusion of soluble polysaccharides can greatly enhance product attributes, such as sensory properties, physical stability and shelf life extension.

In this context, whilst these materials can be classified as dietary fibres, they are not typically used for nutritional supplementation in manufactured food systems. This is primarily due to the fact that that the dosage levels to achieve a particular technical effect within a product are usually a few tenths of a per cent of the combined formulation, and thus not considered as being of sufficiently high concentration to achieve a nutritional benefit. That said, there has been increasing interest in recent years as to the function and role of isolated insoluble and soluble fibres during digestion (Lovegrove et al., 2017; Noack, Timm, Hospattankar, & Slavin, 2013). The inability to be digested in the stomach and small intestine and retention of native structure can enable the water structuring characteristics of polysaccharides to be at least partially retained during the digestion process. As discussed earlier, gastrointestinal motility can be manipulated in relation to the material properties of the digesta. Viscous or gelled materials can slow down the rate of gastric emptying and increase transit time within the small intestine, positively influencing satiation. The viscous properties of digesta can also serve to influence the rate of diffusion of nutrients to the epithelium. Such effects have been explored for a number of different polysaccharide systems. The digestive properties of sodium alginate, for example, have come under particular scrutiny for its ability to enable varying structural states in the stomach and small intestine, ranging from varying degrees of viscosity through to formation of gelled structures, for example, in the presence of calcium (Hoad et al., 2004) or (under acidic conditions in the stomach) through synergistic interactions with other polysaccharides such as pectin.

These effects have been mainly considered for pharmaceutical applications for which the polysaccharide is the primary structuring material, allowing the structural and material properties to be highly defined, as well as potentially bypassing consideration such as oral processing. Specific functions have included the controlled delivery of drugs, and a particularly novel application as a gastric raft (i.e. forming a structured environment of the surface layer of the gastric fluid), that has been demonstrated as being efficacious at mitigating the symptoms of acid reflux (Jang, Lee, Ryu, Son, & Kang, 2014). Other physiological effects, such as manipulating satiety have also been extensively studied for alginate as well as other polysaccharide systems, and whilst the use of model systems has demonstrated that effects can be generated that are influenced by the material properties of the polysaccharide during digestion, challenges remain in translating these effects into actual food systems, where the maintenance of the target structure through oral processing, gastric and small intestinal residence can be difficult to achieve, whilst still retaining the expected eating properties of the food in question.

Research interest in the digestive behaviours of polysaccharide carbohydrates has also tended to focus on those systems already permitted for use in food or pharmaceuticals. This is partly due to the challenges of bringing new materials through the clearance processes required for utilisation in food manufacturing. However, it is also the case that there are still countless materials naturally present in flora and fauna that may have specific functional digestive behaviours that could be of utilisation in both food and drug systems. One recent particular example relates to the potential use of a native New Zealand gum in reducing food intake (Wee, Lentle, Goh, & Matia-Merino, 2017). The gum in question is extracted from the mamaku black tree fern. The polysaccharide composition within the fern is quite complex, being predominantly a glucuronomannan comprising a backbone of 4-linked methylesterified glucopyranosyl uronic acid and 2-linked mannopyranosyl residues, branched at 0.3 of 45% and at both 0.3 and 0.4 of 53% of the mannopyranosyl residues with side chains likely comprising terminal xylopyranosyl, terminal galactopyranosyl, non-methylesterified terminal glucopyranosyl uronic acid and 3-linked glucopyranosyl uronic acid residues (Wee, Matia-Merino, Carnachan, Sims, & Goh, 2014).

This polysaccharide shows some unusual rheological behaviour when isolated and solubilised. Dependent on concentration and ionic environment, it behaves as a pseudoplastic fluid at shear rates typically greater than 4–10 s^{-1}. However, before onset of shear thinning behaviour, there is an intermediate shear rate region for which the polysaccharide demonstrates rheopectic properties. This is unusual behaviour for a polysaccharide system, and represents a material that can potentially undergo shear thickening under the shear conditions present in the stomach. As part of an in vivo study, Wee and co-authors investigated the food intake of rats that had been gavaged with a solution of the gum and evidenced a reduction in extent of gastric emptying with accompanying suppression of appetite and food intake relative to a control group that had not been gavaged with the gum solution. Whilst these findings demonstrated a clear impact of the gum on eating behaviour, there are acknowledged challenges associated with the progression of these effects into food systems for human consumption with comparable physiological outcomes. Nevertheless, these findings not only support prior research highlighting the impact of water structuring on digestive behaviours but also demonstrate the opportunities arising from exploration of materials outside of the scope of those currently used in food manufacture.

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From Bite to Nutrient: The Importance of Length Scales

Didier Dupont and Françoise Nau

1 Introduction

Unravelling the fate of food in the gastrointestinal tract is essential to better understand the health effects of the food and to fight against diet-related pathologies such as cardiovascular diseases and type-2 diabetes. Digestion is the process that transforms food into nutrients that will be available to maintain homeostasis. The first step of digestion occurs in the mouth, where mastication transforms solid and semi-solid foods into particles while mixing with saliva allows for bolus formation and initiates digestion of carbohydrates. Then the bolus is transferred into the stomach, where acid conditions and specific enzymes (pepsin, gastric lipase) start hydrolyzing macronutrients like proteins and triglycerides. The next step occurs in the small intestine, where other digestive enzymes further degrade macronutrients allowing for their absorption. In the small intestine, proteins are hydrolyzed by trypsin, chymotrypsin, elastase, carboxypeptidase, etc., lipids by the pancreatic lipase and its colipase, and carbohydrates by the pancreatic amylase. Small intestinal digestion is finalized by the enzymes of the brush border membrane to release small nutrients that can pass through the epithelial barrier to reach the bloodstream. Undigested material, fibers for example, reaches the large intestine where it is further metabolized by the intestinal microbiota.

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2 Does the Food Matrix Structure Play a Key Role in Food Digestion?

Food digestion and absorption has been extensively studied over the last decades and the impact of the food matrix structure on the bioaccessibility (fraction of a nutrient that has been released in the gastrointestinal tract by the digestion process and is available for absorption) and the bioavailability (fraction of a nutrient that has been digested and absorbed and is available for the metabolic functions of the organism) of nutrients has been clearly demonstrated. The food matrix concept is based on the fact that "nutrients are contained into a larger continuous medium that may be of cellular origin (in fruits and vegetables) or a microstructure produced by processing where they may interact at different length scales with the components and structures of the medium" (Parada & Aguilera, 2007). Therefore, bioavailability of a micronutrient highly differs if it is free and soluble in a liquid, bound to plant organelles or entrapped into a complex macromolecular matrix. One of the best examples to illustrate this is the bioavailability of carotenoids. Carotenoid absorption requires release from the food matrix, solubilization in the oil phase and mixed micelle formation, uptake into intestinal mucosal cells, packing into chylomicrons, and secretion into the lymphatic system, critical steps that are influenced by a complex set of factors. Carotenoids in fruit and vegetable matrices are located inside the chromoplast organelles in a specific sub-structure of crystalline, membranous or globular nature that is embedded in a cellular structure (Schweiggert, Mezger, Schimpf, Steingass, & Carle, 2012). Consequently, the chromoplast sub-structure and the cell wall are the two main natural structural barriers that govern carotenoid release from the matrix (Jeffery, Holzenburg, & King, 2012; Palmero et al., 2013). Food structure, as modified by food processing, influences the absorption of these micronutrients from the diet, for example, disruption of the natural matrix during food processing may increase their bioavailability. The impact of carrot cooking on carotenes bioavailability has been investigated in several in vivo studies (Livny et al., 2003; Tydeman et al., 2010). At identical composition, the release of carotenes in the gastrointestinal tract is linked to the disintegration of cellular structures and it is therefore favored by a heat treatment applied to the food. Another example is the impact of the lipid droplet size of an emulsion on the digestion and absorption of lipids. By changing the homogenization conditions, Armand et al. modulated the mean lipid droplet size of the emulsion (0.7/10 μm). They found that 20–37% of triglycerides were hydrolyzed in the stomach for the 0.7 μm droplet size material, whereas this range decreased to 7–16% for the 10 μm droplet size system (Armand et al., 1999). In the duodenum, important differences were also found, with 57–73% of triglycerides being hydrolyzed for the fine emulsion, compared to only 37–46% for the coarse one. When the same triglycerides were given to humans in emulsified form versus non-emulsified form as part of a standardized breakfast, the kinetics of postprandial plasma triglycerides was faster and higher for the emulsified form, especially in obese subjects (Vors et al., 2013). The influence of food structure on carbohydrate digestion is also well known. For instance, the glycemic index (GI) of

a bread depends on its density (d) (Saulnier et al., 2010). A basic French baguette ($d = 0.16$) presented a GI of 75, whereas another one with identical composition but a higher density ($d = 0.24$) exhibited a GI of 55, because of its lower starch accessibility. Indeed, GI is not considered to be a characteristic of the human being but rather a property of the food item itself (ISO, 2010).

The case of proteins has been studied more extensively. A comparison between proteins digested as pure compounds (molecular scale), protein aggregates (supramolecular scale), protein gels (microscopic and/or macroscopic scale) is possible and gives information about the structural parameters driving the digestion of this macronutrient.

3 Investigating Protein Digestion at Different Length Scales

3.1 Molecular Scale: Digestion of Purified Proteins

The ability of a single protein to be hydrolyzed by digestive enzymes in the gastrointestinal tract highly depends on different physicochemical and structural characteristics of the protein and, in particular, on its sequence of amino acids. Indeed, in order to be degraded, the protein of interest will have to contain peptide bonds that can be cleaved by the digestive proteases. The specificity of cleavage of the major pancreatic enzymes, that is, trypsin and chymotrypsin, that are both serine-proteases, is well known; trypsin will cut the Lys-X and Arg-X bonds, whereas chymotrypsin will hydrolyze the Tyr-X, Trp-X, Phe-X, and Leu-X bonds. In contrast, pepsin, which is an aspartic protease, has a broader specificity that is harder to predict. Based on the pepsin digestion of 39 proteins and the exhaustive identification of the peptides generated, (Hamuro, Coales, Molnar, Tuske, & Morrow, 2008) have proposed a table summarizing the probability for all the peptide bonds to be hydrolyzed by the enzyme. The limit of this pioneering work is that pepsin was immobilized onto a support which could affect its specificity.

However, the presence of a cleavage site within the sequence does not mean necessarily that the protein will be digested. As an example, β-lactoglobulin, the major bovine whey protein, has several potential cleavage sites along its sequence but it is not hydrolyzed, in its native form, by pepsin (Mandalari et al., 2009). β-lactoglobulin is a globular protein with a very compact structure and the major cleavage sites are buried inside the structure and they are not accessible by the protease (unpublished data). Once the protein is denatured by heat-treatment, its unfolding exposes the cleavage sites and the protein becomes degradable. Therefore secondary and 3D structures of a protein are considered as key factors to explain its susceptibility towards digestion. Using the synchrotron-based Fourier transform infrared microspectroscopy, Doiron, Yu, McKinnon, and Christensen (2009) showed that the protein structure α-helix to β-sheet ratio had a significant positive correlation with total intestinally absorbed protein supply and negative correlation with degraded protein balance (Doiron et al., 2009).

The comparative in vitro digestion of three model proteins with different structures emphasized the role played by the structure on the kinetics of proteolysis (Dupont, Mandalari, Molle, Jardin, Leonil, et al., 2010). β-casein was chosen as a representative of natively unfolded proteins whereas β-lactoglobulin was selected because of its resistance to gastric digestion caused by its compact structure. Egg white ovalbumin was expected to exhibit an intermediate behavior. Results obtained after submitting these three model proteins to in vitro digestion confirmed the link between the 3D structure of a protein and its ability to be hydrolyzed in digestive conditions. During the gastric phase, β-casein was fully hydrolyzed within 10 min whereas β-lactoglobulin was not affected by the action of pepsin and ovalbumin was partly hydrolyzed. The intestinal phase did not change the ranking of these three proteins (Fig. 1).

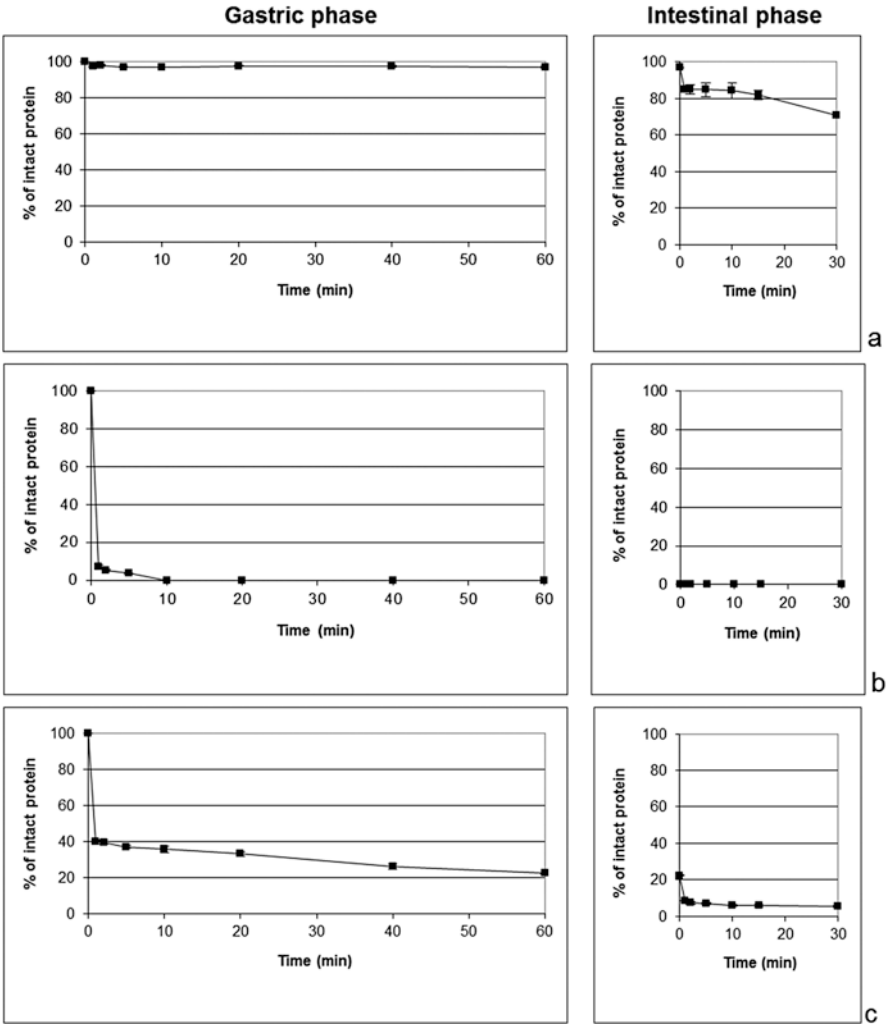


Fig. 1 Gastric and intestinal digestion of β-lactoglobulin (**a**), β-casein (**b**) and ovalbumin (**c**)

One of the main interests of scientists for studying purified protein digestion is to evaluate allergenicity. Indeed, it is generally considered that for eliciting an allergic reaction, a dietary protein has to, at least partly, survive the gastrointestinal tract in order to stimulate the immune system. The protein does not have to be intact when reaching the epithelial cells and peptides generated by the digestion process and long enough to contain at least two epitopes could be responsible for sensitization (Mills, Jenkins, Alcocer, & Shewry, 2004). Therefore, the general opinion appears to be that the lower limit for allergenicity of peptides is a molecular weight of approximately 3.5 kDa (Lack et al., 2002). Astwood, Leach, and Fuchs (1996), using a rather basic incubation test with pepsin, compared the resistance to pepsin digestion of 16 known food allergens, that is, ovalbumin, β-lactoglobulin, Ara h2, β-conglycinin, etc. and nine common plant proteins considered to be non-allergens like Rubisco LSU and SSU from spinach leaf, lipoxygenase from soybean seed, sucrose synthetase from wheat kernel, β-amylase from barney kernel or acid phosphatase, and phosphofructokinase from potato tuber. They showed that while major food allergens in general resisted the digestion process, non-allergenic proteins (mainly enzymes) were in contrast rapidly digested (Astwood et al., 1996). Using sturgeon caviar and parvalbumin, the major fish allergen, as examples, impairment of the digestion process was shown to increase allergenicity of the proteins under investigation in a Balb/c mouse model further supporting the hypothesis of a link between resistance to digestion and allergenicity (Untersmayr et al., 2005, 2003). In contrast, other authors found that there was no clear relationship between digestibility measured in vitro and protein allergenicity (Fu, Abbott, & Hatzos, 2002). Similarly, when reviewing all the literature available on digestibility studies of pure allergens, Bøgh and Madsen did not find clear evidence of such a link (Bøgh & Madsen, 2015). The overall controversy can certainly be explained by the different experimental conditions (enzyme–substrate ratio, pH and duration of the gastric phase, etc.) that were used in those different studies and also by differences in analytical techniques that were used to characterize the digested product.

Digestion of purified proteins has also been carried out in order to identify bioactive peptides that could be released in the gastrointestinal tract. This type of experiment has been conducted in vitro (Capriotti, Caruso, et al., 2015; Capriotti, Cavaliere, et al., 2015; Caron et al., 2016; Dupont, Mandalari, Molle, Jardin, Leonil, et al. 2010a; Ferranti et al., 2014) but also in vivo and recently the identification of peptides in the jejunum of adults fed with caseins or whey proteins has been published (Boutrou et al., 2013). In this study, the opioid β-CN 60–66 peptide and the β-CN 108–113 antihypertensive peptide were quantified in the lumen by mass spectrometry. Concentrations measured were found to be sufficient to exert a biological activity, that is, higher than the half maximal inhibitory concentration (IC_{50}); however, to do so, the peptides would have to cross the epithelial barrier and concentrations that would reach the bloodstream would be much lower. Also, the time of survival of a dietary peptide has been shown to be short and it is difficult to predict whether the peptides will stay long enough in the bloodstream to reach their targets. More work is really needed to demonstrate that bioactive peptides are able to go through the intestinal epithelium, reach the bloodstream and their targets in sufficient concentrations to generate a biological effect on the host. Such evidence could make acceptance of health claims by EFSA easier.

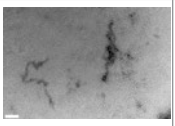
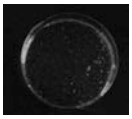
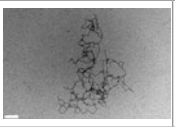
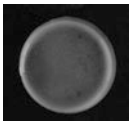
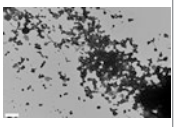
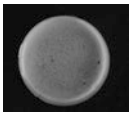
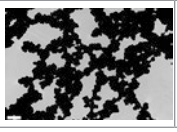
3.2 Nanoscale: Digestion of Heat-Induced Protein Aggregates

The impact of heat-induced aggregation on protein digestibility has been studied on different model food proteins and the results are sometimes conflicting. While some authors reported a decrease in digestibility for milk caseins (Dupont, Mandalari, Molle, Jardin, Rolet-Repecaud, et al., 2010b), meat (Bax et al., 2012), or wheat proteins (Petitot, Abecassis, & Micard, 2009), others found an increase in digestibility for proteins like β-lactoglobulin or concanavalin A (Peram, Loveday, Ye, & Singh, 2013; Takagi, Teshima, Okunuki, & Sawada, 2003). One possible explanation of these differences can be attributed to the structure of the protein itself. Milk for instance contains two types of proteins with opposite structural characteristics: whereas caseins are natively unfolded proteins with a very loose and flexible structure, whey proteins are globular and exhibit dense and compact structures. Application of a heat treatment to unfolded proteins that are extremely sensitive to proteolysis in their native state can induce aggregation and provide a structure to these proteins limiting the access of digestive enzymes in the gastrointestinal tract. In contrast, globular proteins that are traditionally resistant to proteolytic enzymes are denatured before being aggregated and denaturation results in an opening of the compact structures making them more sensitive to digestion. Differences between studies can also be attributed to differences in experimental conditions and particularly to the intensity of the heat treatment applied to the proteins. For instance, at 70 °C, meat proteins underwent denaturation that enhanced the speed of pepsin digestion by increasing enzyme accessibility to protein cleavage sites whereas above 100 °C, oxidation-related protein aggregation slowed pepsin digestion (Bax et al., 2012).

We investigated the impact of heat-induced aggregation of proteins on their digestibility using egg white ovalbumin as a model (Nyemb et al., 2014). Heat-induced aggregation was performed using different combinations of pH and ionic strength in order to obtain a range of different aggregate morphologies. The different aggregated ovalbumin structures are shown in (Table 1).

At pH 5 and ionic strength 0.8 M, the protein solution appeared as a suspension of solid particles within a clear aqueous phase; the particle size ranged from 20 to 250 μm, with a mean size of 80 μm. At pH 9 and ionic strength 0.03 M, size of the measured particles ranged from 10 to 140 nm with a mean size of 33 nm. Aggregates formed at pH 7 were more similar to the aggregates formed at either pH 9 or pH 5 depending on the ionic strength of the pH 7 solution. For example, for the pH 7 solution with an ionic strength of 0.03 M, the particle size of the aggregates ranged from 7.5 to 140 nm (with a mean size of 16 nm) and the solution was virtually transparent, which were characteristics closer to the aggregate solution prepared at pH 9. In contrast, for the aggregate solution prepared with an ionic strength of 0.3 M at pH 7, the particles were larger (10–80 μm, with a mean size of 30 μm) and the appearance of the solution was more opaque, which was more similar to the aggregate solution formed at pH 5. The microstructure of the four aggregate solutions was examined using transmission electron microscopy (Table 1). Linear aggregates

Table 1 Summary of the different treatment conditions, macrostructure, microstructure, morphology, particle size, and predicted net charge of the different aggregates

Treatment	Macrostructure	Microstructure (TEM)	Morphology	Mean particle size	Predicted net charge
pH 9/IS 0.03 M			Linear	33 nm	−63
pH 7/IS 0.03 M			Linear-branched	16 nm	−38
pH 7/IS 0.3 M			Spherical	30 μm	−38
pH 5/IS 0.8 M			Spherical-agglomerated	80 μm	+16

were present in the solution prepared at pH 9/ionic strength 0.03 M, while linear-branched aggregates were observed in the solution prepared at pH 7/ionic strength 0.03 M. At pH 7.0, the increase of ionic strength from 0.03 to 0.3 M led to completely different morphological features, with the formation of spherical particles of about 30 μm diameter. At pH 5/ionic strength 0.8 M the aggregates consisted of clusters of large spherical-agglomerated particles.

When submitted to in vitro digestion the native and the different aggregated forms exhibited different behaviors (Fig. 2). The native form was shown to be the most resistant to the digestive process with 70% and 40% of the protein remaining intact after the gastric and intestinal phase respectively. In contrast, linear aggregates were almost totally hydrolyzed after only 10 min of gastric digestion. Spherical-aggregated assemblies showed a significant resistance to in vitro digestion, with c.a. 30% of the protein remaining intact after gastrointestinal digestion. Finally, linear-branched and spherical aggregates exhibited intermediate behaviors. From these data, it appears that kinetics of digestion will depend on both the extent of aggregation of the protein (native more resistant than aggregated) and the morphology of the aggregates (linear more hydrolyzed than spherical); the surface area to volume ratio of the aggregates, and the degree of protein unfolding have been proposed as the major contributing factors driving the extent of ovalbumin in vitro digestion. Similar data were obtained by Macierzanka et al. on another model protein, that is, β-lactoglobulin, (Macierzanka et al., 2012). These authors found that β-lactoglobulin gels formed close to the isoelectric point of the proteins were very

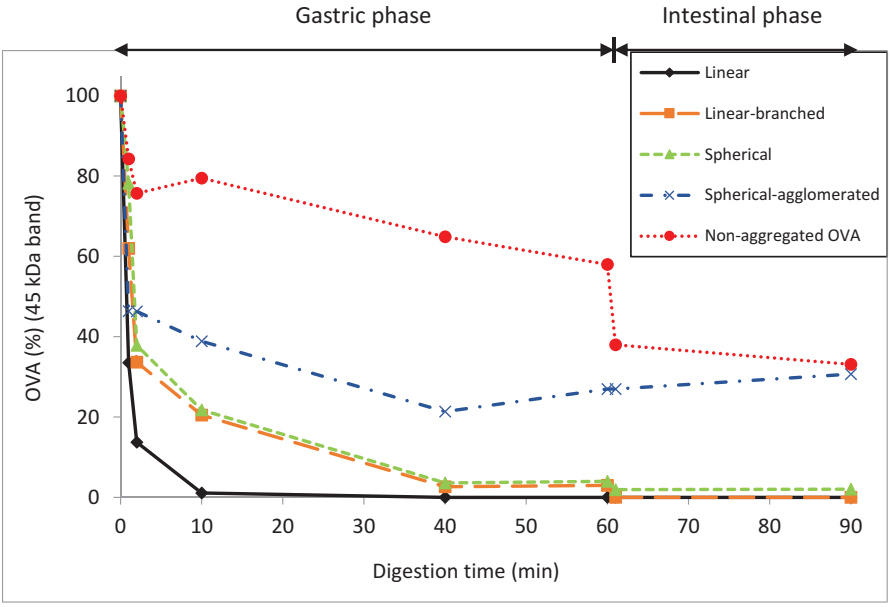


Fig. 2 Extent of ovalbumin digestion during in vitro simulated gastric and small intestinal digestion estimated by densitometry on SDS-PAGE gels of the non-aggregated and aggregated ovalbumin

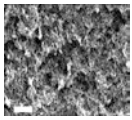
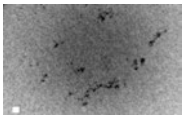
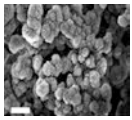
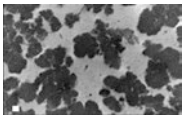
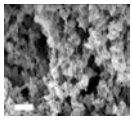
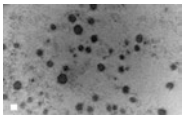
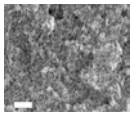
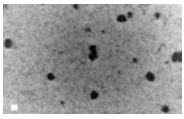
resistant to simulated gastric digestion with more than 85% of the protein remained intact after 1 h in the stomach. This clearly showed the potential of protein gels as delivery systems or for use in modulating uptake. Indeed, incorporating bioactive molecules sensitive to the action of pepsin or to the low pH of the gastric secretions into those gels would protect them from degradation in the stomach and allow for their release in the small intestine where they could be absorbed.

3.3 Microscale and Macroscale: Digestion of Protein Gels

In the previous paragraph, it was shown that heat-induced aggregates of pure globular proteins were more extensively digested than the native protein and that spherical aggregates were more resistant than linear ones. To investigate a higher scale, thermotropic egg white gels (composed of several proteins) were manufactured in different physicochemical conditions (pH and ionic strength) in order to induce four different types of aggregates and gel microstructures (Table 2).

The microstructure of the four different gels was examined using SEM and cryo-TEM. SEM was used to examine the overall gel network organization and to obtain information about the aggregate morphology (Nyemb et al., 2016). Cryo-TEM was used to examine the detailed aggregate morphology and organization. At high ionic

Table 2 Summary of the treatment conditions, morphology, macrostructure, microstructure of the different egg white gels

Treatment	Macrostructure	Microstructure (SEM)	Microstructure (CRYO-TEM)	Schematic Network
pH 2/IS 0.05 M				
pH 5/IS 1 M				
pH 7/IS 0.05 M				
pH 9/IS 0.05 M				

strength and pH 5 close to the isoelectric point of ovalbumin, the protein net charge and the electrostatic repulsions between proteins are minimized. Protein aggregation is dominant over protein unfolding and the predominance of attractive forces over denaturation favors the formation of particulate gels made of spherical aggregates (Croguennec, Nau, & Brule, 2002). Thus, the granular-spongy gel appears as large aggregates more or less interconnected with large pores between gel particles. The smooth-rigid (pH 9) and intermediate (pH 7) gels were comprised of both spherical aggregates (black spots on the cryo-TEM micrographs, likely corresponding to ovotransferrin aggregates) and linear ovalbumin aggregates; the relative amounts and organization of these two kind of aggregates differ between smooth-rigid and intermediate gels, making the pore size range of the mesh greater for the intermediate gel than for the smooth rigid gel. Finally, at pH 2 and low ionic strength, the protein net charge and electrostatic repulsions are as high as at pH 9, and proteins tend to denature rather than aggregate. The formation of a filamentous gel made of linear aggregates is then also favored in these conditions. The fracturable gel therefore presents all the characteristics of a dense and homogeneous gel network.

It was observed that at the end of in vitro gastric and intestinal digestion phases, the extent of digestion was positively correlated with the pore size of the egg white gels. Thus, the microstructure of the gels may be a factor that influences the extent of protein digestion, and it could be therefore hypothesized that the larger pores of the granular-spongy and intermediate gels enhanced enzyme diffusion compared to the smooth-rigid and fracturable gels.

It is noticeable that the smooth-rigid, intermediate, and granular-spongy gels manufactured in conditions similar to that leading to linear, linear-branched, and spherical-agglomerated ovalbumin aggregates, respectively, showed the opposite behavior than the original aggregates regarding the extent of protein digestion. For instance, whereas linear aggregates were the most sensitive to digestion, they were able to form very dense networks of proteins that inhibited the diffusion of digestive proteases limiting proteolysis. These examples emphasize the importance of the length scale on the digestive process.

Nevertheless, more research is needed to better understand the mechanism of action of digestive enzymes on their substrates in the different compartments of the gastrointestinal tract. In the case of protein gels, we are currently trying to determine whether an enzyme like pepsin is able to penetrate the gel particles to digest them from the interior or if it acts through an erosion mechanism (Thévenot, Cauty, Legland, Dupont, & Floury, 2017).

3.4 Macroscale: Comparative Kinetics of Digestion of Liquids and Gels of Identical Composition

The only way to really demonstrate the effect of food structure at the macroscopic level on hydrolysis in the gastrointestinal tract is to compare the behavior of food of identical composition and caloric charge but different macrostructures. As an example, six liquid, gelled, or semisolid dairy matrices (skim raw or heat-treated milks, stirred and unstirred acid gel, raw or heat-treated rennet gel) manufactured from the same milk powder (Fig. 3) were given to six mini-pigs fitted with two cannulas in the duodenum and mid-jejunum, and equipped with a catheter in the abdominal aorta. The gastric emptying half-time was shown to be directly influenced by the food structure; it was 98 min for the liquid matrices and increased up to 148 min for the acid gel (Le Feunteun et al., 2014). Gel stirring led to an intermediate viscous liquid structure and, consequently, to an intermediate half-time of 124 min. Rennet coagulation increased significantly the gastric emptying half-time. It was shown that rennet gel was transformed into a dense curd in gastric conditions. The curd was slowly eroded by pepsin in the stomach explaining a longer retention of the matrix in the stomach and the dramatic increase in gastric emptying half-time (Barbe et al., 2014). This definitely demonstrates that, at identical composition and caloric charge, the food matrix structure regulates gastric emptying.

Quantification of milk proteins in the duodenal effluents showed significant differences in kinetics of proteolysis in the small intestine between samples (Fig. 4). After ingestion of milk, caseins and whey proteins entered massively and rapidly in the duodenum and their respective concentrations decreased rapidly after 30 min to go back to the basal level. In contrast, acid gel led to a lower increase of milk proteins, which lasted much longer than that of milk (Barbe et al., 2013). Similarly to gastric emptying, stirred gel exhibited an intermediate behavior between milk and non-stirred acid gel. Finally rennet gel led to low concentrations of milk proteins

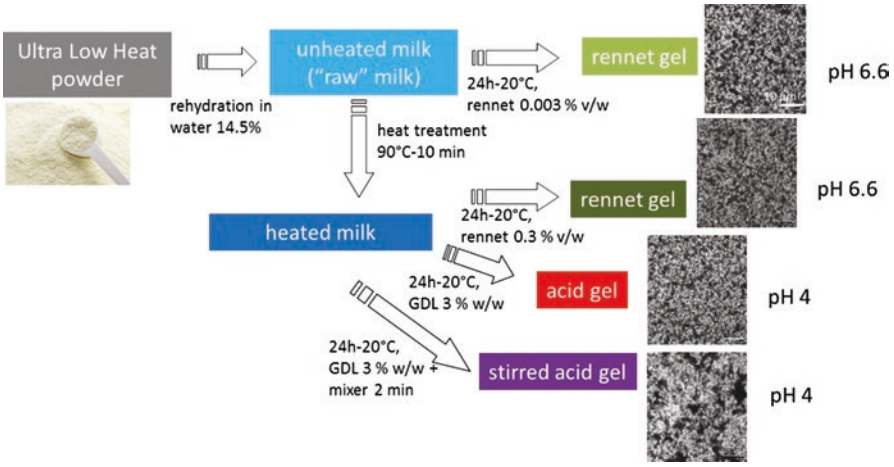


Fig. 3 Design of six isocaloric dairy matrices of identical composition but different microstructures

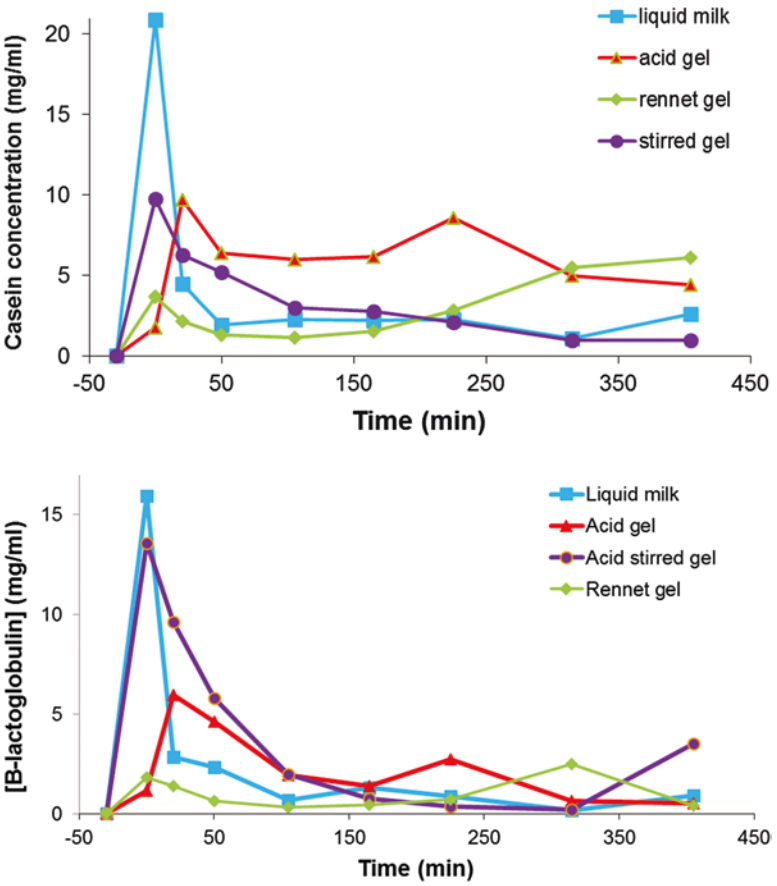


Fig. 4 Evolution of the casein (top) and β-lactoglobulin (bottom) concentrations as measured in the duodenum of six mini-pigs fed a heated milk, acid gel, stirred acid gel, and rennet gel of identical gross composition

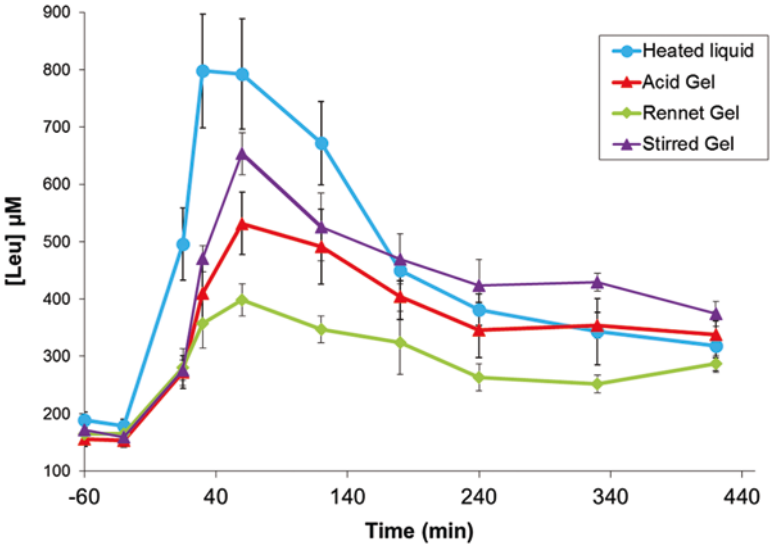


Fig. 5 Evolution of the leucine concentration in plasma of mini-pigs fed a heated milk, acid gel, stirred acid gel, and rennet gel of identical gross composition

throughout digestion with a slight increase after 7 h of digestion confirming the slow erosion by pepsin of the curd formed in the stomach (Barbe et al., 2014).

Quantification of plasma amino acids showed similar trends than the ones observed for milk proteins in the duodenum. For leucine (Fig. 5) as well as for the other amino acids, milk led to a sharp and early peak, whereas the peak was lower and appeared later for dairy gels. Again, stirred gel showed an intermediate behavior between milk and non-stirred gel, and rennet gel led to very low levels of amino acids in the bloodstream. Quantification of ghrelin in the plasma showed a strong decrease in the concentration of the hormone in the hours following an acid gel ingestion compared to milk ingestion indicating that acid gel consumption could favor satiety.

4 Conclusion

The present chapter shows that digestion of a micronutrient or macronutrient will highly depend on the structure that is adopted by this molecule within the food matrix, its interactions with the other food constituents and its level of structuration. A protein like ovalbumin in its native form is quite resistant to digestion but can generate linear aggregates that are themselves highly sensitive to hydrolysis but can constitute blocks of protein gels highly resistant to digestion. It is crucial to consider all the length scales for characterizing the digestion of a food in a proper manner. Indeed, milk rennet and acid gels of identical composition that exhibited similar

characteristics at the macroscopic level were shown to behave very differently during digestion. Whereas acid gels were rapidly disintegrated in the stomach and easily emptied into the small intestine, rennet gels were shown to form large aggregates in gastric conditions that stayed longer in the stomach and were slowly eroded by pepsin. These opposite behaviors were certainly due to differences at the molecular and/or the microscopic level.

This is the reason why at INRA we have been developing for many years a multiscale characterization of food structures. It consists of analyzing food at the molecular, mesoscopic, microscopic, and macroscopic level. The approach has been recently applied to food digestion with great success. For instance, in vitro and in vivo digestion of human milk has been characterized using this strategy (de Oliveira et al., 2017; de Oliveira, Bourlieu, et al., 2016; de Oliveira, Deglaire, et al., 2016). SDS-PAGE, mass spectrometry, and chromatography were used to quantify proteins, peptides, and amino acids respectively, whereas gas chromatography allowed for lipid characterization at the molecular level. Confocal laser scanning microscopy was used to characterize the organization of the different food constituents at the microscopic scale, whereas laser light scattering allowed to determine particle size distribution. It is a powerful way to perfectly understand how foods are disintegrated in the gastrointestinal tract.

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Part III
Fundamental Understanding of Digestion Processes

Tools/Methods for Quantifying Digestion: Medical Imaging Aspect

Carolyn Costigan and Luca Marciani

1 Background

Over a century has passed since movements of foods in the gastrointestinal tract were first "observed by means of the shadows cast on a fluorescent screen" (Cannon, 1904). New medical imaging technologies have been invented and developed, revolutionizing the way one can look inside the body. More commonly used in clinical practice and biomedical research, the use of medical imaging tools to look at foods and beverages in the body, and to help quantifying processes of digestion, has recently increased. This new field is looking very promising and the opportunities are vast. Each imaging technique is different, with intrinsic strengths and weaknesses, but basic imaging principles cut across all of them. The following paragraphs describe briefly the in vivo imaging techniques available and provide some of the many examples from the literature of the outcome variables that can be measured.

2 Radiographic Methods

X-ray imaging is well over 100 years old. The technique is relatively simple. It is based on a beam generated by an X-ray tube in front of the subject, travelling straight through the body. Inside the body different organs will attenuate the X-ray beam differently. The beam is then detected on the other side of the subject and an image of the insides of the body, based on the different attenuation of the beam, is

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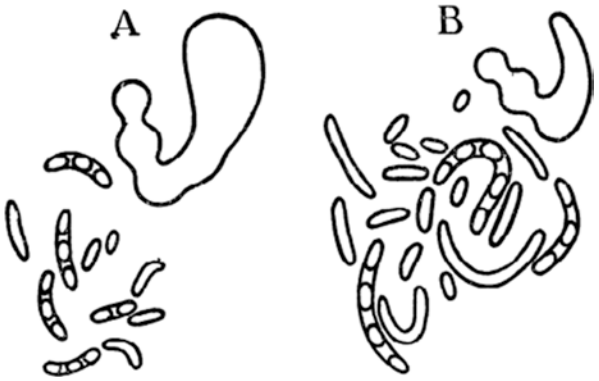

Fig. 1 The "tracings of the shadows" as drown in the original Cannon paper showing the stomach and intestine of a cat after feeding boiled lean beef (**a**) or boiled rice (**b**). Reproduced with permission from Cannon (1904)

formed on a detector (once comprising a scintillator and film but nowadays most often a digital capture plate or panel).

This imaging technique provided the first pioneering dynamic images of the movement of different foods from the stomach and through the small intestine in a feline model (Cannon, 1904), Fig. 1.

The technology is relatively cheap and accessible though when considered for the study of digestion it suffers from limitations. One limitation is the inherent nature of the X-rays which are ionizing radiation capable of inducing biological damage in the subjects. The other limitation is that many foods and beverages need to be mixed with radiopaque materials such as bismuth salts or barium sulfate to allow for radiographic assessment. For these reasons their use to study food digestion has been limited and mostly confined to the earlier part of the twentieth century (McCance, Prior, & Widdowson, 1953). There has been recent interest in using CT to measure bowel gas volumes [Bendezu et al. (2015) and references therein].

3 Nuclear Medicine

The X-ray technique uses an external source and forms an image after the radiation beam has passed through the body. Nuclear medicine methods instead place a radioactive label on materials that are injected or ingested and later detect the radiation emitted from inside the body. Gamma scintigraphy is the oldest imaging technique. It uses gamma ray emitting labels and has been used to quantify gastric emptying since the late 1960s (Griffith, Owen, Kirkman, & Shields, 1966). Unprecedented information about digestion emerged from the early scintigraphy studies (Fig. 2). This includes the differential emptying of liquids and solids

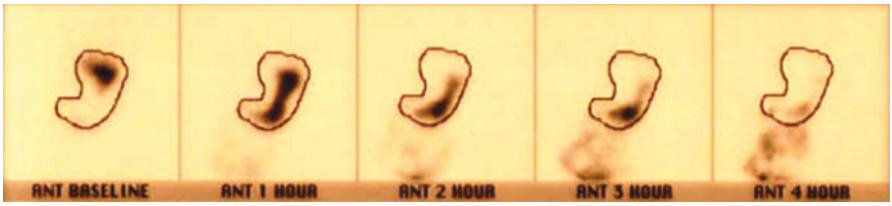

Fig. 2 Gamma scintigraphy images of a standard 255 kcal bread and ^{99m}Tc-sulfur colloid labeled egg test meal emptying from the stomach of a subject. Reproduced with permission from Lin et al. (2005)

(Campbell et al., 1977; Heading, Tothill, McLoughlin, & Shearman, 1976), the size of emptied particulates (Meyer, Elashoff, Porterfink, Dressman, & Amidon, 1988), and fat being retained selectively in the proximal stomach (Edelbroek, Horowitz, Maddox, & Bellen, 1992). A more recent evolution of gamma scintigraphy is single photon emission computed tomography (SPECT) which is able to acquire multiple projections around the body that can be reconstructed in a three dimensional map of the distribution of the radioactive label. Positron emission tomography or PET is a more sophisticated technique, using positron tracers and exploiting the radiation emitted by the positron annihilation to reconstruct the projections form the body. The nuclear medicine medical imaging techniques usually have poor spatial resolution but are very sensitive so the amount of radiation needed for detection is small which, in turn, provides a smaller radiation dose to the subjects.

Nuclear medicine applications to the gastrointestinal tract expanded rapidly to the pharmaceutical industry and the gastroenterology community to image and quantify digestion and small bowel and colonic transit (Camilleri, Hasler, Parkman, Quigley, & Soffer, 1998; Szarka & Camilleri, 2012). Gamma scintigraphy can label separately the liquid and solid component of a meal using different isotopes such as ^{99m}Tc and ^{111}In with separate photopeak energy. The dual-isotope technique was used for example to assess gastric emptying of white bread and wholemeal bread, showing no differences in emptying for the solid phases but significant difference in the gastric emptying of the liquid phase (Grimes & Goddard, 1977).

Another classic application of nuclear medicine is transit through the gut. For example gamma scintigraphy was used to determine delayed gastric emptying and accelerated small bowel transit of bran (McIntyre, Vincent, Perkins, & Spiller, 1997). SPECT has also been used to measure gastric volumes (Bharucha et al., 2007) and simultaneous volumes and emptying (Simonian et al., 2004).

4 Ultrasound Imaging

Another method to image gastric contents and assess gastric emptying is ultrasonography (Darwiche, Almer, Bjorgell, Cederholm, & Nilsson, 1999; Ricci, Bontempo, Corazziari, Labella, & Torsoli, 1993). Ultrasound imaging uses a probe

that sends sound waves (typically of a few megahertz) inside the body of a person. The sound waves reflect (echo) differently from different tissues and materials and the reflected waves are detected and reconstructed as an image. One of the drivers for the development of intragastric ultrasound of foods and beverages has been the need to assess residual gastric volumes before anesthesia (Soreide, Hausken, Soreide, & Steen, 1996; Van de Putte & Perlas, 2014). The main advantages of ultrasound are the lack of ionizing radiation, the relatively low cost and its wide availability. Limitations include the difficulty in obtaining full cross sections of the proximal stomach due to the common presence of air inside the stomach, the deeper location in the body and the presence of the rib cage (Van de Putte & Perlas, 2014), and a certain degree of dependence on the operator.

Ultrasound provides best views of the gastric antrum (Fig. 3). Different beverages can appear anechoic or hypoechoic while air mixed with solid food during mastication can give to the meal a "frosted-glass pattern" (Van de Putte & Perlas, 2014). Various studies correlated the area of a single cross section of the antrum with total gastric volume to allow a representative measure of total gastric volume (Ricci et al., 1993) and gastric emptying assessment.

Due to the low cost and availability ultrasound has been used in many digestion studies. Examples of research use include imaging gastric emptying of bread with added sodium propionate (Darwiche et al., 2001), the effect of cinnamon on gastric emptying of rice pudding (Hlebowicz, Darwiche, Bjorgell, & Almer, 2007), and the emptying of pasta with added inulin (Russo et al., 2011).

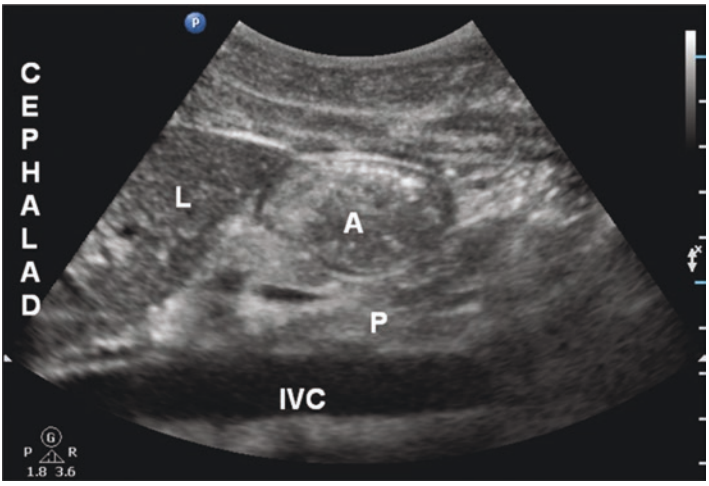

Fig. 3 Sonographic image of the gastric antrum with solid meal content. *A* antrum, *L* liver, *P* pancreas, *IVC* inferior vena cava. Reproduced with permission from Van de Putte and Perlas (2014)

5 Magnetic Resonance Imaging

Magnetic resonance imaging or MRI exploits a different principle to image the body, the ability of hydrogen nuclei to receive and transmit a radiofrequency signal when placed in a strong magnetic field. Spatial encoding can be superimposed to this physical phenomenon so that images of the location of the transmitting hydrogen protons can be reconstructed. Different environments surrounding the hydrogen atoms affect the signal differently so that contrast, say between healthy and diseased tissue or thick and liquid food, is generated inherently due to the nature of the materials imaged. The MRI scientist can manipulate and exploit this to look at a range of properties of the body or foods, such as geometric volumes, fat content, and flow. The technique uses non-ionizing radiation so it can be used for longer, serial, crossover studies of digestion (Marciani, 2011). This ability, plus the multiplanar imaging and good spatial resolution are being increasingly used to study the gastrointestinal fate of foods and beverages in gastrointestinal tract within an undisturbed, physiological state.

5.1 Stomach

The abdomen can be imaged by MRI with good in plane resolution (e.g., 1.5–2 mm) and 5–10 mm slice thickness in one breath-hold. The choice of contrast (T1 weighted or T2 weighted) is determined by the kind of food and which properties one wants to observe. Digestion is a relatively slow process hence the subjects are generally scanned at intervals over a period of time. They are asked to lie down briefly for the imaging and then are taken out of the bore of the magnet and asked to sit upright in a quiet room nearby, until the following data point is needed. Gastric content volumes or total gastric volumes (meal + gas) can be calculated by drawing manually or semiautomatically on dedicated software. The data are then plotted as gastric emptying curves (Schwizer, Maecke, & Fried, 1992). Gastric volume measurements are a validated and reproducible outcome measure (Boulby, Gowland, Adams, & Spiller, 1997; Feinle, Kunz, Boesiger, Fried, & Schwizer, 1999; Fidler et al., 2009; Fruehauf et al., 2007; Schwizer et al., 1992).

The ability to look serially at food materials inside the stomach during digestion has provided new insights that were previously impossible (Fig. 4). These include for example observing self-structuring of gelling alginates in the stomach (Hoad et al., 2004; Marciani et al., 2002). The intragastric retention and decay of aerated foam beverages (Murray et al., 2015). The impact of the fat emulsions with different intragastric stability (Marciani et al., 2007) and layering and retention of isocaloric liquid or structured meals (Mackie, Rafiee, Malcolm, Salt, & van Aken, 2013) on physiological and satiety outcome measures.

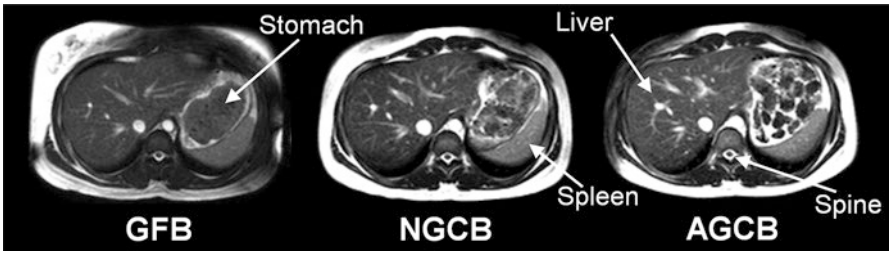

Fig. 4 MRI images showing different intragastric behavior of the three breads types: gluten free bread (GFB), normal gluten content bread (NGCB) and added gluten content bread (AGCB). GFB bread appears as a mixed, homogeneous mass, AGCB as discrete ensemble of bread pieces with sharp edges surrounded by fluid and NGCB a less defined and more mixed volume of bread and fluid. Reproduced with permission from Coletta et al. (2016)

5.2 Small Bowel

Recent work has focused on quantifying luminal volumes of freely mobile small bowel water content. This outcome measure has been validated against duodenal infusions (Hoad et al., 2007). The fluid volumes environment in the undisturbed bowel had been little explored beforehand. MRI provides a unique tool to look at fluids as shown for example by the effect of bran (Marciani et al., 2010) and the large volumes generated by fructose supplementation compared to glucose or inulin, Fig. 5 (Murray et al., 2014).

In the past few years MRI methods to quantify the motility of the small bowel have also been refined. The same imaging plane is imaged rapidly and repetitively (approximately every second) and later the images can be viewed as cine-MRI movies. The series can also be analyzed to extract quantitative metrics of motility (Hahnemann, Nensa, Kinner, Gerken, & Lauenstein, 2015; Hahnemann, Nensa, Kinner, Maderwald, et al., 2015; Menys et al., 2014; Odille et al., 2012). This is most commonly done in the clinical setting, distending the small bowel with substantial amounts of contrast media to help the visualization but it is also a rather unphysiologic stimulus compared to feeding. Recently these techniques have been applied to follow serially the small bowel motility response to feeding a simple canned soup meal (Khalaf et al., 2017). This study in healthy volunteers shows a large and rapid increase in small bowel motility from fasted baseline to immediately after the participants ate the soup meal (Fig. 6) in conjunction with the postprandial hormonal and appetite responses.

5.3 Colon

The colon has also been the focus of recent MRI work. MRI can measure the volumes of this organ using manual or semiautomated segmentation methods (Pritchard et al., 2014; Sandberg et al., 2015). The ability to measure serially and

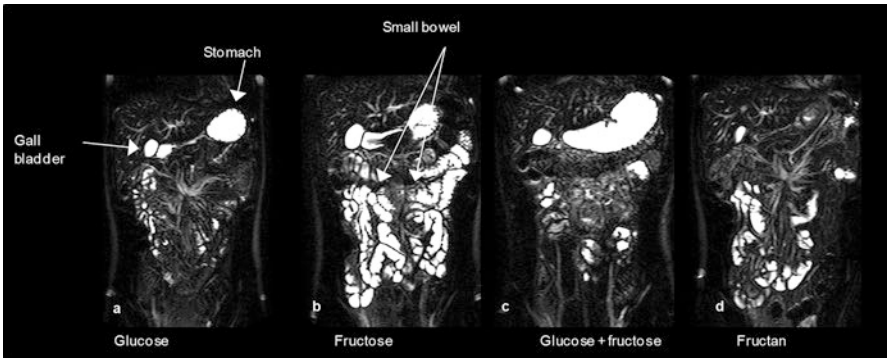

Fig. 5 A representative example of coronal images of the small bowel from a single volunteer 75 min after drinking each of the following test drinks, on separate days 1 week apart: (**a**) glucose, (**b**) fructose, (**c**) glucose + fructose, and (**d**) fructan. In this type of images the contrast is weighted so that fluids appear very bright and most of the body tissues very dark. The small bowel enhanced secretory response after the fructose drink can be clearly seen, while the addition of glucose negates this response as expected, because of the activation of the glucose transport receptor. The stomach and gall bladder are also visualized in these images. Reproduced with permission from Murray et al. (2014)

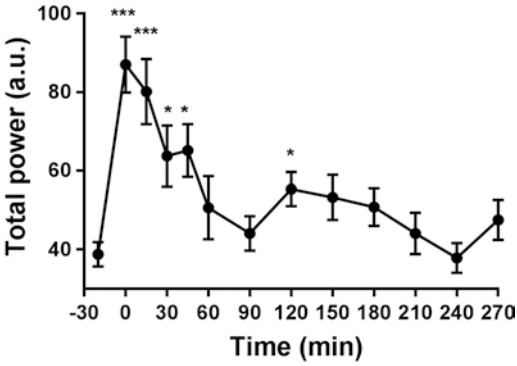

Fig. 6 Changes in total power measure of small bowel motility in $n = 15$ healthy volunteers who ate a soup meal. Data are (mean ± SEM). $^{***}p \leq 0.00$, $^{**}p \leq 0.001$ and $^{*}p < 0.05$ Reproduced with permission from Khalaf et al. (2017)

during digestion the colonic volumes, gas volumes, biomass volumes, and parameters relating to the physicochemical environment in the lumen is powerful. The relation between the microbiota, digestion, and the origin of symptoms is currently a topic of great interest. An example of how this can be used was given in a recent study following the gastrointestinal fate of an inulin challenge (Murray et al., 2014). Another study showed the inability of the ascending colon in patients with irritable bowel syndrome with diarrhea (IBS-D) to accommodate postprandial inflow (Pritchard et al., 2014). Intestinal gas does not provide any MRI signal hence gas pockets can be very easily seen on abdominal MRIs. One clear example is provided by measures of colonic gas after supplementation with inulin, Fig. 7 (Murray et al., 2014).

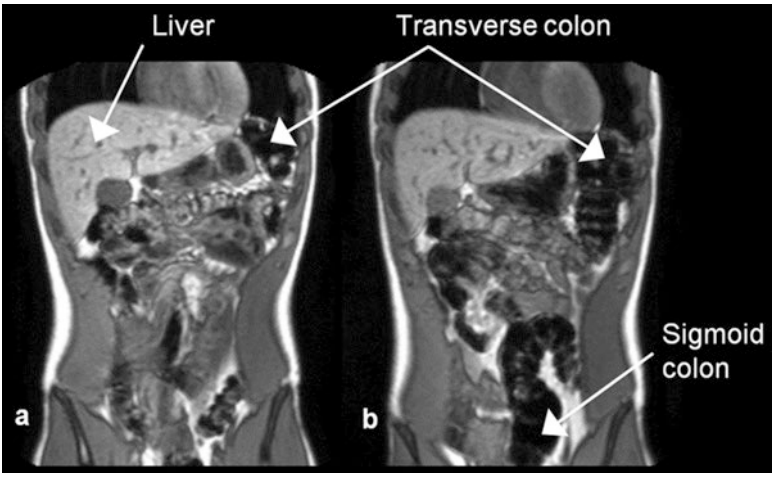

Fig. 7 The image on the right shows large amounts of gas (seen as black because it provides no MRI signal) in the colon of a subject who was fed a drink of inulin. The image on the left shows the corresponding image at baseline where little gas can be seen. Reproduced with permission from Murray et al. (2014)

6 Conclusions

Medical imaging techniques have been providing novel insights in the physiological processes of digestion and have been helping to quantitate various aspects. Medical imaging is also being used to investigate mechanisms of appetite and of symptoms in health and in various gastrointestinal diseases in response to feeding complex meals and also individual food materials of interests.

Among the different imaging methods available, MRI is rapidly taking a central role due to the lack of ionizing radiation, good spatial and temporal resolution, and ability to measure a battery of outcome parameters of interest in the quantitation of digestion in a single experimental study day. The experience so far shows that every advance brought in by imaging digestion has opened more research questions and we expect this field to be very active in the years to come.

Beyond the clever imaging methodologies and the fine abilities of the imaging experts, one simple fact needs also to be kept in mind: people like pictures. Images deliver messages easily, and they can help diverse, multidisciplinary audiences to understand better the fascinating processes of digestion. The new field of in-body imaging of foods and beverages has only started to show its potential.

7 Future Directions

Imaging of gastrointestinal function and of foods and beverages in the lumen is rapidly evolving and the number of users worldwide is increasing. This is positive and from the technical point of view will lead to a refinement and higher

standardization of the methods and of the outcome variables. Developments will encompass particularly the measurements relating to physicochemical properties of food at the various stages of the passage through the gastrointestinal tract.

Beyond the quantitation of physiological digestion processes, medical imaging tools will be increasingly used to generate human data to inform and increase in vitro/in vivo relevance of bench models of digestion and also to support patent applications. For example in vivo MRI data were used to design and validate functional aspects of the Dynamic Gastric Model and support its international patent (Wickham & Faulks, 2012). In another example in vivo MRI was used to support a patent relating to methylcellulose materials designed to increase satiety (Adden, Anderson, Huebner, & Knarr, 2011). The ability of imaging to elucidate the mode of action of food materials and supplements will also be increasingly used to support food industry's health claims with the regulatory authorities. For example MRI measures of intestinal gas are explicitly mentioned as appropriate outcome variable by the EFSA guidelines on the scientific requirements for some health claims relating to the gastrointestinal tract (EFSA, 2016).

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Quantitative Characterization of Digestion Processes

Lotti Egger, Olivia Ménard, and Reto Portmann

1 Analysis of Digestion Processes In Vivo Versus In Vitro

Digestion of food is a highly complex process, involving a multitude of biochemical reactions, and although it is occurring every day, many open questions remain on how complex food structures are degraded, released from food, and absorbed into the circulation. Food digestion can be studied by conducting in vivo studies in humans or animals, or using in vitro models. In vivo studies are restricted for ethical reasons, are expensive, and sample collection may be difficult. In vitro studies are easy to conduct and allow for running several samples in parallel, but may oversimplify the complex physiological digestion processes. The growing interest in understanding the molecular mechanisms of digestive processes has led to the development and use of many in vitro digestion protocols (Hur, Lim, Decker, & McClements, 2011). Different analytical methods were applied to monitor the degradation processes, making the comparison of experimental and analytical results difficult.

The research undertaken during the COST action Infogest Network (http://www.cost-infogest.eu/) was a major step towards improved comparability of in vitro results. The main goals of this network of researchers was establishing a harmonized in vitro digestion method and validating it for its physiological relevance. Specialists from the field of food digestion agreed on a simple static digestion protocol based on physiological data from literature, published in 2014 (Minekus et al., 2014). Improvements in harmonization were analytically assessed in several interlaboratory trials performed with skim milk powder (SMP) at the level of protein

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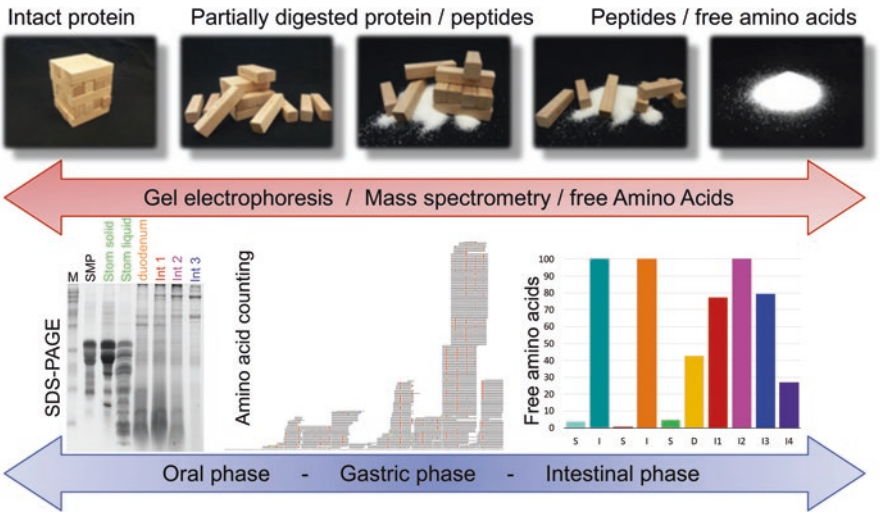

Fig. 1 Schematic drawing of methods used for the analysis of protein hydrolysis during digestion

degradation (Egger et al., 2016). The comparability of protein degradation between in vitro and in vivo digestion was addressed in further work with the same SMP used for interlaboratory trials fed to pigs. To assess protein digestion during the interlaboratory trials and during the pig experiment, different analytical methods were developed and are described in this chapter.

In the first part of this chapter, the harmonized static in vitro digestion protocol is introduced. In the second part of the chapter, the analytical methods applied for assessing the degradation of the three macronutrients proteins, fat, and carbohydrates are illustrated, with a main focus on protein digestion. SMP was used for all trials as a model food for the study of protein hydrolysis.

The analytical methods for protein hydrolysis from whole proteins to peptides and free amino acids include protein separation by SDS-PAGE for whole proteins and large peptides (>5 kDa), analysis of peptides (>5 aa), and analysis of free amino acids (FAA) (Fig. 1). These same analytical methods were used to investigate the improvement in harmonization and to compare in vitro with in vivo experiments.

Lipids are a more heterogeneous group of nutrients, including triglycerides, phospholipids, and steroids. The methods described in this work focus on triglycerides, which are quantitatively the most abundant class of fats in most foods. Triglyceride digestion occurs in three steps. The first two steps of hydrolysis use a thin-layer chromatography method, allowing for the quantification of triacylglyceride hydrolysis to diacylglycerides and monoacylglycerides. The third step of hydrolysis uses gas chromatography, allowing for the quantification of individual and total free fatty acids (FFA).

Carbohydrates are the third group of macronutrients described in the chapter. Dairy products are devoid of starch, and not appropriate substrates for amylases

present in saliva and the duodenum. Disaccharide lactose is the main carbohydrate in dairy products, which arrives untransformed at the ileum before being hydrolyzed by lactase to glucose and galactose. In most in vitro digestion (IVD) systems, these brush-border enzymes are not included, and these hydrolytic steps are not investigated. The final part of this chapter offers a selection of analytical results showing the relationship between food digestion and nutrition.

2 General Aspects of the Harmonized IVD Protocol

The static harmonized IVD method developed by the Infogest Network (Minekus et al., 2014) is based on several previously published IVD protocols (Hur et al., 2011; Kopf-Bolanz et al., 2012; Versantvoort, Oomen, Van de Kamp, Rompelberg, & Sips, 2005), and it is separated into three compartments. The protocols provide the enzyme activities for each enzyme in U/ml of digesta using enzymes from different suppliers to improve the comparability between experiments from different laboratories. Methods for these enzyme activity determinations are provided in the supplementary material of the original publication (Minekus et al., 2014). To achieve valid comparability of experimental results, it is necessary to determine the enzyme activities for each batch of enzyme, and after storing the products for long periods.

The oral digestive phase comprises a mechanical processing step and contains α-amylase, which initiates the degradation of starch. Protein coagulation and hydrolysis starts in the acidic gastric digestive phase at pH 3, and in the presence of pepsin. Finally, the presence of pancreatin in the intestinal digestive phase together with bile leads to degradation of all macronutrients to the level of absorbable molecules. The workflow leaves the decision to include an oral digestive phase for liquid meals available (Fig. 2). In later experiments, it was observed that the oral digestive phase was needed even for liquid food (Egger et al., 2016). After the short, 2-min oral phase, incubation at 37 °C is performed by gentle mixing using a rotating wheel, lasting for the gastric and intestinal digestive phases of 2 h. Neutralizing the pH to 7 using NaOH improved the stopping conditions for the gastric phase leading to inactivation of pepsin, prior to snap-freezing in liquid nitrogen (Egger et al., 2016).

3 Protein Hydrolysis Testing with Skim Milk Powder as a Model Food

The IVD protocol was designed for the digestion of different complex food with variable nutrient content. As a protein rich and stable food, SMP was chosen to validate protein digestion from whole proteins to peptides and free amino acids. SMP was digested in vitro and the previously described analytical techniques (Fig. 1) were applied after the three endpoints of IVD (oral, gastric, and intestinal).

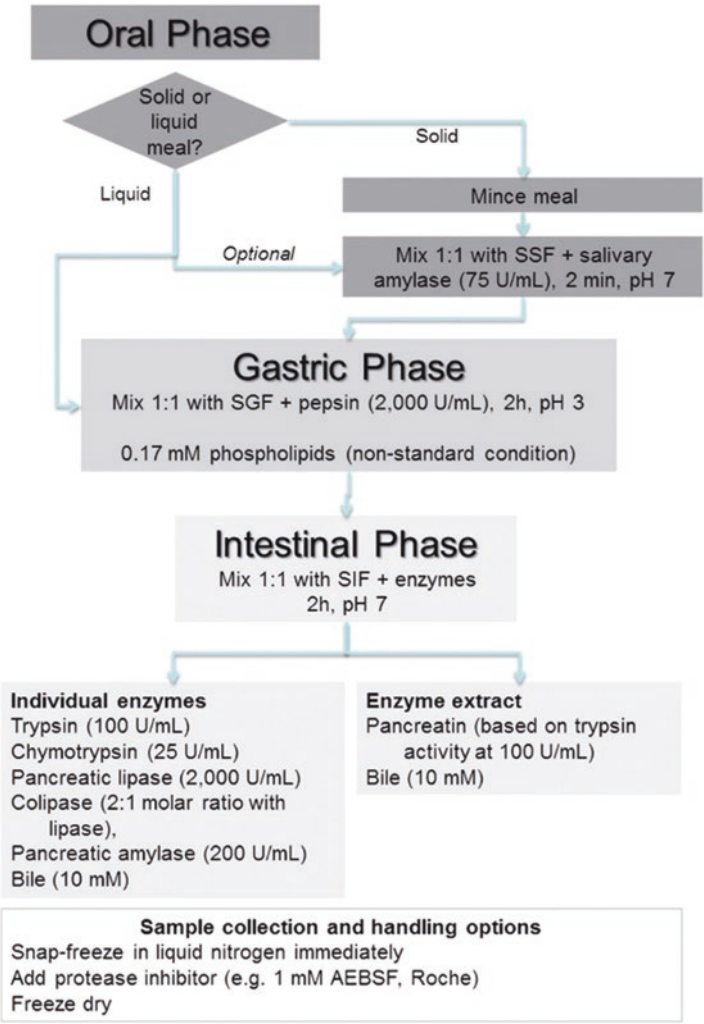

Fig. 2 Schematic workflow of the harmonized in vitro digestion protocol (Minekus et al., 2014)

4 Protein Separation by SDS-PAGE and Digestive Enzyme Identification by Mass Spectrometry

Protein separation by SDS-Page, although providing only a qualitative pattern on the cleavage of proteins rather than a quantitative assessment, is highly useful for visualizing differences between samples. For example, SDS-Page was performed with samples analyzed after three interlaboratory trials, where the participants applied their in-house digestion protocols in the first trial, and the harmonized

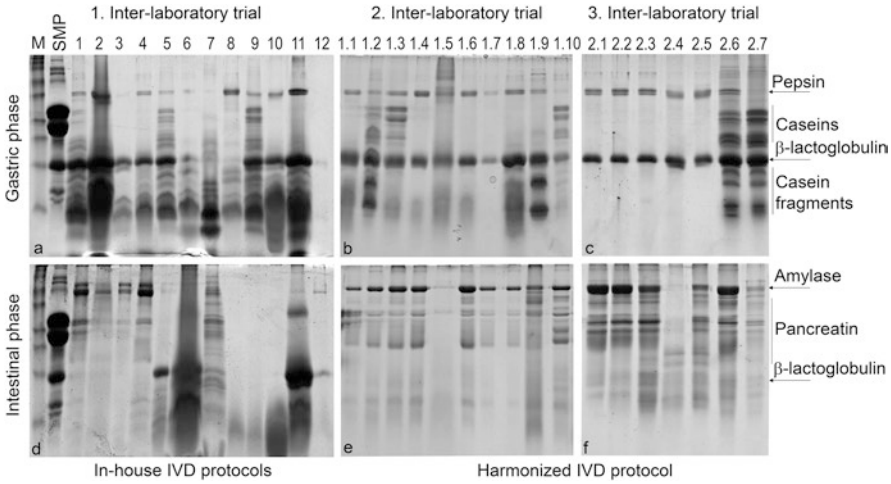

Fig. 3 SDS-Page of digested SMP samples after the gastric and intestinal phase of in vitro digestion. Samples used either In-house IVD protocols (**a**, **d**), or the harmonized IVD Infogest protocol (**b**, **c**, **e**, and **f**) (Egger et al., 2016)

protocol in the second and third trials (Fig. 3). The heterogeneity of the protein bands visible in the different samples illustrates the improvement in harmonization achieved by the consensus protocol. For example, digestion of β-lactoglobulin, previously shown to be resistant to pepsin in the gastric phase (Kopf-Bolanz et al., 2012), was degraded after the gastric phase in some samples of the first interlaboratory trial, while it was still present after the intestinal phase in other trials (Fig. 3, In-house IVD protocols). These differences were clearly absent after introduction of the harmonized IVD protocol (Fig. 3, Harmonized IVD protocol). Caseins previously shown to be degraded in the stomach (Sanchón et al., 2018), were still present at the end of the gastric phase in some cases, possibly indicating insufficient pepsin activity in some of the samples (Fig. 3, In-house IVD protocols).

In a previous work by Kopf-Bolanz and coworkers, the bands on the gel not attributed to major milk proteins when compared to the undigested pasteurized milk were cut from the gel and identified by mass spectrometry (Fig. 4) (Kopf-Bolanz et al., 2012).

5 Peptide Length Analysis by Size Exclusion HPLC

At the end of the intestinal digestion step, no dairy proteins could be identified from SDS-Page gels, indicating that they were hydrolyzed to smaller peptides than can be separated by this method. The smaller peptide fragments present after the different digestion steps were analyzed by size exclusion HPLC.

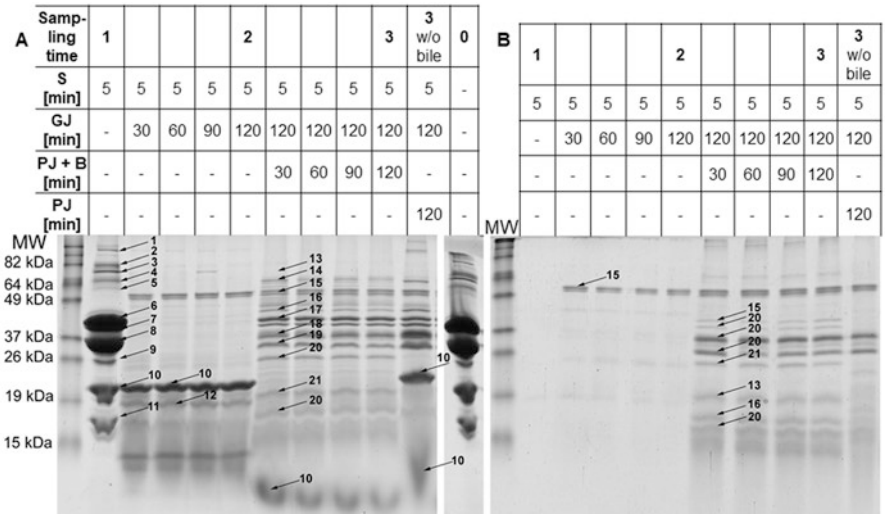

Fig. 4 SDS-Page of digested pasteurized milk samples after the oral (lane 1), gastric (lanes 2–5), and intestinal phases (lanes 6–10) of in vitro digestion and undigested pasteurized milk. Numbered bands were cut and identified by mass spectrometry according to Table 1

Elution of in vitro-digested samples after the intestinal phase was compared to intact milk proteins (Fig. 5a, Standard 1: αs_1-casein, Standard 2: αs_2-casein, Standard 3: α-lactalbumin), and also to smaller peptides (Standard 4: miraculin, 20 aa in length, and Standard 5: VPP, 3 aa in length). In the digested milk sample, ST3, the main peaks eluted between Standards 4 and 5, indicated that the peptide size in SMP after the intestinal phase corresponded to a peptide size between 3 and 20 aa in length. The evolution in peptide hydrolysis after the different digestion steps (Fig. 5b) showed no peptides after the oral phase (ST 1), protein fragments and larger peptides after the gastric phase (ST 2), and no fragments larger than 20 aa after the intestinal phase (ST 3). In addition, the presence of bile increased protein hydrolysis (ST 3 versus without bile).

6 Peptides from 5 to 20 Amino Acids: Protein Identification by LC-MS

Size exclusion HPLC only provides the size of the total present peptides after IVD, and requires a mass spectrometry analysis to obtain a specific peptide pattern for each individual protein. For this analysis, the digested samples were passed through cutoff filters with a pore size of 30 kDa, and subsequently separated by high pressure liquid chromatography (solvent gradient H_2O (A) to acetonitrile (B), both with 0.1% formic acid, 0–15 min: 5–60% (B), 15–20 min: 60–95% (B)), coupled to a mass spectrometer using an electron spray ionization interface (Kopf-Bolanz et al.,

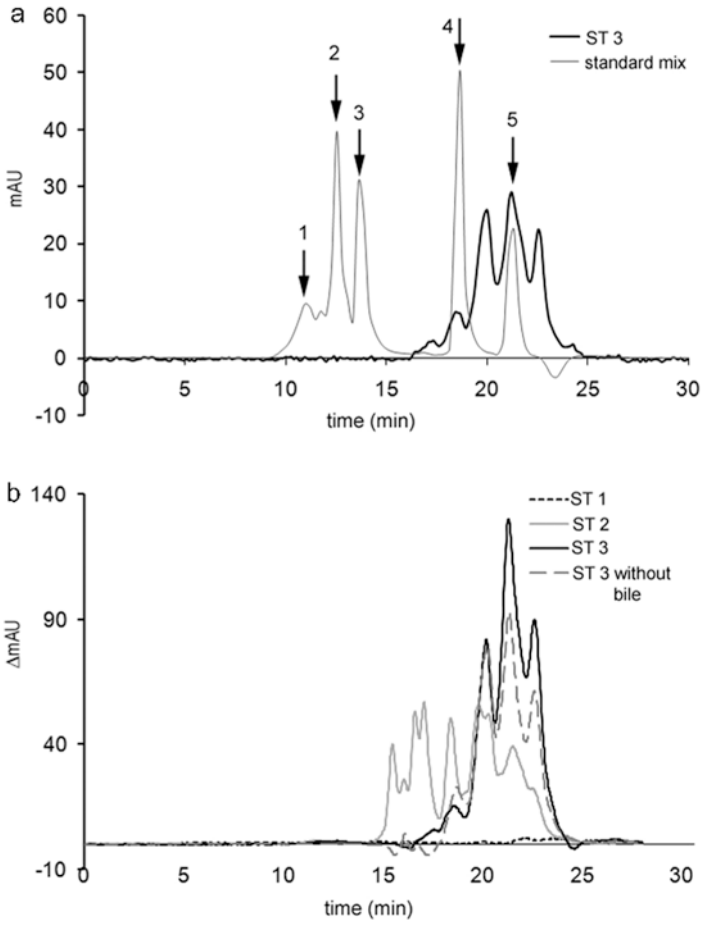

Fig. 5 Intestinal phase (ST3) comparison with standards (**a**); evolution of proteolysis in the different digestion phases ST1-ST3 (**b**). Size exclusion HPLC of digested SMP samples compared to standard proteins and peptides (Kopf-Bolanz et al., 2012). Standard 1: α-s_1-casein, Standard 2: α-s_2-casein, Standard 3: α-lactalbumin; smaller peptides (Standard 4: miraculin, 20 aa in length, Standard 5: VPP, 3 aa in length). ST1: oral phase, ST2: gastric phase, ST3: intestinal phase, and ST3 without bile

2012). The samples were measured in four overlapping narrow-mass windows for peptide fragmentation over a total range of 290–1300 m/z (i.e., 290–410, 390–610, 590–910, and 890–1300). The minimal signal intensity was set to 500 for MS/MS spectra generation. The obtained raw files were merged with Mascot Daemon, prior to the identification search with Mascot, using a milk protein database from different species. Peptides with a minimal length of 5 aa and an ion score cutoff of 20 were considered, and identified peptides aligned according to the protein sequence (Egger et al., 2017a). Peptides identified twice in a triplicate analysis were plotted to obtain an overview of the total identified peptides within a protein (Fig. 6). An

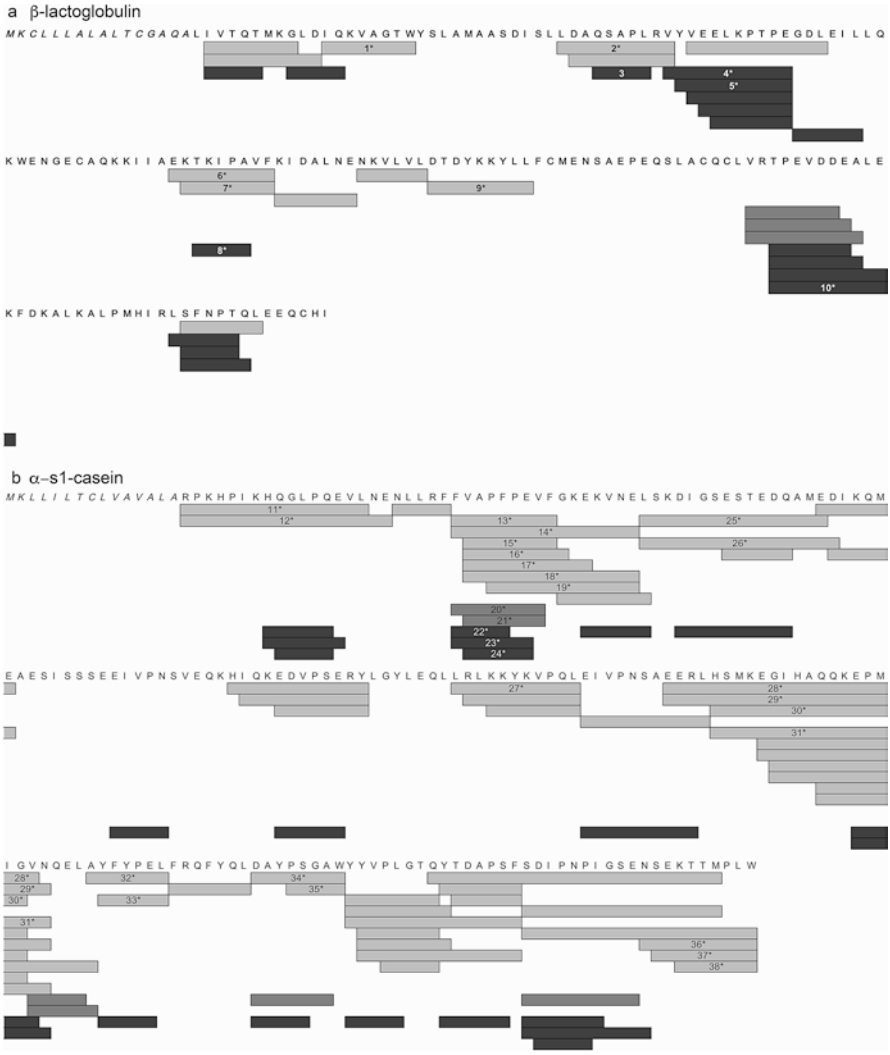

Fig. 6 Peptides of β-lactoglobulin (**a**) and α-s1-casein (**b**) identified after IVD. Light grey: Peptides identified after the gastric step; Dark grey: Peptides identified after the intestinal step; Numbers and asterisks: Peptides with reported bioactivity. ACE-inhibitory (1, 2, 4–7, 11, 12–24, 34–38); Antioxidative (4, 5); DPP-4-inhibitory (6–8, 13–18, 20, 21); IgE binding (10, 28–31); Mineral binding (25, 26); Antimicrobial (27), Immunocyto-modulatory (36–38) (Kopf-Bolanz et al., 2012)

example representation showing all peptides identified within the sequence of β-lactoglobulin (Fig. 6a) or αs_1-casein (Fig. 6b) was used for this qualitative visualization. Sequences with known bioactivities are highlighted with an asterisk (Kopf-Bolanz et al., 2014).

Typically, a peptide is identified multiple times per MS/MS run, and includes a relative quantification of protein stretches summed as the times an amino acid is

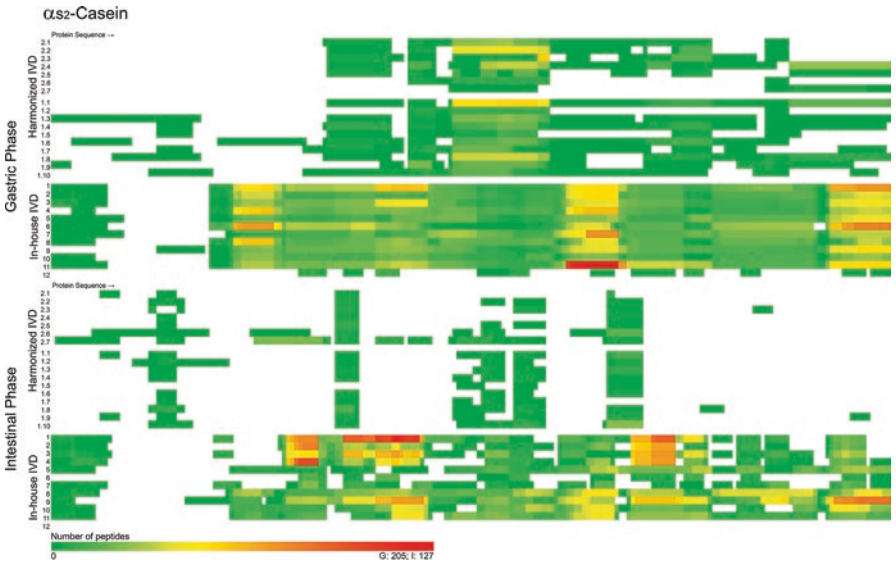

Fig. 7 Heatmap showing the abundance of amino acids identified within the sequence of αs_2-casein (Egger et al., 2016). White stretches indicate regions where no peptides were identified. Green segments indicate low abundance graduating to red segments showing high abundance (gastric phase max. 205, intestinal phase max. 127) of aa identification within the protein sequence

identified within the milk peptide, defined as amino acid count. The amino acid count can be represented in a heatmap using a color code from low abundance (green) to high abundance (red), with white stretches of non-identified sequences, such as the example comparison of peptide patterns of αs_2-casein between different laboratories (Fig. 7). This representation is especially useful when comparing many different samples.

Variability of peptide patterns between replicate samples can be visualized and quantified using an x-y-representation of the same amino acid count, where the protein sequence is represented on the x-axis, and the number of identifications within a peptide sequence is represented on the y-axis. A quantitative indication for the variability between samples can be obtained using a Spearman correlation analysis, such as the variability between pigs within the different sampling sections during SMP digestion. Individual samples are drawn in color with a calculated average pattern shown in black (Fig. 8). The figure includes the Spearman coefficient for each section, indicating the correlation between individual pigs.

The same x-y-graphs may be used to compare digestion models, such as between the harmonized IVD at the gastric (G) and intestinal (I) digestion endpoints with the corresponding in vivo pig sampling sections (S or D, and I1-I4), allowing for visualization of similarities, for example, β-casein protein patterns (Fig. 9).

A statistical evaluation showing the variability between samples (such as samples from different laboratories shown in Fig. 7) can be performed by calculating a

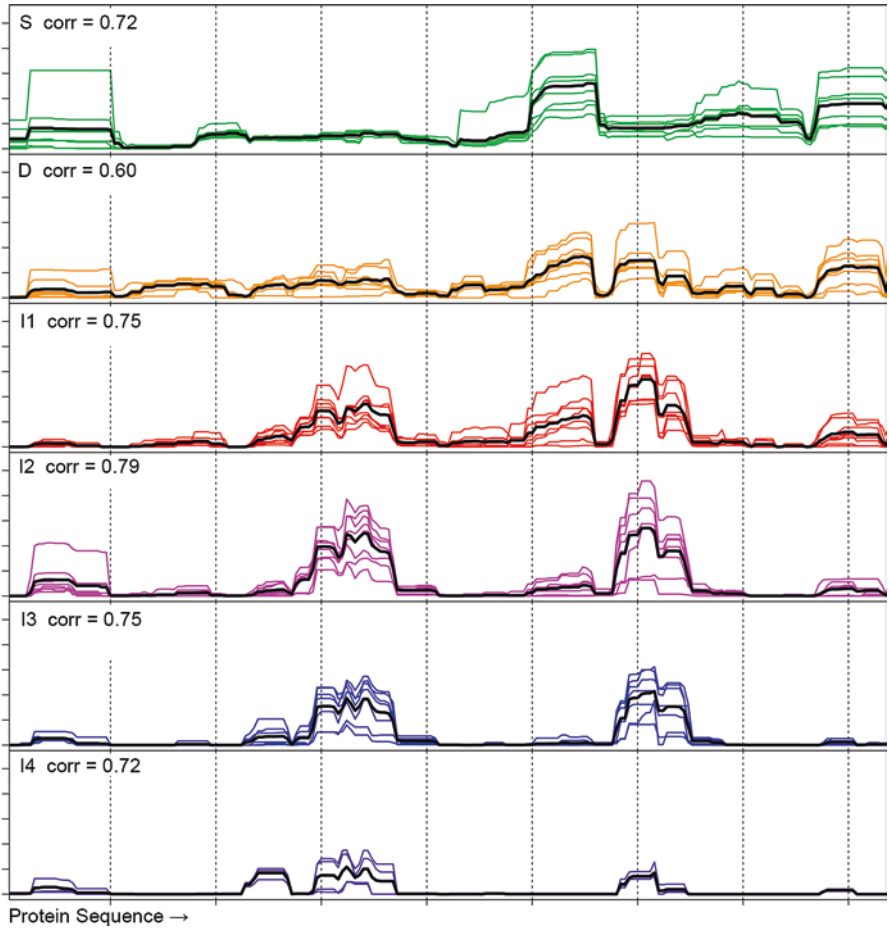

Fig. 8 Variability of peptide patterns from β-casein among different pigs after SMP consumption at different sampling sections, including Stomach (S), duodenum (D), proximal ileum (I1), median ileum (I2), distal ileum (I3), jejunum (I4). The calculated average peptide drawn in black, and correlation coefficients cited (Egger et al., 2017a, 2017b)

Spearman correlation matrix considering the peptide patterns of the major milk proteins (αs_1-, αs_2, β-, κ-casein, and β-lactoglobulin) between all different samples (Fig. 10).

A partial least squares analysis can be performed to visualize the similarities and differences among samples from several digestion models, such as SMP digestion in pigs, the harmonized IVD, and the individual protocols of IVD (Bohn et al., 2017). For this analysis, the peptide patterns previously described in Figs. 7 and 8 of the major milk proteins were compared among the different digestion models. An average peptide pattern was calculated from individual pigs, and compared to the

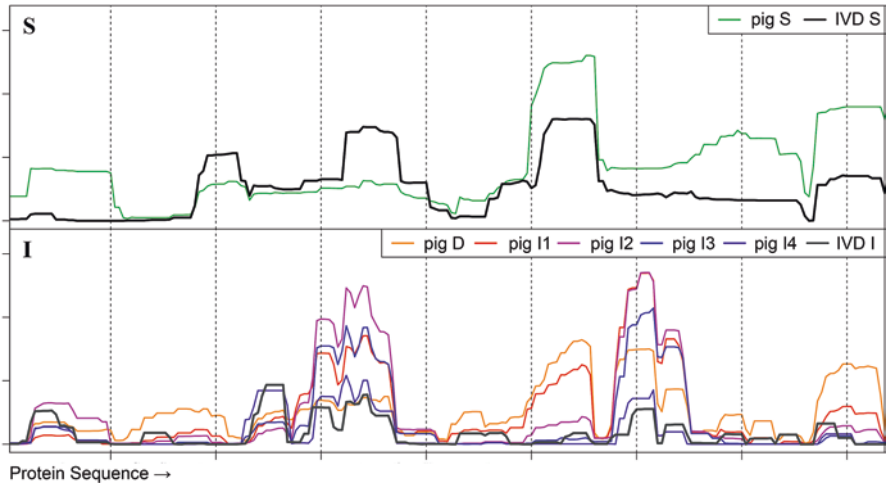

Fig. 9 Comparison of average peptide patterns between IVD and in vivo pig digestion. Overlay of peptide patterns from β-casein after IVD (black) with pig (green) samples after the gastric phase (S) and IVD samples (grey) with pig samples (D-I4) of intestinal (I) samples (Egger et al., 2017a, 2017b)

results from the interlaboratory trials using different in-house protocols (Fig. 11, In-House Stom, In-House Intestin) or the harmonized Infogest protocol (Fig. 11, Harmo Stom, Harmo Intestin). The different sampling sections in the pigs show an evolution from the stomach to duodenum, the proximal ileum (Pig Int 1), and the medial ileum (Pig Int 2). The harmonized gastric samples clustered with the pig gastric samples, and the harmonized intestinal samples clustered with the pig medial intestinal samples (Pig Int 2). In contrast, the samples using the in-house protocols were clearly separated from the other models, indicating that their peptide patterns were different.

7 Analysis of Total Free Amino Acids with the OPA Method

At the end of digestion, most proteins are degraded to FAA. Total FAA can be analyzed using the OPA method, where primary amine groups are derivatized with o-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol (Kopf-Bolanz et al., 2012). However, this method does not discriminate between single AA or dipeptides and tripeptides present in the solution after the precipitation step. Additionally, as the OPA reagent reacts with all primary amines present in a solution, some amino acids with two primary amines will result in a higher OPA value. These imprecisions must be considered when using this method for analysis of total free amino acids, making the method suited for comparison purposes rather than absolute quantification. Comparison of digestion experiments between different laboratories

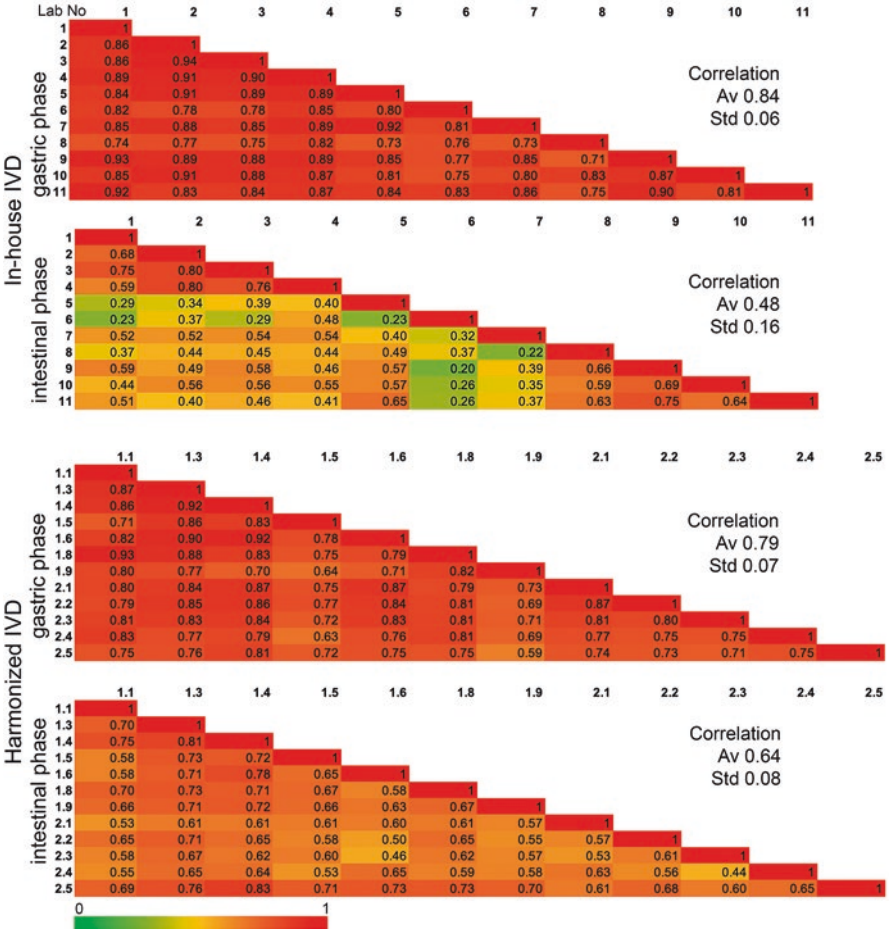

Fig. 10 Spearman correlation matrix calculated considering the major milk proteins (αs_1-, αs_2-, β-, κ-casein and β-lactoglobulin) including three different interlaboratory trials performed before harmonization (In-house IVD), and after harmonization (Harmonized IVD) of the protocol (Egger et al., 2016)

indicates that only a minor amount of FAA (<1%) is present after the gastric phase, with >90% of the proteins hydrolyzed to FFA and small peptides at the end of digestion (Fig. 12). The figure shows the maximal value (100%) release of FAA from SMP, considering the total protein content analyzed by the Kjeldahl method (ISO (8968-3:2007/IDF 20-3:2007) Milk. Determination of nitrogen content. 2007) and the six most abundant milk proteins (αs_1, αs_2-, β-, κ- casein, β-lactoglobulin, and α-lactalbumin) according to their relative distribution (Eigel et al., 1984).

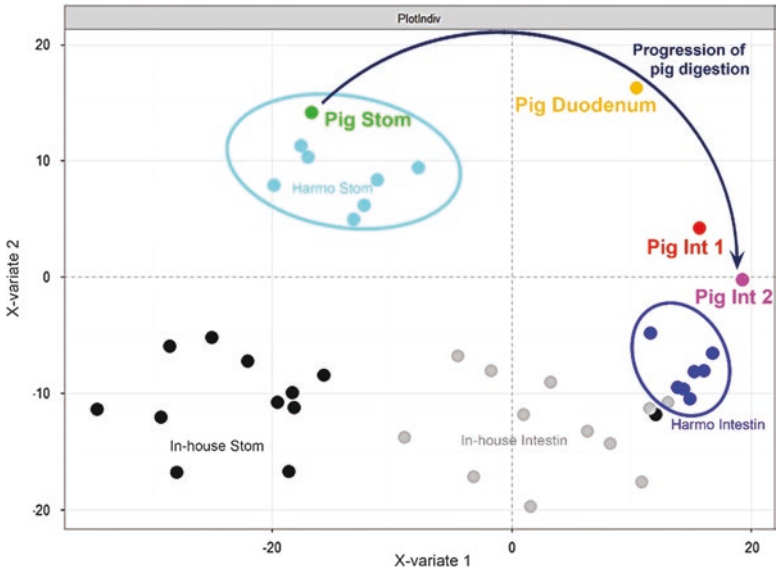

Fig. 11 Partial least squares analysis performed with samples from different digestion protocols (Bohn et al., 2017). SMP was digested in vivo in pigs with the harmonized IVD protocol, or with individual in-house digestion protocols during interlaboratory trials

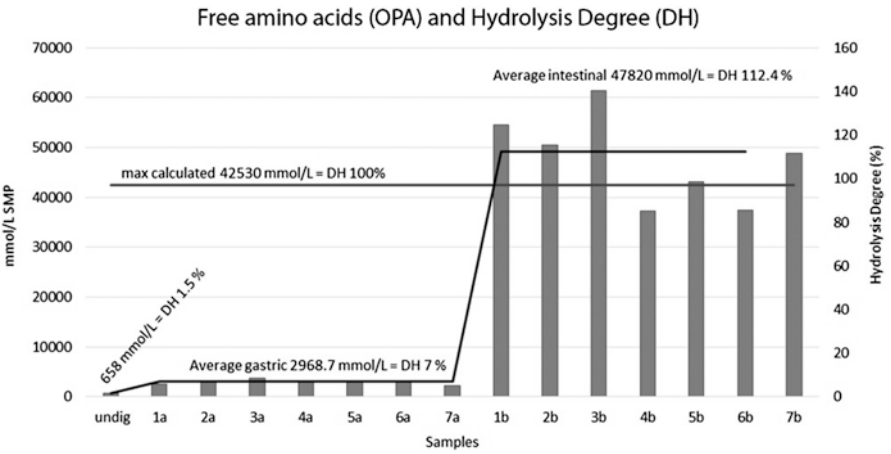

Fig. 12 Analysis of total FAA and degree of hydrolysis with the OPA method after gastric and intestinal phases of the harmonized IVD of SMP digestion. Samples 1–7 are from different laboratories, a: gastric phase, b: intestinal phase (Egger et al., 2016)

8 Determination of the Degree of Hydrolysis of Digested Protein

Several methods exist for the determination of the degree of hydrolysis (DH) of the digested proteins, defined as the percentage of hydrolyzed peptide bonds in a protein (Adler-Nissen, 1986). Most often, the DH is analyzed by pH stat, based on the NaOH consumption at a constant neutral pH, and the calculation of the released amino acids (Spellman et al., 2003). Other methods are based on compounds that react with amino groups, such as trinitrobenzene-sulfonic acid (TNBS) or the OPA method (Nehir El et al., 2015; Nielsen, Petersen, & Dambmann, 2001; Spellman, McEvoy, O'Cuinn, & FitzGerald, 2003). The degree of hydrolysis is then calculated based on a reference amino acid (Std.AA), for example, serine (Nehir El et al., 2015; Nielsen et al., 2001), or glutamic acid (Fig. 11b), multiplying the delta A between sample and standard (Std.AA) according to the formulas (Fig. 13).

9 Analysis of Individual Free Amino Acids by HPLC

The analysis of individual FAA is more precise, and gives additional information on the liberation of essential versus nonessential amino acids of a certain food. For example, during digestion of pasteurized milk, it has been observed that essential AA were preferentially released (Kopf-Bolanz et al., 2012) (Fig. 14). This analysis also indicates whether certain amino acids are more resistant to hydrolysis than others. Proline, for example, was found to be highly resistant to enzymatic hydrolysis (Hernandez-Ledesma, del Mar Contreras, & Recio, 2011), and is found in many proline-rich peptides after the digestion process, such as VPP and IPP.

(1) $$Std.AA - NH_2 = \frac{A_{sample} - A_{blank}}{A_{Std} - A_{blank}} \times conc.Std \frac{mmol}{L} \times 0.1 \times \frac{100}{Sample\ (g)} \times protein\ (\%)$$

(2) $$h = (Std.AA - \beta)/\alpha\ mmol/protein(g)$$

(3) $$DH = \frac{h}{h_{tot}} \times 100$$

Fig. 13 Formulas for the calculation of DH: The absorption (A) is measured at 340 nm, values for α and β were calculated for a ratio of casein to whey of 85.3/14.7%, found in SMP, where $\alpha = 1.033$, $\beta = 0.386$ (Adler-Nissen, 1986). The h_{tot} is the total number of peptide bonds that can be hydrolyzed calculated for SMP with a ratio of 45.7% β-casein, 14.1% αs_1 casein, 14.1% αs_2 casein, 10.2% κ casein, 11.3% β lactoglobulin, and 3.2% α-lactalbumin

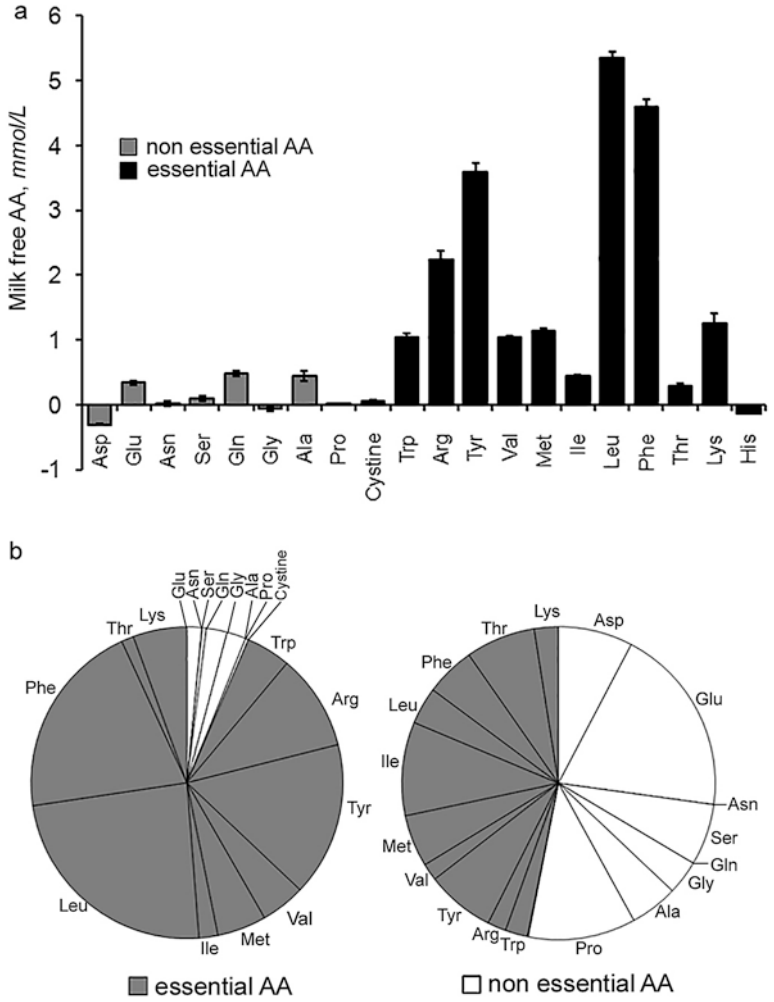

Fig. 14 Analysis of individual FAA by HPLC after the intestinal step of IVD. Essential and non-essential free AA after the intestinal step of IVD (**a**). Ratio of essential/non-essential AA after the intestinal phase of SMP digestion (**b**, left panel), versus calculated values in milk (**b**, right panel) (Kopf-Bolanz et al., 2012)

10 Methods for the Quantification of Fat Digestion

Fat digestion during IVD was previously investigated by Bourlieu and coworkers (Bourlieu et al., 2015) and Kopf-Bolanz and coworkers (Kopf-Bolanz et al., 2012). The digestion of triglycerides, the main dietary fat contained in food, starts to a small extent with the action of gastric lipase in the gastric step. Due to the cost and

limited availability of human gastric lipase, most IVD protocols omit this enzyme (Minekus et al., 2014). Fat digestion occurs mostly in the intestinal step through the emulsifying action of bile and pancreatic lipase. Triglycerides first undergo hydrolysis to diacylglycerides and monoacylglycerides before FFAs and glycerol are released.

The first hydrolysis steps can be analyzed by thin-layer chromatography.

11 Thin-Layer Chromatography

Understanding digestion of lipids from either human milk or infant formula is relevant in the context of infant nutrition. Studies dealing with infant lipid digestion are presented to focus on methods employed to assess lipolysis.

Thin-layer chromatography (TLC) is employed to follow and visualize the evolution of different classes of lipids during the digestion process compared to lipids present in undigested food, providing a qualitative view of the products of lipolysis and concomitant residual triglycerides.

Bourlieu et al. (2015) evaluated the impact of technological treatments, such as homogenization and heat treatment, on gastric lipolysis and structure of infant formula. Three different matrices were submitted to in vitro gastric digestion using rabbit gastric extract as a source of gastric enzymes containing pepsin and lipase. The matrices were obtained after (1) applying minimal treatment (skimming and standardization) with low impact on native milk fat globules, shown as M1 in Fig. 15; (2) homogenization of M1 using a two-stage, high-pressure homogenizer, shown as M2 in Fig. 15; or (3) pasteurization treatment applied to M2, shown as M3 in Fig. 15.

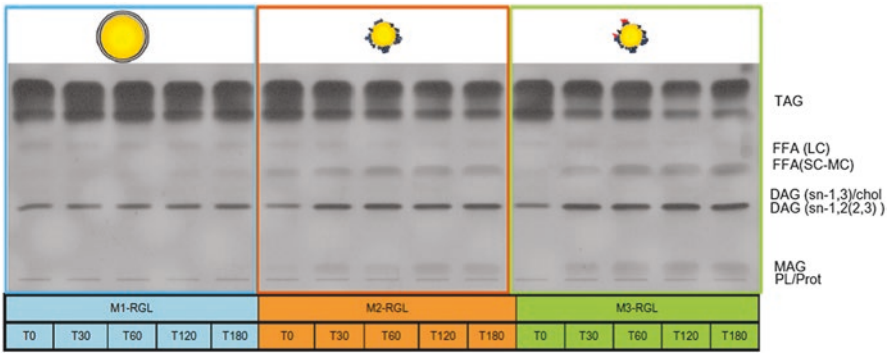

Fig. 15 Thin-layer chromatography study of the evolution of lipid classes during in vitro gastric digestion. Abbreviations: *TAG* triacylglycerol, *FFA* free fatty acids, *LC* long chain, *SC* short chain, *DAG* diacylglycerol, *chol* cholesterol, *MAG* monoacylglycerol, *PL* polar lipid, *prot* protein (Bourlieu et al., 2015)

These three matrices, similar in terms of composition, mimic the structure of either human milk (M1) or infant formula (M2, and M3). The objective was to assess the impact of the fat droplet structure on the kinetics of lipolysis and matrix disintegration. During the in vitro digestion assays, aliquots of samples were withdrawn and direct lipid extractions performed. After the lipid extraction, the chloroform phase was spotted on silica gel plates using an automatic TLC sampler, as described previously (Bourlieu, Rousseau, Briard-Bion, Madec, & Bouhallab, 2012).

After in vitro gastric digestion, the lipid classes of the three matrices were dominated by residual TAG, FFA, and sn-1,2(2,3) DAG lipolysis products. At 30 min of digestion, traces of MAG appeared in the two most processed matrices, indicating that a small fraction of sn-1,2(2,3) DAG was further hydrolyzed to MAG in the gastric step.

The three matrices had different behaviors when comparing the disappearance of the TAG, and the appearance of the lipid products (FFA, DAG, and MAG). For the M2 and M3 matrices, bands of FFA and DAG were more intense, and TAG bands were clearer than the bands observed for M1 (Fig. 15). The specific surfaces of the lipid droplets of the three matrices differed due to the different surface adsorption available for gastric lipase. In terms of infant nutrition, this study highlights that the structure of the emulsion is a key parameter governing the kinetics of the gastric lipolysis. Infants fed with either human milk or infant formula have different patterns of lipolysis, and potentially different fatty acid bioaccessibility.

Densitometric analyses of the plates by measuring the grey intensity of each band provides a semiquantitative analysis of the different lipid classes observed before and during digestion. The lipolysis level is calculated by comparison to the band representing the undigested matrix. Maintaining a linear relationship between the amount loaded on the plates and the grey intensity prevents interpretation issues.

A more appropriate method to evaluate and quantify the FFA uses thin-layer chromatography coupled to a flame ionization detector (TLC-FID), such as IATROSCAN equipment (Carriere et al., 2005).

De Oliveira et al. (2016) evaluated the impact of holder pasteurization of human milk on the kinetics of lipolysis during in vitro dynamic digestion at a term newborn stage. The evolution of lipid classes was analyzed using IATROSCAN MK5 equipment. To allow for quantification, the mass detection data were converted into moles using the average molar masses (g/mol) calculated from the FA composition of raw human milk (RHM). The instantaneous lipolysis degree during digestion was expressed as the percentage level of FFA (in moles), versus the total acyl chains present in residual glycerides and FFA quantified at a given time, shown as the following equation:

$$LD = \frac{100 * [FFA]}{\left(3 * [TG] + 2 * [DG] + [MG] + [FFA]\right)}$$

The lipolysis degree (LD) is calculated as a percentage using [FFA], [TG], [DG], and [MG], respectively, representing the free fatty acids, triacylglycerides, diacylglycerides, and monoacylglycerides in molar concentration (mol/L) (Fig. 16).

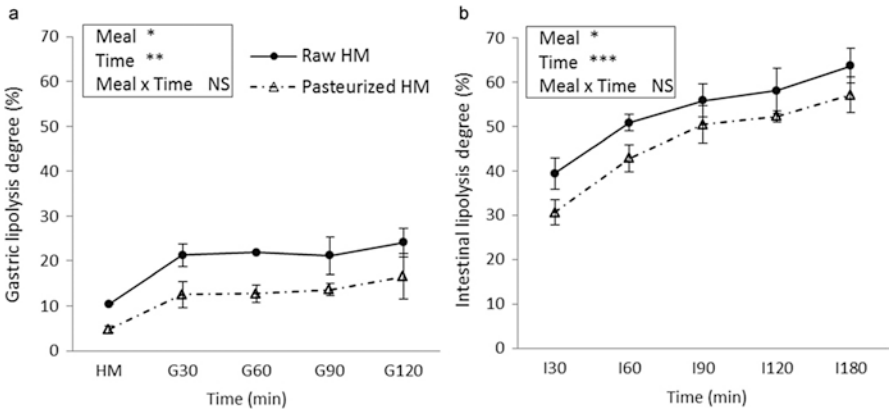

Fig. 16 Kinetics of lipolysis of RHM (Raw Human Milk) and PHM (Pasteurized Human Milk) during gastric (**a**) and intestinal (**b**) in vitro dynamic digestion simulating newborn term conditions. The lipolysis degree was determined based on TLC–FID for digesta and on gas chromatography for undigested milk. Data points represent means ($n = 2$ for RHM and $n = 3$ for PHM) ± SD. $p < 0.001$ (***), $p < 0.01$ (**), $p < 0.05$ (*), and $p > 0.1$ (NS) (de Oliveira et al., 2016)

Only 10–20% lipolysis was measured at the end of the gastric digestion step, as lipolysis occurred mainly in the intestinal step, where the level improved up to 60% for the RHM. A meal effect was observed, pasteurization led to a lower extent of lipolysis due to inactivation of the endogenous bile salt stimulated lipase present in human milk by pasteurization.

12 Analysis of Total and Individual Free Fatty Acids by Gas Chromatography

TLC coupled to FID allows for quantification and monitoring of the kinetics of lipolysis, but cannot identify the profiles of the released FFA. FFA can be analyzed after solid phase extraction by gas chromatography (GC), using internal standards (for example, C5, C11, and C17) added to the aliquot prior to solvent extraction, as previously described by Bourlieu et al. (2012) (Fig. 17).

The profiles of FFA released during digestion were compared to the profile of the FA initially esterified (Fig. 17). Regardless of the type of meals, during lipolysis, FFA (C18:1, c9, and C18:0) were selectively released as the relative amounts were higher in the released FA fraction than in the total FA initially esterified, while the proportion of released C16:0 tended to be lower when compared to the total FA initially esterified. GC is also a suitable method to quantify FFA if internal standards are added at the lipid extraction step. A similar analysis of FFA by GC after the intestinal step of IVD revealed that SCFA were hydrolyzed at a higher rate (Fig. 18a)

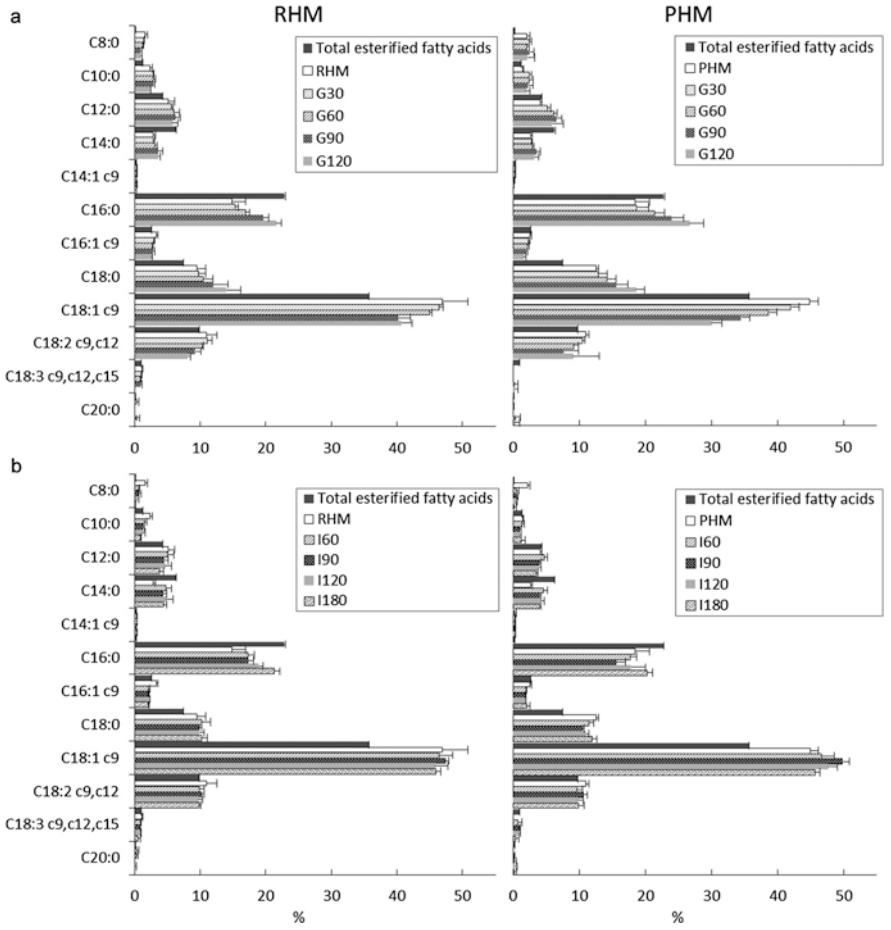

Fig. 17 Acyl chain profiles initially esterified in human milk compared to free fatty acids released from RHM and PHM during gastric (**a**) and intestinal (**b**) in vitro dynamic digestion. Fatty acids were determined by gas chromatography. Data points represent means ($n = 2$ for RHM and $n = 3$ for PHM) ± SD (de Oliveira et al., 2016)

than the medium- and long-chain FA, compared to the original product (Fig. 18b). The authors explained this observation by the preferential location of SCFA at the sn3-position, similar to the sn1-position, the preferred substrate for hydrolysis by pancreatic lipase (Kopf-Bolanz et al., 2012). To evaluate lipid digestion, the combination of different qualitative and/or quantitative methods allows for an interesting overview of lipolysis events.

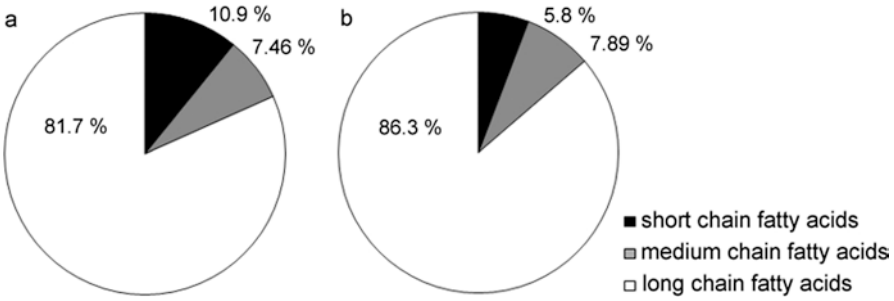

Fig. 18 Analysis of classes of individual FFA after the intestinal step of IVD of pasteurized milk by GC (**a**) and total FA present in undigested pasteurized milk (**b**) (Kopf-Bolanz et al., 2012)

13 Digestion of Fat and Carbohydrate-Rich Food

The digestion of carbohydrate-rich food was not included in the interlaboratory comparisons of the Infogest Network. Dairy products, especially SMP as food, are not suited for the study of carbohydrate digestion as their main carbohydrate is disaccharide lactose. Lactose is hydrolyzed by lactase, an enzyme secreted by the cells of the brush border membrane, which are not integrated in most of the IVD models. One of the models including brush border enzymes has been described by Picariello et al. (2015).

Digestion of complex carbohydrates, such as starch and dextran, is initiated in the oral phase by salivary amylase. During the gastric phase, salivary amylase further acts on the bolus only during the initial step of gastric digestion, due to the decrease in pH at later stages of digestion (Minekus et al., 2014). Most of the starch is hydrolyzed through the action of pancreatic amylase, secreted into the duodenum, and integrated in the intestinal step of most of the IVD protocols as an individual enzyme or as a component of pancreatin (Kopf-Bolanz et al., 2012; Minekus et al., 2014; Versantvoort et al., 2005). A recent work on starch digestibility described by Romano et al. applied the AACC International Approved Method 32-40.01 (Resistant Starch in Starch Samples and Plant Materials) (Romano et al., 2016). Hasjim and coworkers compared in vivo and in vitro starch digestion by applying the AACC International Approved Method 76-13.01 Total Starch Assay Procedure (Megazyme Amyloglucosidase/alpha-Amylase Method) (Hasjim, Lavau, Gidley, & Gilbert, 2010). Warren and coworkers used two methods for the quantification of hydrolysis rates by α-amylase and amyloglucosidase from potato and maize starch, during in vitro digestion experiments (Warren, Zhang, Waltzer, Gidley, & Dhital, 2015). In the first method, released glucose was enzymatically quantified with glucose-oxidase and peroxidase (GOPOD-method), and the second method quantified the reducing sugar with para-hydroxybenzoic acid (PAHBAH), (Moretti & Thorson, 2008). Both methods led to comparable

results, although the PAHBAH assay seemed to overestimate the initial rate of starch digestion (Minekus et al., 2014).

14 Food Digestion and Nutritional Aspects

14.1 In Vitro Digestion and Allergenicity Studies

The allergenic potential of proteins in the diet has been related to their digestibility (Bossios et al., 2011). One example of digestion-resistant protein is the whey protein β-lactoglobulin, previously shown to be resistant to gastric digestion (Egger et al., 2016; Kopf-Bolanz et al., 2012, 2014; Mandalari et al., 2009) (Fig. 19). Different dairy products were analyzed by SDS-PAGE after gastric digestion (Fig. 19b), and intestinal digestion (Fig. 19c), and the bands identified by mass spectrometry. After the gastric step, all products contained different levels of full-length or short version β-lactoglobulin with the exception of Gruyère cheese, which does not contain any whey proteins. Although no β-lactoglobulin was visible on gel after the intestinal step, peptides were present at this stage (Fig. 20). Peptide patterns of β-lactoglobulin after gastric and intestinal in vitro digestion analyzed by mass spectrometry showed the presence of digestion-resistant stretches in the protein. Some of these digestion-resistant stretches (Fig. 20, black frame) correspond to highly allergenic sequences identified in cow's milk allergy (CMA) (Miller, Meredith, Selo, & Wal, 1999; Selo et al., 1999; Wal, 1998).

14.2 Release of Bioactive Peptides

Protein digestion is not the complete breakdown from whole amino acid sequences to single free amino acids. Both in vivo and in vitro studies have demonstrated that the digestion process releases smaller peptides (Aluko, 2015; Nongonierma & FitzGerald, 2015a, 2015b), and larger peptides (Regazzo et al., 2010), that are eventually absorbed by a mechanism that is not completely understood. Several studies report the identification of milk protein-derived peptides in human serum after their consumption, confirming the bioavailability of these peptides (Caira et al., 2016; Sanchez-Rivera et al., 2014, 2016). Physiological effects after oral administration of the pentapeptide HLPLP from β-casein were observed in spontaneously hypertensive rats (Sánchez-Rivera et al., 2016). The recent increase in related publications shows the high interest in bioactive peptides as nutraceuticals without the side effects of commonly used drugs. A summary of milk-derived peptides and recent research is presented in a recent review by Egger & Ménard (2017).

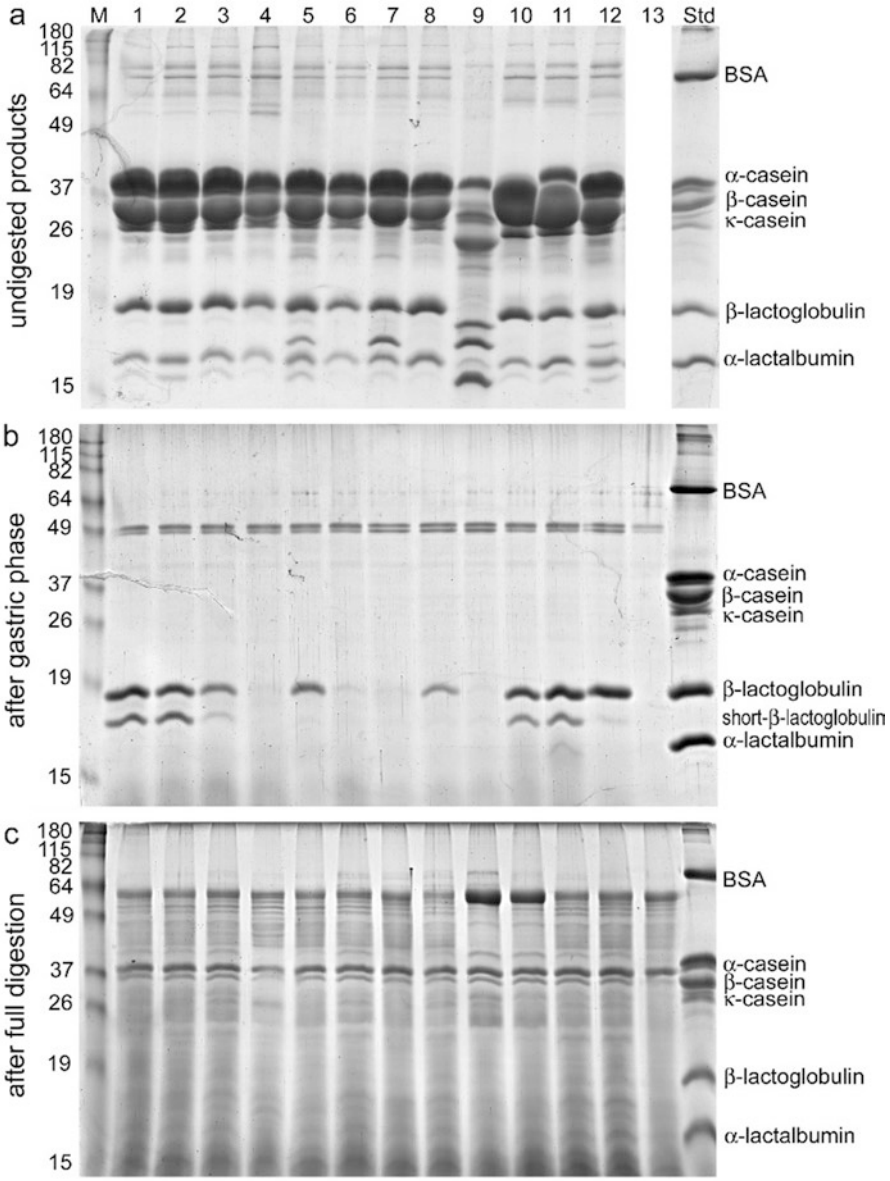

Fig. 19 SDS-PAGE of IVD samples from different dairy products after the gastric phase, showing the resistance of β-lactoglobulin to pepsin (Kopf-Bolanz et al., 2014). Lanes include (1) raw milk, (2) pasteurized whole milk, (3) UHT whole milk, (4) UHT cream (35% fat), (5) skimmed UHT milk, (6) yoghurt, (7) curd cheese, (8) kefir, (9) Gruyère cheese, (10) sheep milk, (11) goat milk, and (12) acidified pasteurized milk (pH 4.6)

Fig. 20 Heatmap showing amino acids identified within the sequence of β-lactoglobulin (Egger et al., 2016). White stretches indicate regions where no peptides were identified. Green segments indicate low abundance graduating to red segments showing high abundance (gastric step max. 29, intestinal step max. 54) of AA identification within the protein sequence. A black border identifies two highly allergenic regions (Miller et al., 1999; Selo et al., 1999; Wal, 1998)

14.3 Release of Essential Amino Acids After Digestion of SMP

In vitro digestion experiments with SMP showed that essential amino acids were preferentially released (Fig. 14). This phenomenon was also observed to a lesser extent in a pig trial where the same SMP was digested in vivo and samples were taken along the digestive tract from the stomach, duodenum, proximal jejunum, and median jejunum (Fig. 21).

15 Conclusions

This overview on analytical methods for monitoring protein, fat, and carbohydrate digestion showed that for each nutrient, multiple methods are required to visualize the different levels of hydrolysis in different compartments. Comparisons between

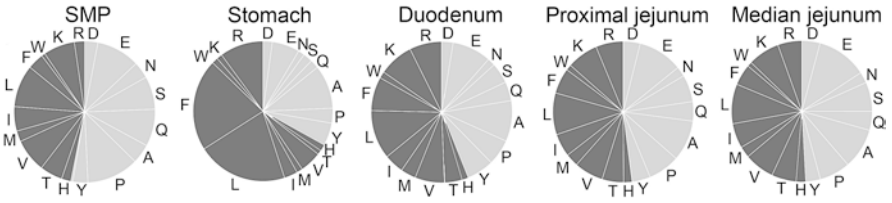

Fig. 21 Release of essential and non-essential free amino acids after digestion of SMP in pigs along the digestive tract in samples from the stomach, duodenum, proximal jejunum, and median jejunum. SMP indicates the calculated distribution of amino acids

laboratories or different digestion models yield the most successful results, by performing interlaboratory studies, using the same methods, or using the same food. The applicability of the presented methods of protein hydrolysis to other protein-rich food or to food in general must be demonstrated in each case.

The development of the harmonized IVD, and in parallel the development of the interlaboratory trials with SMP were possible thanks to the Infogest Network (http://www.cost-INFOGEST.eu/), where specialists in food digestion from over 25 countries worked together.

The principal focus of this overview is on protein hydrolysis, especially SMP digestion, as the harmonized IVD protocol was developed with this focus. However, recent work has expanded the research to include lipid and carbohydrate digestion, and future work may show a more precise picture of step-by-step hydrolysis of these nutrients.

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Consumer Psychology and Eating Behaviour

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1 Introduction

Intuitively, one might think that the amount of food taken to the mouth during a meal depends solely on the calorie content and eventually on whether we like the food or not. Of course, if we taste a soup, say, and we dislike it, we will not consume it to its entirety, neither will we do the same if the portion is too large for us. However, there are many more factors concerning the surroundings of the food (i.e. how the food is presented) that play a major role in food intake. I will refer to these as extrinsic parameters since they are not linked to the caloric content or the sensory properties of the food but more to presentation aspects that affect primarily our cognition about the food (expectations) and eventually may impact our perception and digestion process.

There are several visual cues that might give rise to expectations about the portion size or the satiating ability of the food served, these range from written information about the food's calorie content or other nutritional information, through to the way in which the food items or contents are displayed (mixed, grouped by colour or size). Additionally, there is also evidence suggesting that even the colour and haptic cues, specifically, the weight of the container, might also lead people to believe that what they are about to ingest will be lighter or heavier.

Throughout this chapter I will review the empirical evidence on these research areas, I will then describe some of the most common methods used to assess how people create expectations about the satiating properties of foods, and then I will discuss the potential theoretical explanations behind these phenomena. Let us start with what is probably the most established effects, those of written labels, specifically health-related information.

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2 Health-Related Labels

As one would expect the information about fat content of a product might influence our decision about how much to consume of it. In which direction? In the early 1990s a study was conducted where people ate more vanilla ice-cream when it was accurately labelled as "high-fat" than when inaccurately labelled as "low fat" (Bowen, Tomoyasu, Anderson, Carney, & Kristal, 1992). However several more recent studies have documented an opposite pattern of results. For instance, Provencher, Polivy, and Herman (2009) reported that people (regardless of whether they were restrained eaters or not) ate a significantly greater number of cookies (35% more) when they were described as made with "healthy" ingredients compared to those supposedly made with "unhealthy" ingredients instead. These behavioural responses are mostly observed among younger and female participants (e.g. Piqueras-Fiszman, Ares, & Varela, 2011). There are some interesting cross-cultural differences to be noted when it comes to explaining why we would decide to eat more, or less, from a food categorised as being "healthy" or "unhealthy": Werle, Trendel, and Ardito (2013) found that the French implicitly associate "healthy" with "tasty" and equate "unhealthy" with "untasty" when shown images of foods that are obviously healthy and unhealthy (such as salad and pizza, respectively), as well as when given a neutral food (a mango milkshake) labelled as either "healthy" or "unhealthy". North Americans, by contrast, tended to associate "unhealthy" with "tasty" instead (see Raghunathan, Naylor, & Hoyer, 2006).

Cavanagh and Forestell (2013) extended this study to look at snack brands that are typically associated with healthy or unhealthy eating. In this study, consumers rated cookies given a healthy brand label as being more satisfying and as having a better flavour, when actually the biscuits they were eating were identical in both conditions. Furthermore, restrained eaters consumed more of the healthy brand than of a brand that had been described as less healthy. By contrast, unrestrained eaters did not seem to care much about the label that was given to the food. This might help explain the contradictory results that have now been documented across a variety of foods. For instance, Wansink, Ittersum, and Painter (2004) reported that a meal description including "healthy" or "diet" gave rise to higher liking ratings for hedonic foods (e.g. desserts) than for side dishes or starters.

On occasion, the label "healthy" is not needed for a certain dish to convey healthiness in the mind of the consumers. Irmak, Vallen, and Robinson (2011) presented participants with a pasta salad (a mixture of chopped tomatoes and other vegetables, pasta shells, salami, and mozzarella, served on romaine lettuce and dressed with a vinaigrette). Indeed this dish could be identified as either a salad or as a pasta dish. However, when it was identified as "pasta", the participants who were dieters rated it as being less healthy and less tasty than did the non-dieters. Conversely, when described as a salad, the dieting status of the participants had no effect on their overall evaluation of the dish. In this case, changing the description of the food resulted in a change in consumption. The authors explained that the non-dieters might have been insensitive to food cues, relied on cues indicating a lack of

healthiness and tended to use a heuristic strategy when they evaluated the foods. A similar follow-up study revealed that participants ate twice as much fruit candy when it was labelled as “fruit chews” rather than as “candy chews”.

So, is labelling or categorising a food product as healthy, or highlighting its healthier properties, leading to higher calorie intake? Wansink and Chandon (2006) have claimed that including low-fat nutrition labels might actually contribute to the growing obesity crisis. Their suggestion was that people might eventually consume more because they believe that a food is light (but see Miller, Castellanos, Shide, Peters, & Rolls, 1998). As the literature reviewed in this section has made clear, the health-related information given to consumers not only created expectations but actually affected their reported sensory perception, their liking of the product (which heavily depends on consumers attitudes towards their diet), and how much they serve themselves and eventually consume.

3 Container Characteristics

Apart from foods’ label, other non-verbal cues extrinsic of the food, such as the colour of the container or its weight, might also modulate the way people perceive the food and eventually its consumption. Regarding the effect of certain colours of the extrinsic elements of food on people’s intake, it is worth considering the fact that the colour red has been shown to elicit avoidance motivation across a variety of behavioural contexts [e.g. Mehta & Zhu, 2009; though Singh (2006), once reported that red stimulates appetite]. Several studies have investigated the effect that the colour red has on the consumption of both snack foods and soft drinks (e.g. Genschow, Reutner, & Wänke, 2012). For instance, the participants in Genschow et al.’s study drank less from a cup with a red label than from a cup with a blue label. They also ate less snack food from a red plate than from a blue or white one. The authors concluded that red might function as a subtle stop signal that works even outside of a person’s consciousness and may thereby reduce incidental food and drink intake. Bruno, Martani, Corsini, and Oleari (2013) extended this line of research by looking further into this phenomenon, demonstrating that red plates reduced the consumption of food (and the use of hand cream). Interestingly, this behaviour occurred independently from liking, since all the samples were rated similarly liked. In addition, Bruno et al. also demonstrated that their results were not dependent on the Michelson (luminance) contrast nor on the colour contrast either. From these findings the authors concluded that it might simply be that the colour red was associated with the meaning of avoidance and that this was influencing people in those conditions.

In addition to any effect that colour and shape of the plateware has on the perception of food, when it comes to calculating the volume (amount) of food to serve and/or to consume, we are also influenced by the size of the plateware (Levitsky & Youn, 2004). This is particularly relevant considering the fact that the average size of tableware or food containers has increased by around 20% in the last century (Wansink, 2010). Wansink et al. (2006) demonstrated in a social event that when

participants were given a larger bowl, they served themselves 50% more ice-cream than those given a much smaller bowl. Furthermore, since the participants nearly always finished their food, those eating from a larger bowl ended up consuming far more ice-cream. Van Kleef, Shimizu, and Wansink (2012) obtained similar results when the bowls that differed in size were the everyday containers from which a pasta was served in a canteen setting; the difference in capacity in this case was nearly double (3.8 versus 6.8 l). Despite the fact that the people's individual plates were all of the same size (approximately 23 cm in diameter), those who served themselves from the larger communal bowl ended up with 77% more pasta and felt more satiated than the others who had a smaller common bowl. Wansink and his colleagues attempted to account for these results in terms of the Ebbinghaus–Titchener size-contrast illusion and/or the Delboeuf illusion (Lyman, 1989). Such visual illusions may result in a given amount of food being perceived as much smaller on a larger bowl and vice versa (Wansink & Cheney, 2005). It is however important to note here that the effects of the size of the plateware on a people's consumption behaviour are somewhat inconsistent. For instance, participants in Rolls, Roe, Halverson, and Meengs's (2007) studies did not eat less from smaller plates than from larger plates (the different sizes being 17, 22, or 26 cm).

The weight of the dish could also influence how much we eat. Piqueras-Fiszman, Harrar, Alcaide, and Spence (2011) explored whether the weight of the bowl from which people tasted a yoghurt exerted any influence on their multisensory flavour perception. Three bowls, identical except for their weights, were filled with exactly the same amount of yoghurt. Participants were instructed to hold each of the bowls in their hand while rating the taste and flavour of the yoghurt on four scales. The food sampled from the heaviest bowl was rated as being significantly denser, more expensive, and more liked than when sampled from the lightest bowl. Why could this be observed? Since weight properties are often used to describe the density of a food (e.g. as when we describe a food or meal as being "heavy"), the attributes that we associate with the heavier bowl may well have been transferred (subconsciously or otherwise) onto the participant's perception of the qualities of the food in the bowl itself (Spence & Gallace, 2011). As such, these results can perhaps be best explained in terms of psycholinguistic and metaphoric transfer effects. In a follow-up study, Piqueras-Fiszman and Spence (2012) corroborated these findings even when yoghurt was served from light (20 g) versus heavy (95 g) plastic bowls. Despite the smaller variation in the weight of the plateware, the yoghurt in the heavier bowl was nevertheless still estimated as being significantly more satiating (prior to consumption), and denser (as in the previous study).

4 Display of a Single Food Product Variety

The way in which foods that vary only in terms of colour are laid out also influences acquisition or behaviours related to portion selection. In particular, Kahn and Wansink (2004) reported a series of studies on both adults and children using foods

and non-food products. They demonstrated that increasing either the actual (or perceived) variety of flavour and/or colour in a selection resulted in a significant increase in consumption behaviour. In their first two studies, around 40 children (6–10 years old) picked up, and ate, significantly more jelly beans (and coloured beads), more than twice as many, when the assortment presented 24 differently coloured items as compared to when the assortment presented only 6 different colours, always organised in separate compartments. However, the difference in the number of colours did not have an effect on the amount chosen and their consumption when the colours were mixed into a single compartment. In another study conducted with adults, the participants were given bowls of M&Ms of either seven or ten colours and either presented with an equal distribution of colours (symmetric condition) or with 30% dark-brown M&Ms (asymmetric condition). The results of this final study showed that only for the asymmetric assortments did increasing the actual colour variety from seven to ten lead to a significant increment in the quantity consumed (of 77%).

The fact that the same effects were reported for both food (jelly beans and M&Ms) and non-food items (beads and small toys) in Kahn and Wansink's (2004) study suggests that it is not specifically colour variety what affects consumption behaviour for food, but rather an impact of visual variety more generally. The influence of colour variety on people's consumption behaviour highlighted by this research would therefore likely operate via another mechanism whereby the structure of an assortment moderated the effect of actual variety on perceived variety. Kahn and Wansink further demonstrated that it is perceived variety that, in turn, influences consumption (or selection) quantities through anticipated consumption utility.

Redden and Hoch (2009) conducted a series of experiments that may help to explain why perceived variety increases consumption (or acquisition). They demonstrated (Study 3) that variety in an assortment can reduce its perceived quantity; this was shown in a study where the participants were asked to match the quantity of a sample of 52 g of brown M&Ms contained in a transparent bowl by pouring M&Ms into another transparent bowl, knowing that they would not consume them afterwards. In one condition, the M&Ms to pour were a mix of three colours, while in another condition, the M&Ms had only a single colour. The results showed that participants poured 12% more into a bowl when the candy was multicoloured. Thus, beyond any separate effects variety might have on choosing an amount to consume, variety caused people to pour more candy into a bowl, presumably because variety made them perceive that they had placed less in the bowl in the first place. In another study (Study 4), the participants were asked to match quantities of M&Ms (as in Study 3), but this time the number of colours in the sample bowl also varied (single colour vs. four colours). Not only were the results from Study 3 replicated, but there was also the predicted main effect of the number of colours in the sample, whereby people poured less when matching a sample that had multiple colours versus a single colour, probably because a sample appeared to have less quantity when it had a variety of colours. Thus, it would appear as though a variety of colours reduces perceived quantity whether in the sample or the poured items, and, consequently, could lead people to estimate smaller serving portions and eventually to repeat servings.

5 Variety of Food Components Within a Meal

Let us move forward to meals comprised of different food items and the effect of this type of variety on how full do we (expect to) feel. Most studies of expected fullness or satiation have focused on several different foods but few have considered meals that comprise different components. In studies of intake behaviour, the "variety effect" refers to an increase in the amount of food consumed when exposed to multiple foods with different sensory characteristics (taste, texture, odour, and appearance) compared to when exposed to a constant sensory feature. During the consumption of a meal this effect would lead to meal termination. Wilkinson, Hinton, Fay, Rogers, and Brunstrom (2013) showed that the variety effect occurs in the context of meal planning, since the participants in their study anticipated the variety and selected more food for a second course when its sensory characteristics differed from those of the first course.

More recent research has studied how people estimate satiation when a meal comprises multiple items. Keenan, Brunstrom, and Ferriday (2015) showed participants a set of pictures of meals with different degrees of variety and measured the perceived volume and expected satiation. The hypothesis was that under conditions of increased food variability judgments of expected satiation would be based on the perceived volume rather than on prior experience. What was observed was that expected satiation decreased as variety increased. However, the results also showed that when variability was low (less than three different items), evaluations based on perceived volume were significantly lower than those based on expected satiation, and when food variability was high (4–5 items), these judgments coincided. The authors therefore proposed that increased variety induces the use of perceived-volume heuristic, which decreases expected satiation, and this may affect food intake volume and actual satiation.

It is relevant to note that the expected satiation of a food is the extent to which that food is expected to deliver fullness and that these beliefs may not exactly reflect the actual satiation effect of foods, but may give indications as to factors in foods that are important in appetite regulation (de Graaf, Stafleu, Staal, & Wijne, 1992).

6 Measurements of Intake, Satiation, and Satiety

6.1 Manipulations

Top-down effects on intake and sensations of fullness are normally measured in controlled intervention tests that take into account factors such as body weight, age, gender, habitual diet, alcohol consumption, and the physical activity or dietary restraint of the participants, among others (Tarrega & Fiszman, 2017). In behavioural research areas, however, many studies are conducted in real-life settings, where, for instance, in a buffet context, containers are weighed before and after consumption.

Researchers usually collect evaluations of satiation by making subjects consume an experimental food (weight in grams or energy) ad libitum under standardized conditions (e.g. Blundell et al., 2010). Of course, an issue with these set-ups is that subjects that come to the lab often would not really eat as much as they can. Satiety, on the other hand, is commonly measured using an experimental technique known as the preload-test meal paradigm (Blundell et al., 2010), with a control group given either no preload or a placebo treatment. In this type of satiety study, the subjects are presented with prepared food(s) matched for taste, appearance, and other sensory properties, but varying in energy and/or macronutrient composition. These are called preloads because they are given before a test meal, with which variables such as intake are measured. The preloads can be manipulated in a way unknown to the subjects to assess the physiological responses to the preload, or overtly manipulated to test both physiological and cognitive responses. After a specific amount of time, the effects of the preload are measured by monitoring spontaneous food intake of test meal(s), or alternatively, by collecting participants' self-reports of their own food intake. Subjects evaluate their hunger on scales prior to, and at predetermined time intervals after the preload and the test meal. In many of these experiments, food intake for the remainder of the day is also self-recorded by the subjects.

6.2 Tools

This section will discuss methods that are used in laboratory settings to measure in a controlled way the effect of specific foods or information on expected satiety and satiation, be it through matching tasks or self-reported evaluations. There are two main ways of presenting the food stimuli: (1) looking at the food (either the real product or a picture) or (2) tasting the food (normally just a bite or spoonful). Either way, the food is rated by comparison or matching with a "standard", or scales. Showing pictures (in physical or digital form) is undoubtedly simpler and less time-consuming than having to prepare real food, mostly when they are meals. However, attention should be paid to the quality of the photographs (or screen), illumination, angle of observation, etc., so that food items are easily recognisable. However, when the food items being evaluated have a similar appearance, looking at images alone would not elicit the ability to distinguish the foods reliably on the basis of expectations. This approach is therefore preferred when the manipulations involve purely visual cues or written labels, for example. When using real food, sensory appraisal of the taste, odour or texture of the samples will contribute considerably to the judgement of expectations. People normally report no difficulty in making the assessment regardless of how the food is presented.

Regarding the comparison of foods, there are some methods developed to assess the expected satiety or satiation. One is the Constant Stimuli Method. It involves using digital images and was developed for comparing expected satiety measurements across a large number of foods. It was first proposed in an experiment where one food with a known fixed energy content was shown on a computer screen

(Brunstrom, Shakeshaft, & Scott-Samuel, 2008). Next to this reference item, pictures of different amounts of a second common food were displayed one at a time and the participant was asked to indicate which of the two foods would prevent them from feeling hungry for the longest period. Probit analysis indicated the amount of the comparison unit (i.e. energy) that was expected to be as equally satiating as the standard. The authors considered that "expected satiety" refers to the relative satiety that a subject expects from different foods when compared on a calorie-for-calorie basis, derived from calculations after having compared the expectations generated by several different foods and everyday decisions about their portion size. The authors showed that decisions about meal size are also largely motivated by reasons such as the learned post-ingestive consequences of consuming that (or a similar) food.

Another method based on the same concept is the Method of Adjustment (Brunstrom & Rogers, 2009). As with the previous method, a food of known fixed energy content is displayed on a computer screen. Next to this reference food, a different food is displayed. The participants are asked to "Imagine you are having this food for lunch today; look at the picture on the left and match the picture on the right so that both foods will leave you feeling full to the same extent immediately after eating them." The participants can then change the amount of this second "comparison" food accordingly. This method provides a "point of subjective equality" that represented the amount of the comparison food (i.e. the energy content) that was expected to be equally as filling as the standard (see also Wilkinson & Brunstrom, 2009).

Apart from these methods, scales are often used. Researchers can show images and ask participants for instance, to estimate the time, in units of 15 min, which would pass between eating the foods in the picture and the subsequent emergence of feelings of hunger (called satiating time). Researchers have also asked participants to rate the foods shown in each picture on a structured scale called the satiating strength rating, anchored with 1 = "weakly satiating" and 20 = "strongly satiating" (de Graaf et al., 1992). So no comparisons or matching is done but rather evaluations on scales. Marcano, Morales, Vélez-Ruiz, and Fiszman (2015) measured expected satiation when tasting a food in a study that used cheese pies with different kinds of visible particles added (wheat bran, ground coconut, whole flaxseeds, and oatmeal). The expected satiation ratings were obtained on 9-point scales (labelled from 1 = "If I ate this whole pie it would not fill me at all" to 9 = "If I ate this whole pie it would fill me a lot").

A study that combined tasting food and comparison food pictures in an assessment of satiety expectations of equicaloric milk-based snacks was that of Tarrega, Martínez, Vélez-Ruiz, and Fiszman (2014). Before tasting a sample, each participant was provided with the four printed cards. Each card showed seven pictures representing increasing amounts of one of the four comparison foods: apple, chocolate bar, ham and cheese sandwich, and doughnut. Participants were asked to indicate the amount of each food (from one to seven) they would eat if "It is 5 p.m. and you want to eat something to keep you going until dinner at 9 p.m.". The snack sample was then served and after consuming a single spoonful, the participant had

to select "the amount of food on each of the four scales that would be equally as satiating as the snack you are tasting, considering the serving size" (a 125 ml-capacity white plastic cup was provided). The "same expected satiety" score that each participant awarded each snack on each scale was transformed into the corresponding energy load in kcal. Consequently, the authors proposed the use of "relative expected satiety" (RES) values to indicate how satiating consumers expect the food to be relative to the amount of food that they would eat in those circumstances.

7 Factors Affecting Expectations of Satiation and Satiety

As mentioned at the beginning of this chapter, satiation and satiety are initially affected by sensory and cognitive factors, including expectations about what will be consumed, the taste, texture, and smell of the food or drink and any associations with previous experiences (Benelam, 2009). Throughout our lifetime and experiences of eating we learn and acquire expectations about the consequences of consuming different foods (Brunstrom, 2011). These expectations develop from the moment we are firstly exposed to a novel food, when an individual creates associations between the food's sensory properties and its ability to elicit satiation (Gibson & Brunstrom, 2007). On subsequent exposure, these learned associations influence portion size selection and thus determine subsequent energy intake (Brunstrom & Shakeshaft, 2009). As a result, a memory of expected satiation may also be important for eliciting energy compensation.

There are now several pieces of evidence suggesting that satiety expectations can influence the actual experience of satiety post consumption. For example, three familiar foods were shown on a computer screen and participants had to assess their expected satiety and ideal portion size (Wilkinson et al., 2012). A real bowl of one food was then presented and the participants self-selected an ideal portion size before consuming the portion ad libitum. The results showed that expected satiety was a good predictor of both virtual and physical self-selected portions and actual food intake, demonstrating that satiety expectations can drive aspects of portion selection and influence the actual post-consumption experience of satiety. However, other methods could be used to avoid demand effects, that is, people acting as they "promised" they would or wanting to demonstrate they were accurate in their own estimation.

7.1 Familiarity and Learning

As it has become obvious at this point in the chapter expectations are based on familiarity. The exact role of familiarity was first related to expected satiety in a study that measured the satiety expected from 18 different foods using constant

stimuli methodology[14]. The results showed that expectations differed considerably across food categories and some foods were expected to confer 5–6 times greater satiety than others (calorie for calorie). However, these differences were not only due to visual cues (physical properties observed or the size of the food in the picture). The findings showed that familiar foods were generally expected to be more filling, and also seemed to indicate that when we face a novel or less familiar food we first tend to assume a lower satiating capacity until we learn from experience otherwise.

Subsequently, several other studies have focused on determining the role of familiarity, learning, and exposure on expected satiety or satiation. For instance, using the method of adjustment, O'Sullivan, Alexander, Ferriday, and Brunstrom (2010) also highlighted the satiation that a food is expected to deliver depends in part on the subject's familiarity with the food. Interestingly, these results have also been observed among children. Hardman, McCrickerd, and Brunstrom (2011) carried out a study on expectations of satiation across energy-dense snacks with children. They showed that not only children who were very familiar with a number of snack foods expected them to deliver greater satiation but also those who knew the foods but who never or rarely consumed them. However this finding is not so robust across all foods; the relationship between familiarity and expected satiation was stronger for foods that were higher in energy content, while with the two products in the study with a lower energy content the link was not observed. Based on these results, the authors suggested that learning occurs predominantly with high-energy foods because they are more likely to be eaten until fullness is experienced, whereas when products are not consumed to fullness the possibility of learning about their satiating consequences is limited.

Familiarity can explain the differences among consumers concerning their expected satiation from the same product. Brunstrom, Shakeshaft, and Alexander (2010) demonstrated this with data pooled across foods and also in data for one product (sushi). Expected satiation increased with familiarity, confirming that with novel foods people are more conservative when it comes to estimating satiation. Considering this, Irvine, Brunstrom, Gee, and Rogers (2013) explored the relationship between expected satiety and familiarity with eating a food to fullness. In a first experiment, self-reported measurements of expected satiation, liking, and other 11 attitudinal aspects towards seven foods were collected. Familiarity was significantly correlated with expected satiety, but only when it was linked to the frequency of having eaten the food to fullness. In a second experiment, the familiarity of participants with a food (wine gum) was manipulated by an episode of eating wine gums to a substantial level of fullness. The results showed that participants on day 1 who experienced eating gums until they were full expected wine gums to be significantly more filling on day 2. In contrast, participants who did not eat the wine gums expected the same satiation level between days 1 and 2. These results showed again that there is a learned basis to satiety expectations and added that one occasion of eating a food to satiety (despite not normally consumed in such a manner) is enough for the participants to modify or reconsider their satiety expectations for that food.

7.2 Beliefs and Memory

We know already that, without information about a food, we will know when it will make us feel full because we have learned the filling sensations associated with those sensory properties delivered. However, more related to the focus of this chapter, research has shown that actually what consumers "believe" they have consumed might overrule the actual consumption when it comes to estimating portion sizes in the next consumption occasion. Yeomans, McCrickerd, Brunstrom, and Chambers (2014) studied how the memory of a recently consumed meal influenced post-meal hunger and fullness and the expected satiating effects of the food when eaten on a subsequent occasion. The authors manipulated the "actual" and "perceived" amount of soup that the participants consumed. Before lunch, half the participants were shown 300 ml of soup and half were shown 500 ml. During lunch, half of each group consumed 300 ml and the other half 500 ml, inversely. Immediately after lunch, self-reported hunger decreased as expected and the differences observed were related to the portion of soup actually eaten. However, two and three hours after the meal, the participants' hunger was determined by the portion of soup they had been shown before lunch, that is, those who thought they had consumed the larger 500-ml portion reported significantly less hunger than those who had seen 300 ml of soup. After 24 h, the expected satiation delivered by a 400 ml bowl of soup was significantly higher for those who had previously seen 500 ml of soup, regardless of the amount eaten. This seems to confirm that post-ingestive experience and, in this case, the memory of recent eating, can influence subsequent expected satiation assessments (see also Higgs, 2002, 2008 for additional evidence on the effect of memory on subsequent intake]. Similarly but in the opposite direction, Wansink, Painter, and North (2005) manipulated people's beliefs of how much they were consuming by making them consume soup in a bowl that was constantly being refilled. Thus, people's intake was much more than what they saw. Those participants who were unknowingly eating from self-refilling bowls ate more soup than those eating from normal soup bowls. However, despite consuming 73% more, they did not believe they had consumed more, nor did they perceive themselves as more sated than those eating from normal bowls. They argued that people use their eyes to count calories, and estimate fullness, and not their stomachs (though note that the question about fullness was asked immediately after in this case). It may be pointed out that an increasing number of studies have not measured actual satiation or satiety but only expectations.

8 Conclusions

A vast number of studies have investigated the impact of cognitive cues on the perception and behaviour of participants/consumers. In the domain of eating behaviour, and particularly on estimations about satiety, satiation, portion size, and

eventually intake, many factors beyond the sensory properties or energy density of the food itself play an important role. These range from the information provided to participants through to aspects of product presentation that influence people's estimations of volume and calories. While the majority of those studies have tended to focus on studying these expectations and estimations from the immediate surroundings of the food (e.g. containers, display) or the beliefs about the food itself, in a controlled setting, it is important to note that cues from the environment also play a big role in modulating our food and drink related behaviour (see Higgs, 2016; Spence & Piqueras-Fiszman, 2014). However, reviewing those influences will have to remain as the subject of another review.

What is evident at this point is that learning processes as well as our tendency to use heuristics to estimate volumes (and calories when it comes to food) make us serve ourselves and consume based on what we sensorially perceive (visually, haptically, etc.) rather than on real amounts and fullness (if we can ever accurately tell!).

In this chapter I also describe some methods used to collect information about people's estimations of fullness and satiety, by comparison methods and scales. However, one should be cautious in relying only on what people report feeling, since as cited in this chapter, people do not always behave as they report.

Taking all the findings together, this body of research paves the way to novel and exciting opportunities to possibly help people make responsible healthier food decisions, while not necessarily having to modify the properties of the product itself.

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Tools and Methods to Quantify the Digestion of Protein, Lipid, Starch and Fibre from a Chemistry/Microbiology Perspective

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1 Introduction

Foods generally contain a mixture of different nutrients. To determine the nutritional value of a food, it is common to measure its nutrient content. However, some of these nutrients will not be available to the body. The manner in which the nutrient is located within the food structure (e.g. amylose or amylopectin in starch), the formation of a matrix with other structures (e.g. protein–starch–fibre), and the presence of antinutritional factors (e.g. protease- and amylase-inhibitors in legumes) in the food may, for example, interfere with the digestion and subsequent absorption of the nutrient. Moreover, many foods that are consumed by humans are processed, exposing them to heat, pressure and other materials such as alkalis. This processing may also affect the nutritional availability of some nutrients (e.g. lysine).

The proportion of nutrients that will be digested and absorbed by the body will vary between sources of the nutrient and can even vary between batches of the same food ingredient (Hendriks et al., 2002; Hendriks, Cottam, Morel, & Thomas, 2004). Therefore, to better understand the nutritional value of foods, it is necessary to determine where and how foods are digested in the digestive tract and the nutrients absorbed.

Foods can be digested in different ways in different parts of the gastrointestinal tract (GIT). For example, while dietary protein is digested in the upper GIT (stomach and small intestine) and absorbed as amino acids or oligopeptides, dietary fibre

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appears to be mainly digested in the lower GIT and absorbed mainly as short-chain fatty acids (SCFAs). A variety of methodologies, including in vivo and in vitro methodologies, have been developed to determine how each food component is digested and nutrients are absorbed along the length of the GIT. For example, different in vitro models (e.g. in vitro pepsin–pancreatin digestion) have been developed to study the gastro-small intestinal digestion of dietary proteins, while other in vitro models (e.g. microbial enzymes, living bacteria) have been developed to study the digestion and fermentation of dietary fibre.

In this chapter tools and methods that have been developed to study the digestion of protein, starch, lipids, and fibre are described. The first part of the chapter provides an overview of digestion throughout the GIT for each food component and the methodologies commonly used to study digestion. The methods used to study the digestion of protein, lipid, and starch have mostly been described previously, while the digestion of dietary fibre is an area which is still being elucidated with many recent studies giving new information (Montoya, Henare, Rutherfurd, & Moughan, 2016; Montoya, Rutherfurd, & Moughan, 2017). Therefore, the second part of the chapter provides a more detailed description of the methods currently being used, and those in development, to determine dietary fibre digestion and fermentation.

2 Overview of Digestion

The digestion of food begins in the mouth with particle reduction by mastication and the hydrolysis of starch with salivary α-amylase and lipids with lingual lipase (Dawes et al., 2015; Pedersen, Bardow, Jensen, & Nauntofte, 2002). However, due to the short retention time of the bolus in the mouth, this digestion is generally not considered to be significant (Dawes et al., 2015; Pedersen et al., 2002).

The arrival of the bolus in the stomach from the mouth stimulates the secretion of hydrochloric acid, pepsinogen and gastric lipase from cells present in the gastric mucosa (O'Connor & O'Morain, 2014). The stomach harbours a diverse microbial population (Bik et al., 2006; Gu et al., 2013; Zentek et al., 2013). Microbes possess a wide range of enzymes that are able to cleave nutrients including complex carbohydrates (http://www.cazy.org/) (Lombard, Golaconda Ramulu, Drula, Coutinho, & Henrissat, 2014). The microbes then use the cleaved nutrients as a source of energy and different end-products are produced through fermentation (e.g. SCFAs). Based on the production of fermentation end-products (Højberg, Canibe, Knudsen, & Jensen, 2003; Laerke, Jensen, & Hojsgaard, 2000; Pieper, Boudry, Bindelle, Vahjen, & Zentek, 2014; van Winsen et al., 2001; Zentek et al., 2013) it appears that fermentation occurs in the stomach.

Food compounds (protein, starch, and lipids) are partially digested in the stomach before entering the small intestine. In the small intestine, compounds are digested through the action of several pancreatic and brush border enzymes. Throughout the small intestine there is also a diverse microbial population (Booijink et al., 2010; Frank et al., 2007; van den Bogert et al., 2013; Wang, Ahrné, Jeppsson,

& Molin, 2005) with fermentation end-products being present (Awati, Williams, Bosch, & Verstegen, 2006; Højberg et al., 2003; Holtug, Rasmussen, & Mortensen, 1992; Laerke et al., 2000; Pieper et al., 2014; van Winsen et al., 2001; Zentek et al., 2013). Therefore, the nutrients that enter the small intestine from the stomach are digested by both mammalian and microbial enzymes, and simultaneously absorbed. The role of microbial enzymes in the overall digestion of food compounds has, in the past, received less attention than it should.

Food fragments and nutrients that are not fully digested and absorbed by the end of the small intestine (the terminal ileum) are released into the large intestine (or hindgut) where the majority, but with the exception of fatty acids, are digested by microbial enzymes before being fermented (Flint, Scott, Duncan, Louis, & Forano, 2012). While proteins, starch, and lipids are expected to be mainly digested by mammalian enzymes, dietary fibre is digested completely due to the actions of microbial enzymes (Flint et al., 2012).

Digestibility is a measure that is commonly used to estimate the degree of absorption of nutrients. To determine the digestibility of a nutrient, the difference between the amount of the nutrient that was consumed and that in digesta or faeces is determined. Nutrients that have "disappeared" through the digestive system are assumed to have been digested (released) and absorbed.

Digestibility can be determined at different locations within the digestive tract, depending on the requirements of the study and the nutrient being evaluated. For example protein digestibility is normally determined on digesta collected at the last part of the small intestine (the terminal ileum, hence ileal digestibility), whereas fibre digestibility is normally determined at the faecal level, as discussed below.

Calculating the digestibility of a nutrient by subtracting the amount of the nutrient in digesta or faeces from the amount of the nutrient that was ingested in the diet gives a measure of "*apparent*" digestibility. Different compounds diffuse into or are secreted into the GIT during the digestion of food. These compounds are termed endogenous (or non-dietary), as opposed to dietary or exogenous material (Fauconneau & Michel, 1970; Snook, 1973). Endogenous materials include mucus secreted from cells along the entire GIT, epithelial cells that are sloughed off from the intestinal mucosa, secretions containing digestive enzymes produced by the salivary glands, pancreas and the mucosal lining of the stomach and intestines, serum albumin, and bile acids from the liver. The microbial population in the GIT is also part of the non-dietary digesta component and it has been argued by some that it should not be considered as strictly endogenous. A large proportion of the nutrients in the endogenous material are digested and absorbed in the GIT. For example, a large part (70–80%) of the endogenous nitrogen is reabsorbed before the digesta leave the small intestine (Souffrant, 1991).

The components of endogenous origin (e.g. protein, lipids, sugars, calcium, phosphorus) can be determined and used to correct "*apparent*" digestibility values to "*true*" or "*standardised*" digestibility values (Stein, Sève, Fuller, Moughan, & de Lange, 2007). True digestibility values give a more accurate measure of the amount of the ingested nutrient that has been digested and absorbed than apparent digestibility values. However, to determine true digestibility values, it is necessary to

distinguish between nutrients of dietary origin and those present in the endogenous material.

There are several ways that endogenous material may be determined, including feeding diets devoid of the nutrient to be studied, the regression method and the isotope dilution method. Other methods that are specific to particular nutrients (e.g. the peptide alimentation method for endogenous proteins) will be discussed together with the digestion of the said nutrient.

The traditional method to determine the amount of an endogenous nutrient present in digesta or faeces is to determine digesta or faecal nutrient flows following the consumption of a diet devoid of the nutrient under study, in which case it can be assumed that all of the nutrient in the digesta or faeces would be of endogenous origin. Whereas this method is relatively simple to use, it has been criticised as being "non-physiological". For example, when animals are fed a protein-free diet, this has been associated with a reduction in the amount of gastric and pancreatic enzymes that are secreted (Fauconneau & Michel, 1970; Schneeman, 1982) and a decreased rate of protein synthesis in the entire body and gut tissues (Millward, Garlick, James, Sender, & Waterlow, 1976). Thus the protein-free state alters the metabolism of the whole body, which will include gut metabolism (Rodwell, 1985; Rogers & Phang, 1985; Sauer & de Lange, 1992; Sauer, Stothers, & Parker, 1977; Skilton, Moughan, & Smith, 1988; Taverner, Hume, & Farrell, 1981) which is likely to result in decreased levels of endogenous protein secretions and therefore, an overestimation of the true digestibility of the protein (or amino acid).

The linear regression approach to determining endogenous materials involves feeding increasing amounts of the nutrient of interest (at a constant dry matter intake) and extrapolating the nutrient concentrations in the digesta or faeces to a zero dietary nutrient intake. This allows estimation of the endogenous nutrient concentration. The linear regression method allows endogenous nutrient concentrations to be estimated with more normal nutrient intakes. However, this method assumes that endogenous nutrient concentrations do not depend on dietary nutrient intake. The difference in nutrient content in the digesta or faecal samples with increasing dietary nutrient intake is assumed to be due only to an increased (proportionally) amount of undigested dietary nutrient being present in the digesta or faecal sample. Doubt has been cast over this assumption (Hodgkinson & Moughan, 2007; Hodgkinson, Moughan, Reynolds, & James, 2000; Nyachoti, de Lange, McBride, & Schulze, 1997), as several studies have shown that some food components, such as protein and dietary fibre stimulate the secretion of endogenous nutrients (e.g. protein, sugars) (Cabotaje, Shinnick, Lopéz-Guisa, & Marlett, 1994; Ito et al., 2009; Roth-Maier, Machmüller, Kreuzer, & Kirchgessner, 1993; Satchithanandam, Klurfeld, Calvert, & Cassidy, 1996; Schulze et al., 1994; Souffrant, 2001).

The use of isotopes allows endogenous nutrient concentrations to be determined when a nutrient-containing diet is being consumed. The isotope dilution method has been used mainly to study the digestion of dietary protein (Huisman et al., 1992; Schulze, Butts, Moughan, & Verstegen, 1994; Souffrant, Köhler, & Gebhardt, 1982). The food protein can be labelled with a stable or radioactive tracer (Souffrant, 1991; Souffrant et al., 1982). Alternatively, when an animal model is used, the

animal's body nitrogen pool can be labelled (Huisman et al., 1992; Schulze, Butts, et al., 1994). This allows for undigested dietary nitrogen to be distinguished from endogenous nitrogen and the proportion of endogenous nitrogen in the digesta can be calculated from the dilution of the isotope. There are a number of assumptions that need to be made when using isotopes, and these assumptions are not always tenable, leading to inaccuracies, as described below.

To determine the digestion of nutrients, different methodologies (e.g. in vivo and in vitro) have been developed depending on the nutrient under study. Different mammalian and sometimes microbial enzymes are found in different segments of the GIT. Thus, the digestion at each GIT location will differ across nutrients, as described below for each nutrient class.

2.1 Protein

In the stomach, protein is digested by the endogenous enzyme pepsin and is acted upon by hydrochloric acid. Microbes are also present in the stomach, however, and may also contribute to protein digestion. Amino acids are not absorbed in the stomach. This means that gastric digestion cannot be evaluated via the determination of digestibility and a different approach is needed. One option is to determine the degree of hydrolysis. For example, the degree of hydrolysis of different protein sources has been determined in pigs (Bornhorst et al., 2016; Montoya et al., 2014) and rats (Montoya et al., 2014) by measuring the number of amino groups (NH_2) becoming exposed following cleavage of peptide bonds. Protein sources normally contain a mixture of different proteins. The gastric digestion of each protein can be determined by measuring and comparing the relevant band intensity in SDS-PAGE gels of the diet and stomach chyme (Bornhorst et al., 2016; Montoya, Hindmarsh, et al., 2014; Montoya, Rutherfurd, et al., 2014; Rutherfurd et al., 2011), but this approach is largely qualitative.

When the overall digestion of protein is to be determined, rather than basing this on faecal measures, it is based on measures at the ileal level; that is, following the collection of digesta from the terminal ileum. This is because large intestinal microbes metabolise protein, changing both the composition and quantity of amino acids. In species such as the pig, it has been estimated that 80% of faecal protein is of bacterial origin (Mason, 1980). Moreover, few or no amino acids are absorbed intact in the large intestine (Darragh, Cranwell, & Moughan, 1994; Just, Jørgensen, & Fernández, 1981; Schmitz, Ahrens, Schön, & Hagemeister, 1991; Zebrowska, 1973a; van der Wielen, Moughan & Mensink, 2017). Most nitrogen that is absorbed in the large intestine is in the form of ammonia, with some amines or amides. This nitrogen has no nutritional value to the human/animal other than for the synthesis of dietary dispensable amino acids, and most is ultimately excreted in the urine as urea (Just, 1983; McNeil, 1988; Zebrowska, 1973b).

When determining the quantity or proportion of dietary amino acids that are absorbed, true amino acid digestibility needs to be determined rather than apparent

digestibility, with the appropriate correction for the endogenous amino acids. For this reason the 2011 FAO Expert Consultation on Protein Quality Evaluation in Human Nutrition recommended that true ileal amino acid digestibility be used for evaluating protein quality (Food and Agriculture Organization of the United Nations (FAO), 2013).

Endogenous nitrogen and amino acid secretions can be determined following the feeding of a protein-free diet (nutrient-free method), using the regression method, isotope dilution method or by the peptide alimentation method. The protein-free and regression methods have been described above. Endogenous ileal amino acid excretions determined following the consumption of a protein-free diet have been shown to be lower than those determined when a diet containing peptides is consumed by humans (Moughan, Butts, Rowan, & Deglaire, 2005; Moughan & Rutherfurd, 2012), pigs (Hodgkinson et al., 2000), and rats (Deglaire, Moughan, Rutherfurd, Bos, & Tome, 2007). Comparisons between the endogenous ileal amino acid excretions determined with the regression method and the protein-free method have shown that both methods give similar results (Donkoh, Moughan, & Morel, 1995; Taverner et al., 1981), which calls into question the accuracy of the extrapolated (regression) values.

For the isotope dilution method, the most commonly used tracer for the determination of endogenous protein concentrations is the stable isotope, ^{15}N. When labelled diets are consumed, it is assumed that the labelled and unlabelled food amino acids are absorbed equally and that the endogenous nitrogen that is secreted into the GIT does not become labelled to a significant degree during the duration of the study. It has been shown, however, that a proportion of the absorbed dietary amino acids are rapidly synthesised into body proteins and resecreted as (labelled) endogenous proteins (Leterme et al., 1996). Thus there will be a certain level of underestimation of the endogenous nitrogen determined with this method. When an animal is labelled with ^{15}N, this underestimation of endogenous nitrogen will also occur as the unlabelled absorbed dietary nitrogen will be rapidly synthesised into body protein and resecreted as endogenous protein, but as such will not be labelled with ^{15}N. Endogenous ileal nitrogen concentration determined using the isotope dilution method with ^{15}N-labelled pig has been shown to be greater than that determined when a protein-free diet is fed to the animals (de Lange, Souffrant, & Sauer, 1990). With labelling the animals body, there are also concerns as to what is the most appropriate precursor pool.

The peptide alimentation method, also referred to as the enzyme hydrolysed protein method, was proposed by Moughan, Darragh, Smith, and Butts (1990) for the determination of endogenous nitrogen and amino acid concentrations. It involves feeding a diet in which the only nitrogen source is an enzymatically hydrolysed protein, usually enzymatically hydrolysed casein (EHC), containing a mixture of free amino acids and oligopeptides with no peptides being larger than 5,000 Da. Digesta are collected, centrifuged and ultrafiltered to remove material smaller than the molecular weight filtration cut-off (usually 10,000 Da or lower). Endogenous protein will be in the >10,000 Da fraction and any undigested dietary peptides or free amino acids will be in the <10,000 Da fraction. As well as the animal being in

a normal nitrogen balance and thus a normal physiological state, another important advantage with this method is that it allows the direct determination of total nitrogen and all amino acids, simultaneously. The method cannot be used for protein sources containing fibre or antinutritional factors. Also the presence of endogenous proteins with <10,000 Da size cannot be discounted.

The peptide alimentation method has been shown to result in greater endogenous amino acid flows than the protein-free method in the human (Moughan, Butts, Rowan, & Deglaire, 2005) and other species such as the pig (Hodgkinson et al., 2000) and rat (Deglaire et al., 2007). Studies have shown that the presence of dietary peptides in the GIT affect endogenous amino acid flows (Deglaire et al., 2007; Miner-Williams et al., 2014; Skilton et al., 1988), with a greater quantity of endogenous proteins excreted when dietary peptides are present compared with when free amino acids or no dietary protein is present. Therefore, endogenous amino acid excretions determined using the peptide alimentation method are likely to be more similar to those that occur when a "normal" diet is consumed, than those present when a protein-free or free amino acid diet is consumed. It has been concluded that the protein-free method underestimates endogenous ileal amino acid loss, and the peptide alimentation method is more appropriate, for the determination of true ileal amino acid digestibility values (Moughan & Rutherfurd, 2012).

For foods that are processed or stored for long periods of time, chemical reactions may occur between protein-bound amino acids and reducing compounds present in the food matrix, with the results of a decrease in the nutritional availability of some amino acids, especially lysine. The ε-amino group of lysine can react with other compounds present in the feedstuff during processing and storage to form compounds such as deoxyketosyllysine (the Amadori compound), which is partially absorbed from the gut, but has no nutritional value (Hurrell & Carpenter, 1981). When conventional amino acid analysis is used to determine the lysine concentration of the food and ileal digesta, lysine availability is often overestimated in heat-treated feeds (Moughan, 2005; Rutherfurd, Moughan, & Morel, 1997) and will not give an accurate estimation of lysine digestion.

The *O*-methylisourea method or true reactive lysine bioassay was proposed by Moughan and Rutherfurd (1996) to estimate the nutritional availability of lysine. The bioassay has been shown to be more accurate than assays based on conventional amino acid analysis as an indicator of digestible reactive lysine (Rutherfurd, Moughan, & Morel, 1997) and the bioassay has been applied to a range of processed foods (Rutherfurd, Moughan, & van Osch, 1997; Rutherfurd, Torbatinejad, & Moughan, 2006; Torbatinejad, Rutherfurd, & Moughan, 2005).

2.2 Starch

The digestion of starch begins in the mouth with the action of salivary α-amylase. However, due to the short retention time of the food bolus in the mouth, little hydrolysis occurs but salivary α-amylase does cleave starch in the stomach during the first

hours postprandially (Bergeim, 1926; Ivy, Schmidt, & Beazell, 1936), when stomach pH can be as high as 6.2 (Russell et al., 1993) and will continue until the pH of the stomach contents lowers. It appears that the presence of starch and glucose oligosaccharides in the stomach can reduce α-amylase inactivation at a low pH (Rosenblum, Irwin, & Alpers, 1988).

The partially digested starch is released into the small intestine where digestion continues with the action of pancreatic α-amylase and the brush border disaccharidases. Starch is simultaneously digested, mainly by mammalian enzymes, and absorbed (as glucose) until the digesta reach the terminal ileum. An in vitro fermentation model using small intestinal inoculum has shown that starch is also likely to be fermented by microbes (Montoya et al., unpublished). Starch, undigested at the end of the ileum (often referred to as resistant starch) enters the hindgut where it is fermented by the microbial population, producing various end-products (Cummings & Englyst, 1987; Cummings & Macfarlane, 1991; Knudsen, 2001; Williams, Verstegen, & Tamminga, 2001).

Gastric starch digestion can be determined by measuring the quantity of reducing sugars in the gastric contents (Kurahashi & Inomata, 1999). However, this cannot be determined when other carbohydrates are included in the diet. The digestibility of starch in the small intestine is commonly determined by collecting ileal digesta samples. Resistant starch (which is defined as dietary fibre) will be highly fermented by the large intestinal microbial population. Therefore, total tract starch digestibility is usually not determined. The energy contribution of starch digestion to the body will depend on where the starch is digested. The energy from starch digestion in the small intestine (mammalian enzymes) will be mainly obtained from glucose absorption, while the energy from starch digestion in the large intestine (microbial enzymes) will be from the metabolism of absorbed SCFAs.

2.3 Lipids

The digestion of lipids starts in the stomach where both lingual and gastric lipases cleave fatty acids from the triglyceride molecule. The partially digested lipids continue to be digested by pancreatic lipases in the small intestine (Bauer, Jakob, & Mosenthin, 2005). The monoglycerides and fatty acids released from lipid digestion are incorporated into micelles before being absorbed via the lymphatic system. Shorter chain fatty acids can be absorbed via the epithelium (Hofmann & Borgström, 1964). Lipids are usually highly digested and absorbed throughout the small intestine. In contrast to other nutrients, undigested lipids are not fermented by large intestinal microbes, but biohydrogenation can occur with unsaturated fatty acids (Jorgensen, Gabert, Hedemann, & Jensen, 2000).

To determine gastric lipid digestion, stomach chyme can be collected to measure the amount of free fatty acids. However, if the aim is to determine lipid digestibility in the small intestine this can be done at either ileal or faecal level, following the collection of ileal digesta or faecal samples.

2.4 Dietary Fibre

The entire GIT (mouth to rectum) is colonised by a diverse microbial population. These microbes produce enzymes that are able to hydrolyse intermolecular bonds including those found in dietary fibre. Therefore, digestion of dietary fibre may occur throughout the GIT.

As the diet moves distally through the GIT its nutrient composition changes due to the simultaneous processes of dietary nutrient digestion and absorption and the secretion of endogenous compounds. These processes are affected by the transit time of the food material within each GIT location. The change in nutrient composition, transit time, and the reduction in the concentration of oxygen throughout the GIT influence the composition of the microbes present (both in terms of diversity and quantity) and the resultant microbial fermentation at each GIT location. For example, the lower oxygen concentration, a higher microbial concentration and longer retention time in the large intestine compared to the small intestine explain why dietary fibre is commonly believed to be digested mainly in the large intestine. Digestion of dietary fibre can be determined at any GIT location by determining the amount of undigested dietary fibre. However, dietary fibre digestion is usually determined at the ileum or rectum (i.e. total tract digestion).

3 Generalised Methods Used to Determine Digestion

The process of digestion can be studied in vivo either directly with humans or using an animal model, or an in vitro model of digestion.

3.1 In Vitro Methods

In vitro digestion methodologies are commonly used to determine digestion and/or fermentation of nutrients as they are easy to carry out, inexpensive and allow a high-throughput of samples when compared to in vivo methodologies. In addition, they are useful for mechanistic studies.

Due to the complexity of factors with in vivo systems, in vitro models are a simplification of an in vivo situation. Standardised parameters are selected within each in vitro model to simulate digestion. For example, stomach emptying rate is influenced by the protein source of the diet (Montoya, Rutherfurd, et al., 2014). However, in a standardised GIT digestion model the same gastric digestion time will be used for all samples. There are more advanced in vitro dynamic models that can incorporate some factors that affect digestion [e.g. the TNO intestinal model (TIM) (Rajilić-Stojanović et al., 2010), simulator of the human intestinal microbial ecosystem (SHIME) (Alander et al., 1999; Molly, Woestyne, & Smet, 2011)], but these models

are not necessarily more accurate than their simpler counterparts. In general, validation against in vivo digestion results are required to evaluate the accuracy of the in vitro results (Butts, Monro, & Moughan, 2012; Moughan, 1999).

The same principles used to determine the digestion of proteins in vivo (e.g. release of NH_2 and band intensity in SDS-PAGE gels) can be applied to in vitro studies of gastric and small intestinal protein digestion (Montoya, Gomez, et al., 2008; Montoya, Leterme, et al., 2008). For the in vitro gastric and small intestinal digestion of lipids and starch, it is common to determine free fatty acids (Beisson, Tiss, Rivière, & Verger, 2000; McClements & Yan, 2010) and glucose (Montoya & Leterme, 2011, 2012), respectively. To determine the in vitro fermentation of dietary fibre, food samples are commonly firstly digested using in vitro methods to simulate foregut digestion [pepsin to attempt to simulate gastric digestion and pancreatin to attempt to simulate small intestinal digestion (Bindelle, Buldgen, Boudry, & Leterme, 2007; Boisen & Fernández, 1997)] with the assumption made that there is little, if any, fermentation in the foregut. The undigested products collected from the in vitro foregut digestion simulate the material entering the large intestine, and as such are used as the substrate for in vitro large intestinal fermentation, which often uses a pooled faecal inoculum.

3.2 Ex Vivo Methods

Ex vivo methodologies of GIT digestion use tissue samples or parts of organs (e.g. inverted sac technique) that are excised from a specific GIT location or cell preparations (e.g. Caco-2 cells). Ex vivo methodologies (e.g. everted gut sac, Ussing chamber, primary epithelial cells) are mainly used in mechanistic studies to determine nutrient uptake and rate of uptake (Antunes, Andrade, Ferreira, Nielsen, & Sarmento, 2013; Lefebvre et al., 2015). When combined with in vitro methodologies, digestion and nutrient uptake can be determined simultaneously. As with in vitro methodologies, ex vivo methodologies cannot completely simulate the in vivo situation (e.g. the nutrient flow throughout the GIT cannot be replicated in the excised segment). Another limitation of ex vivo methodologies is the short time that tissue samples remain viable. Recently, stem cells from different GIT locations have been harvested to produce 3-D cell line models (organoids) (Mahe et al., 2013; Young & Reed, 2016). Although this emerging methodology has not been used to determine nutrient digestion/uptake, its greater potential ability to mimic the GIT tissue structure compared to other cell line models, makes it appear to be a useful ex vivo approach.

3.3 In Vivo Methods

When ingredients and foods are destined for human consumption, ideally nutrient digestion would be determined in human studies. Thus, total tract digestibility values can be determined directly in humans, requiring the collection of stools.

However, the subjects must consume the same food or ingredients for several consecutive days. This is no straightforward and necessitates a controlled experimental design, which can lead to a lower level of compliance by the subjects and a high proportion of subjects who do not complete the study. These factors complicate the routine application of studies in humans.

Conducting nutritional studies in humans is particularly complicated when samples are required from within the digestive tract, such as the stomach for evaluating gastric digestion, or the end of the small intestine, such as the case for evaluating the digestion of amino acids. Options that can be used to sample digesta in physiologically "normal" humans from these sites include intubation procedures [e.g. nasogastric or naso-ileal intubation (Gausserès et al., 1996; Mahé, Huneau, Marteau, Thuillier, & Tomé, 1992; Oberli et al., 2015)]. These involve the insertion of a small calibre tube via the nose, which is then swallowed and progresses through the digestive tract until the required sampling site is reached. Digesta can then be sampled through this tube (Bos et al., 2007; Deglaire, Bos, Tome, & Moughan, 2009; Mahé et al., 1992), and nutrient flows are calculated relative to the concentrations of indigestible markers. Due to the small calibre of the tube, only finely ground diets, which are usually given as liquids, can be reliably evaluated with this method. This method can be used to collect samples from different GIT regions (stomach, duodenum, jejunum and ileum) (Borgström, Dahlqvist, Lundh, & Sjövall, 1957; Da Costa, 1971; Mahé et al., 1992). Alternatively, the endoscopy retrograde bowel insertion method can be used to sample from more distal parts of the digestive tract (terminal ileum or large intestine) (Danjo et al., 2003; Saito et al., 2005).

Due to the invasive nature of these methods and several assumptions concerning marker behaviour, an alternative method to sample ileal digesta involves the participation of human ileostomates, who have had their large intestine removed for medical reasons, and their terminal ileum externalized. Human ileostomates have been used to study the digestion of amino acids (Moughan, Butts, Rowan, & Deglaire, 2005; Moughan, Butts, van Wijk, Rowan, & Reynolds, 2005), starch and non-starch polysaccharides (Englyst & Cummings, 1986, 1987; Holloway, Tasman-Jones, & Maher, 1983). Although ileostomates have cooperated with this method in several studies, this approach cannot be relied on for routine application. There are also questions, as to whether the ileostomates per se is a valid model for the normal human. Recently, new methods with humans have been investigated using dual isotopes.

Due to the difficulties in determining nutrient digestibility in humans on a routine basis and the high costs involved, other species such as the growing pig are commonly used as animal models in digestion studies (Deglaire et al., 2009; Moughan, Birtles, Cranwell, Smith, & Pedraza, 1992; Moughan, Cranwell, Darragh, & Rowan, 1994; Rowan, Moughan, Wilson, Maher, & Tasman-Jones, 1994). The physiological and anatomical similarities (e.g. transit time, digestive and absorptive processes) in the foregut of humans and that of pigs, suggests that the pig is likely to be a good animal model for studying digestion in humans between the mouth and the end of the small intestine (Deglaire & Moughan, 2012; Miller & Ullrey, 1987; Patterson, Lei, & Miller, 2008). In addition to the similarities described above, pigs are meal eaters and readily consume foods eaten by humans and, when normalised for the difference in bodyweight, pigs have similar nutritional requirements to

humans. Several reviews have discussed the validity of the pig as a model for studying protein digestion (Deglaire & Moughan, 2012), mineral absorption (Patterson et al., 2008) and gut microbiota modulation (Heinritz, Mosenthin, & Weiss, 2013) in humans and concluded in all cases that the pig is a suitable nutritional model. Similar ileal digestibility coefficients have been shown in the growing pig and adult human for different nutrients such as protein sources (Deglaire et al., 2009; Rowan et al., 1994).

The rat is another animal model that can be used to determine digestion. However, while the rat is a more economical model than the pig, the rat (a natural nibbler) is considered an inferior model (Food and Agriculture Organization of the United Nations (FAO), 2013, 2014), for reasons such as its selective feeding habits, which require the food to be finely ground before feeding. This is likely to influence the digestion of the different nutrients. Sampling in rats is conducted following euthanasia and only a small amount of sample can be collected from each animal. Rats also practice coprophagy, which can be difficult to prevent.

Animal models are also used to determine total tract digestion of foods, for which faecal samples are usually collected, but here the pig, having a large caecum, is a less applicable model. Methods that can be used to collect samples of digesta from different sites of the GIT of animal models, including cannulation techniques or following euthanasia, are described in the following section.

4 Faecal and Digesta Sampling Methods

For the collection of chyme, digesta or faecal samples to study digestion, the amount of material to be collected and the timing of the sample collection depend on the aim of the study and the methodology to be used (e.g. in vitro or in vivo). Samples can be collected either at different time points during digestion to study kinetics (Montoya, Leterme, et al., 2008) or at the end of an experimental period at a single time point (Bindelle, Pieper, Montoya, Van Kessel, & Leterme, 2011; Montoya & Leterme, 2012). When samples are required from in vivo studies from positions that are not easily accessible (e.g. the terminal ileum), the collection of samples may require the use of special approaches. These include naso-ileal intubation, the use of ileostomised subjects, or when an animal model is used, following euthanasia or surgical cannulation procedures.

4.1 Collection of Faecal Samples

Faecal samples can be collected relatively easily in intact humans or animal models to determine total tract digestibility (e.g. total gross energy, lipids and fibre) (Baer, Rumpler, Miles, & Fahey, 1997; Coles, Moughan, Awati, & Darragh, 2013a; Holloway, Tasman-Jones, & Lee, 1978; Montoya, Gomez, et al., 2008; Montoya &

Leterme, 2010) and a total or partial collection can be made of the faeces. Total-tract digestibility studies require adaptation to the experimental diet for several days before sample collection to ensure that the faecal sample collected pertains to the test diet. Following this adaptation period, a total collection of faeces can be conducted over a period of several days while the human or animal continues to receive the experimental diet.

Alternatively, a single sample at a point in time can be collected, which assumes that the transit of nutrients is constant during the experimental period. When the single time sample approach is used, an indigestible marker (e.g. celite, titanium dioxide) must be included in the diet (Jagger, Wiseman, Cole, & Craigon, 1992). The indigestible marker to be used must not be absorbed within the GIT and must move through the GIT together with the nutrient being analysed. The concentrations of the nutrient of interest and marker, both in the diet and samples collected, are used to calculate digestibility. Although the use of markers is a relatively simple approach, studies have shown different pitfalls that can occur when they are used (Köhler, Huisman, Den Hartog, & Mosenthin, 1990; Montoya & Leterme, 2009; Mroz et al., 1996). Despite the limitations of indigestible markers, they are important to reduce variability and allow partial collections of faeces or GIT contents, which can reduce the experimental period (Mroz et al., 1996).

4.2 Collection of Gastrointestinal Tract Contents from Animal Models

4.2.1 Sampling Following Euthanasia

A collection of GIT contents in an animal for a specific purpose (e.g. gastric digestion of dietary protein or ileal amino acid digestibility) (Montoya, Hindmarsh, et al., 2014; Nasset & Ju, 1975; Nasset, Schwartz, & Weiss, 1955) can be carried out following euthanasia or using invasive surgical methods. Sampling following euthanasia is inexpensive when compared to other alternatives as there is less need for specialised expertise (e.g. surgeon, care of cannulas) and facilities.

However, sampling following euthanasia allows the collection of a single digesta sample only and at a single time point only. This means that when the kinetics of digestion are to be studied, the number of animals required increases considerably. Moreover, the use of different animals for each time point of the kinetics study may increase the variability during the statistical analysis as it does not allow the use of a repeated measures analysis (i.e. adjusting for the best variance–covariance structure).

When samples need to be collected from different GIT regions, sampling following euthanasia can be a good option as it allows measurements to be conducted over the entire GIT. There is, however, a limited quantity of digesta that can be collected from each location, which may not be sufficient to conduct all analyses that are required. In this case, more animals may need to be sacrificed to pool their samples

and obtain the quantity of digesta necessary. Some studies have suggested that sampling following euthanasia should be carried out preferentially with the animal under terminal anaesthesia to reduce the shedding of epithelial cells (Low, 1980), which can be a problem when the electric shock is used to sacrifice the animals. This method also necessitates the use of indigestible marker compounds.

Methods such as surgical cannulation allow the collection of multiple samples from the same animals over time and the collection of a greater quantity of material.

4.2.2 Cannulation Methods

Different cannulation approaches have been developed, especially in the pig model, to collect contents from the stomach (Gregory, McFadyen, & Rayner, 1990; Rainbird & Low, 1986), small intestine (Montoya & Leterme, 2012; Mroz et al., 1996; van Leeuwen, van Kleef, van Kempen, Huisman, & Verstegen, 1991; Wilfart, Montagne, Simmins, van Milgen, & Noblet, 2007) and hindgut (Farrell & Johnson, 1970; Theodorou, Fioramonti, & Buéno, 1989; Vervaeke, Dierick, Decuypere, & Cosijn, 1985). The insertion of a T-cannula in the GIT site from which digesta are to be collected is considered the simplest cannulation procedure. Once the T-cannula has been surgically implanted, GIT contents can be collected over time and the collection of digesta following the feeding of several different diets is possible in the same animal. Indigestible markers need to be included in the diet as a complete digesta collection will not occur via a simple T-cannula. Modifications to the simple T-cannulation method have been developed with the aim of collecting a greater proportion of terminal ileal digesta (e.g. post-valvular T-caecum, steered ileo-caecal valve) (Mroz et al., 1996; van Leeuwen et al., 1991), but indigestible markers (Yin et al., 2000) must still be incorporated into the diets with these methods.

Another approach that can be used to collect small intestinal (e.g. duodenal, ileal) (Ivan, 1974; Zebrowska et al., 1982) and colonic digesta is re-entrant cannulation (Moughan & Miner-Williams, 2013). With this method, two cannulas are surgically inserted; one for collection of digesta and the second for the return of digesta when collection is not required. The two cannula are connected when digesta is not being collected (Ivan, 1974). An advantage with re-entrant cannulation is that an entire collection of digesta can be conducted, thus indigestible markers are not required. However, blockages in the cannulas are a common occurrence with re-entrant cannulation and the surgery required is invasive.

While cannulation appears to be a good alternative approach for collecting samples from different regions of the GIT, the presence of the cannulas themselves, especially in the case of re-entrant cannulation, is expected to affect GIT motility, potentially affecting the flow of nutrients throughout the GIT (Zimmermann & Mosenthin, 2002).

4.2.3 Ileo-rectal Anastomosis

In this invasive approach, which is perhaps dubious ethically, the terminal ileum (with or without the ileo-caecal valve) is attached either at the side of (end-to-side) or directly to (end-to-end) the rectum (Green, Bertrand, Duron, & Maillard, 1987; Kohler et al., 1992). A complete collection of terminal ileal digesta is made from the anus, thus indigestible markers are not required. Based on the concentration of SCFAs, intestinal adaptation with an increased microbial fermentation seems to occur in the terminal ileum (Kohler et al., 1992). This is an important limitation with this approach as it may result in an overestimation of ileal amino acid and dietary fibre digestibility values. Another possibility is to replicate the anastomosis operation carried out in humans, whose colon has been removed. Here, the ileum is exteriorised to the body wall.

5 Approaches to Determining Digestibility: Dietary Fibre as an Example

As discussed earlier, methodologies and concepts for starch, lipid and protein digestion are well established. However, this is not the case for the digestion of dietary fibre, which is a rapidly developing subject area. In this case, the digestion of dietary fibre is discussed here in more detail.

In addition to the energy that dietary fibre provides through fermentation in the GIT (up to 11% of human energy requirements) (McBurney & Thompson, 1989; McNeil, 1984), fermentation and the supply of SCFAs also have important physiological implications ranging from GIT health to brain function (Goverse et al., 2017; Guinane & Cotter, 2013; Kelly et al., 2016; Krautkramer, Kreznar, Romano, et al., 2016; Patterson et al., 2016).

According to the most recent definition of dietary fibre proposed by the CODEX Alimentarius Commission (2013), dietary fibre comprises a diverse range of complex structures that contain monomeric units linked by bonds that cannot be hydrolysed by mammalian digestive enzymes. Different in vitro and in vivo methodologies have been developed to study the digestion of dietary fibre throughout the GIT.

5.1 In Vitro Methodology

As described above, dietary fibre in the GIT is only digested by microbial enzymes. Thus in vitro models for dietary fibre fermentation must include microbial enzymes. In vitro fibre digestion models commonly use either purified microbial enzymes (Huang, Sauer, He, Hwangbo, & Wang, 2003), or an inoculum with living microbes

that will produce the different enzymes in real time (Bindelle et al., 2011; Coles, Moughan, Awati, & Darragh, 2013b; Montoya, Rutherfurd, & Moughan, 2016).

5.1.1 In Vitro Approach Using Purified Microbial Enzymes to Simulate Hindgut Fermentation

This methodology, mainly used in animal nutrition studies, involves the use of either cellulase (Huang et al., 2003; Van Der Meer & Perez, 1992) or a mixture of different microbial enzymes (arabinase, cellulase, β-glucanase, hemicellulase, xylanase and pectinase) (Boisen & Fernández, 1997). Strong relationships have been reported between digestibility values generated using these in vitro methods and those generated with in vivo studies (R^2 = 0.54–0.93) (Boisen & Fernández, 1997; Huang et al., 2003; Regmi, Ferguson, & Zijlstra, 2009). One of the major limitations with the use of purified enzymes to determine dietary fibre digestibility is that it does not allow the production of microbial fermentation end-products to be determined (e.g. organic acids, SCFAs). These end-products may play important roles in local (e.g. reduction of pathogenic microbiota) and systemic (e.g. gut motility, reduction of cholesterol) health of the host as has been reviewed elsewhere (Bergman, 1990; Guilloteau et al., 2010; Roy, Kien, Bouthillier, & Levy, 2006; Topping & Clifton, 2001; Vinolo, Rodrigues, Nachbar, & Curi, 2011). It is also relevant to energetics, to determine the degree of fermentability of fibre in addition to digestibility.

5.1.2 In Vitro Approach Using Living Bacteria to Simulate Hindgut Fermentation

With this in vitro approach, living bacteria produce the enzymes required to digest substrates in real time, which may include dietary fibre, undigested dietary protein and non-dietary (endogenous) materials (e.g. mucins) (Montoya et al., 2017). The common way used to obtain living bacteria for human and animal studies is to obtain samples (e.g. faeces) from different donors (n = 3–5) and prepare a pooled-inoculum (Aguirre, Ramiro-Garcia, Koenen, & Venema, 2014). While digesta from the caecum or colon can be collected from animal studies to prepare hindgut inocula for in vitro fermentation studies, this is more complicated for humans. For human studies, it is common to use faecal samples to prepare a hindgut inoculum. There is a clear limitation of this approach, as faecal and colonic microbial populations are likely to differ (Stearns et al., 2011), but the effects of this on fermentation are yet to be elucidated.

The most common parameters used to evaluate the in vitro fermentation of dietary fibre (or other substrates) using an inoculum include the production of gas and SCFAs, the disappearance of dry matter, organic matter and organic matter components (e.g. monomeric units of non-starch polysaccharides). Changes in the microbial population following in vitro fermentation are also commonly determined.

5.1.3 In Vitro Foregut Fermentation

Current knowledge of dietary fibre fermentation suggests that dietary fibre is mainly fermented in the large intestine where there is a much greater microbial population (Macfarlane, Gibson, & Cummings, 1992) and longer retention time (Bouchoucha et al., 2015) compared to the foregut. Thus a plethora of in vitro studies have been conducted to evaluate the hindgut fermentation of dietary fibre. However, comparative metagenomic analyses have shown that the microbial population in the small intestinal digesta and mucosa (Bik et al., 2006; Stearns et al., 2011) possess similar pathways and functions related to dietary fibre metabolism as those present in the hindgut or faeces (Cecchini et al., 2013; Langille et al., 2013; Zhao et al., 2015; Zoetendal et al., 2012). In addition, studies have suggested that a considerable degree of fermentation of the soluble fraction of dietary fibre (Abad-Guamán, Carabaño, Gómez-Conde, & García, 2015; Montoya, Rutherfurd, & Moughan, 2015), pectin (Holloway et al., 1983) and resistant starch (Danjo et al., 2003) occurs in the foregut.

Recently, we have developed an in vitro ileal fermentation method, based on the assumption that within the small intestine, fermentation occurs mainly in the last one third. The preliminary results have shown that ileal fermentation is quantitatively significant and the degree of fermentation varies among substrates (Fig. 1).

5.2 In Vivo Methodology to Determine Gastrointestinal Tract Fermentation

Human studies have been conducted to determine both ileal (Holloway et al., 1978; Lien, McBurney, Beyde, Thomson, & Sauer, 1996; Saito et al., 2005) and total tract (Holloway et al., 1978) dietary fibre digestibility values. To determine the ileal

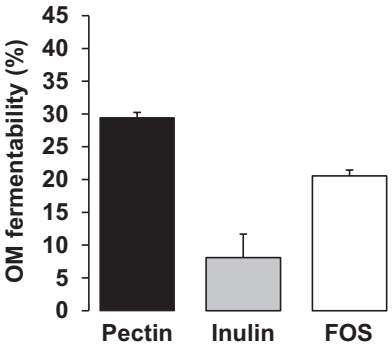

Fig. 1 Organic matter (OM) fermentability (n = 3) of citrus pectin, inulin and fructo-oligosaccharides (FOS) fermented with pooled pig ileal inoculum. The substrates were fermented for 2 h with an inoculum (220 g ileal digesta in 1 L of PBS pH 7) prepared with ileal digesta from five pigs fed a human-type diet (Montoya et al., unpublished)

digestibility of dietary fibre, samples are collected from intact humans (Danjo et al., 2003; Saito et al., 2005) or human ileostomates (Englyst & Cummings, 1985, 1986). One of the limitations of the ileostomate model is that it may allow colonisation of microbiota in the terminal ileum that is different from that present in the "intact" human, including aerobic bacterial species.

In contrast to ileal dietary fibre determination (Holloway et al., 1983; Saito et al., 2005), total tract dietary fibre digestibility determination requires that participants are adapted to the experimental diet for at least 4 days before beginning faecal collections to ensure that the previous diet is not still present in the hindgut. Thereafter, faecal samples are collected over several days (Baer et al., 1997; Forsum, Eriksson, Göranzon, & Sohlström, 1990; Holloway et al., 1983; Wisker, Daniel, Rave, & Feldheim, 1998). The incorporation of indigestible makers in the test diet will depend upon whether an entire or partial collection of faeces is carried out.

It can be complicated to conduct human studies to evaluate dietary fibre fermentation, especially for total tract digestibility, as they require control over parameters that can be difficult to control (e.g. it is difficult to feed a single diet to a person for prolonged periods of time). These limitations can be overcome with the use of animal models (e.g. pigs and rats), as discussed above. In terms of fibre digestion, pigs and humans share similarities in the microbial diversity in their faeces (Heinritz et al., 2013), and there are similar viable counts of *Lactobacilli*, *Streptococci* and *Coliforms* in the ileal digesta of humans (10^7, 10^6 and 10^6/g wet digesta respectively) and pigs (10^8, 10^7 and 10^5/g wet digesta respectively) (Graham & Åman, 1987). However, in contrast to humans, pigs possess a voluminous caecum that may increase the retention time of digesta in the large intestine (29 vs. 37 h retention time for humans (Bouchoucha et al., 2015) and pigs (Latymer et al., 1990; Wilfart, Montagne, Simmins, Noblet, & van Milgen, 2007) respectively). A higher retention time may increase the extent of fermentation of dietary fibre. Thus, when pigs are used as a model for humans to determine hindgut fermentation and/or total tract digestion of dietary fibre, the direction of differences or ranking of foods should be evaluated, as opposed to the determination of absolute values. The dog is likely a better animal model than the growing pig for evaluating hindgut digestion (Hendriks, van Baal, & Bosch, 2012).

Rats are also commonly used to study dietary fibre fermentation (Cabotaje et al., 1994; Levrat, Behr, Rémésy, & Demigné, 1991; Monsma, Vollendorf, & Marlett, 1992; Nyman & Asp, 1982). Although anatomical similarities have also been reported between rats and humans, humans have a greater relative absorptive surface and faster transit time (especially in the hindgut) (DeSesso & Jacobson, 2001; DeSesso & Williams, 2008). These factors may affect the fermentation of dietary fibre. In addition, rats are not meal eaters and may not consume all human diets. Although pigs and rats have similar anatomical, histological and physiological characteristics as humans, there may be subtle unknown differences influencing digestion, and this is an inherent limitation in the use of any animal model.

The duration of the animal studies and the use of indigestible markers depend on the aim of the study (e.g. ileal or total tract digestibility). The most common parameters to determine in the in vivo fermentation of dietary fibre include digestibility,

SCFA concentrations, changes in the microbial population, and effect on the GIT anatomy and morphology. The digestibility of fibre can be either apparent or corrected for endogenous components (Montoya, Henare, et al., 2016; Montoya et al., 2015). In GIT contents and faeces there are several non-dietary components (e.g. bacteria) that interfere with the determination of dietary fibre and therefore in the calculation of dietary fibre digestion. Further work is still required to develop methods to allow a more accurate determination of the digestion of dietary fibre (Montoya, Henare, et al., 2016).

5.3 Combined In Vivo–In Vitro Digestion Methodology to Determine Gastrointestinal Tract Fermentation

Despite the limitations of the in vitro methodologies (e.g. it is not possible to simulate all host–nutrient interactions) (Coles, Moughan, & Darragh, 2005), a combination of in vitro and in vivo methods can be used to determine mechanisms of digestion or fermentation that cannot be easily determined with in vivo studies. For example, the concentration of SCFAs in digesta and faeces only represents the net result of SCFA production and absorption and can be misleading. Therefore, to determine the production and absorption of SCFAs, combined in vivo–in vitro digestion methodologies have been proposed (Christensen, Knudsen, Wolstrup, & Jensen, 1999; Montoya et al., 2017; Montoya, Rutherfurd, & Moughan, 2016).

The combined in vivo–in vitro digestion methodology for studying hindgut fermentation uses human ileostomates (Langkilde, Champ, & Andersson, 2002; McBurney & Thompson, 1989) or animal models (ileal-cannulated or euthanised pigs and rats) (Coles et al., 2013a; Montoya, Rutherfurd, & Moughan, 2016) to obtain ileal digesta for an experimental unit given a human-type diet. These digesta are used to model the end products of small intestinal digestion in humans and represent the material entering the human hindgut. Rather than the estimated material (i.e. substrate) entering the hindgut in a full in vitro digestion approach (or three-step digestion), the material entering the hindgut in the combined in vivo–in vitro digestion methodology will contain both dietary and non-dietary materials that were not digested by foregut mammalian or microbial enzymes. Thus, the digesta substrate from the combined in vivo–in vitro digestion methodology considers the effect of the diet on the foregut and this can give more precise information. For example, in pigs fed a diet containing kiwifruit fibre, non-dietary material (e.g. mucins), rather than dietary fibre that escaped foregut fermentation, was shown to be the main substrate for the hindgut production of SCFAs (Montoya et al., 2017).

The collected ileal digesta are then incubated (in vitro) with a human faecal inoculum to model hindgut fermentation. This hindgut fermentation is carried out in vitro, as SCFA concentrations in hindgut digesta collected in vivo represent only unabsorbed SCFA. This combined in vivo–in vitro digestion methodology has been shown to give accurate predictions of organic matter fermentation in the hindgut of

Fig. 2 Principle of a combined in vivo–in vitro methodology to determine ileal and hindgut fermentations in the same animal model. TJ, terminal jejunal digesta substrate is fermented with an ileal digesta inoculum for 2 h. The ileal digesta could be obtained from human ileostomates or ileal cannulated or euthanised pigs. TI, terminal ileal digesta substrate is fermented with a caecal digesta or faecal inocula for 24 h. The faecal inoculum can be from humans or pigs and the caecal inoculum from pigs. Both ileal and hindgut fermentation are performed under anaerobic conditions (Montoya et al., unpublished)

the adult human (Coles et al., 2013a). Although the combined in vivo–in vitro digestion methodology appears to have several advantages, it does not account for the fermentation of non-dietary material derived from the colonic tissues themselves and from fermentation of dead colonic microbes.

We have recently developed a combined in vivo–in vitro ileal fermentation methodology using the pig model, which assumes that small intestinal fermentation occurs mainly in the last one third of the small intestine (Montoya et al., 2018). This methodology follows the same principle of the combined in vivo–in vitro hindgut fermentation methodology. The digesta entering the last third of the small intestine (i.e. terminal jejunal digesta) are collected to model the substrate available for ileal fermentation, while the ileal digesta from the same group of animals are collected to prepare the ileal inoculum. Both combined in vivo–in vitro ileal and hindgut methodologies can be used to determine ileal and hindgut fermentation in the same animal (Fig. 2). Preliminary results comparing both fermentations in pigs fed a human-type diet suggest that ileal fermentation may be as important quantitatively as is hindgut fermentation. Although nutrients are expected to be absorbed mainly in the first half of the small intestine (Borgström et al., 1957; Nixon & Mawer, 1970), an overestimation of ileal fermentation may occur as some digestible nutrients may be present in the substrate for fermentation (that would otherwise have been absorbed). This potential area is currently being investigated in our laboratory.

In conclusion, there is a range of different methods that can be used to determine or predict nutrient digestion in the GIT. These include carrying out studies in humans directly, using animal models, in vitro methods and in vivo–in vitro combination methods. Different approaches exist within each of these methodologies. Ultimately, an approach is chosen depending on the nutrient(s) to be examined, the aim of the study and the resources available. It is important that the advantages and disadvantages of each approach be recognised to avoid misleading interpretations and conclusions.

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Quantifying Digestion Products: Physicochemical Aspects

Uri Lesmes

1 Introduction

In light of the close link between nutrition and health, numerous studies seek to understand the various and complex processes of digestion governing bioaccessibility, bioavailability and ultimately the bioefficacy of nutrients and food bioactives (see illustration in Fig. 1). This poses a multifaceted scientific challenge that needs to address the physicochemical complexity of food, the spatiotemporal events of breakdown (Bornhorst, Gouseti, Wickham, & Bakalis, 2016) as well as the diversity of digestive physiology of different consumers (Levi et al., 2016; Rémond et al., 2015; Vimaleswaran, Le Roy, & Claus, 2015).

This vivid field of food research has given rise to various strategies to study the bioaccessibility, bioavailability, and bioefficacy of macronutrients, micronutrients, and other bioactive compounds through human trials, animal models, cell cultures, in vitro and even in silico digestion models (Etienne-mesmin et al., 2012; Hur, Lim, Decker, & McClements, 2011; Marze, 2015a, 2015b, 2017; Marze & Choimet, 2012; Payne, Zihler, Chassard, & Lacroix, 2012). While human trials are considered the "golden standard," in vitro models are increasingly used as robust, high-throughput, and practically "ethics-free" platforms for mechanistic investigations. This led to the formation of an international scientific network INFOGEST that devised a harmonized in vitro digestion protocol (Minekus et al., 2014) and evaluated its applicability (Egger et al., 2015). Follow-up efforts have even focused on relating in vitro findings with in vivo results to show the tight correlations of findings in the analysis of macronutrient breakdown (Bohn et al., 2017). Concomitantly, efforts are ongoing to extend the scope of in vitro digestion models to specific human populations, such as infants, the elderly, and gastric bypass patients (Levi et al., 2016).

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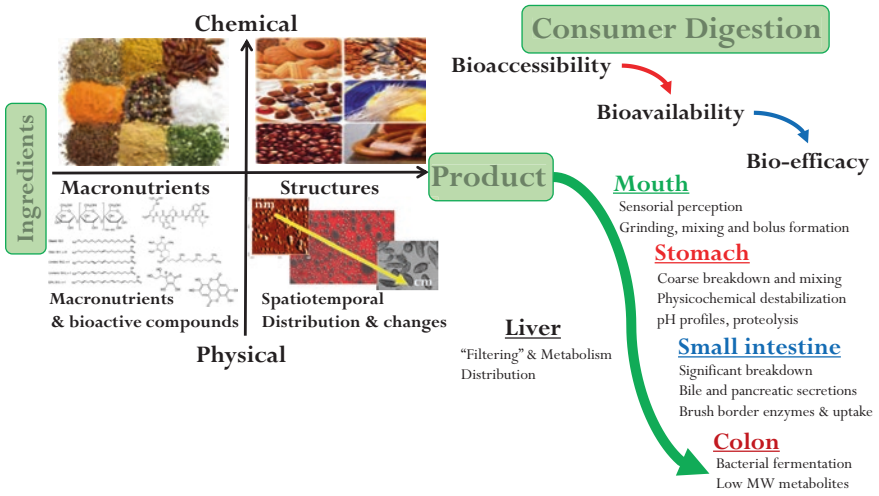

Fig. 1 A schematic illustration of the multifaceted aspects of food's digestive fate

Besides the inherent scientific and technological challenges of physicochemical analysis of digesta, researchers have to face various practical considerations, such as equipment and reagent accessibility (e.g., access to bioreactors or availability of gastric lipase) the mélange of food types and classifications (e.g., solid, semisolid, and liquid foods) (Bornhorst, Ferrua, & Singh, 2015), the multitude of length scales in which food is broken down and the interplay with epithelial and immune cells as well as with the wealth of microbes along the alimentary canal. Therefore, qualitative and quantitative investigation of digesta physicochemical aspects mandates a careful and systematic approach to minimize bias and erroneous interpretations.

One of the first and foremost considerations in the analysis of digesta is the research hypotheses, the selected experimental design and sampling scheme. The latter is selected according to experimental setup, which should be tailored to the research objectives and hypotheses. Thus, clear and well-rationalized objectives and hypotheses are the basis which subsequently determines the properties to be analyzed and the methods for their analysis.

2 What Are the Major Properties Analyzed?

Composition

Most foods and ingested products are complex matrices comprised of various ingredients, in various structures and sometimes even in various physical phases (e.g., the coexistence of gas, liquid, and solid phases in ice cream or other aerated semisolid desserts like mousses). Compositional analyses can focus on macronutrients, micronutrients, and other specific bioactive compounds of interest, such as polyphenols or

other phytochemicals. These analyses may range from holistic and highly generalized -omics analyses down to highly focused and targeted analyses of a specific compound (e.g., lycopene or curcumin), chemical activity (e.g., antioxidant activity), or class of compounds (e.g., polyphenols). The scientific literature is strewn with a myriad of compositional analyses applicable to food research. These depend on the analyte and vary from generalized analyses, such as determination of reducing sugars, lipid or short chain fatty acids content, to highly specific analyses like nuclear magnetic resonance (NMR) techniques or various MS analyses (Tamvakopoulos, 2007). Such analyses can adopt a holistic foodomics or molecular fingerprinting approach to the in situ analysis of digesta, for example by 1H NMR (Bordoni et al., 2014; Vidal et al., 2016). Alternatively, recent studies into digesta samples have also applied advanced methods coupling separation-analysis methods such as liquid chromatography coupled with mass spectrometry (LC-MS) proteomic analyses (Kopf-Bolanz et al., 2014; Shani Levi, Goldstein, Portmann, & Lesmes, 2017; Skinner et al., 2016).

Structure

The structural organization of the components within a product and within digesta may play a detrimental role in food's digestion and the consumer's physiological responses. For example, flocculation and coalescence of emulsion droplets are key players in the digestive lipolysis of emulsions (Marze, 2015a; Marze & Choimet, 2012; Shani-Levi, Levi-Tal, & Lesmes, 2013; Singh & Ye, 2013; Wilde & Chu, 2011). Further, there is a dire need to address the multitude of dimensions at which digestion occurs, ranging from macroscopic digestion down to the molecular level. Therefore, the structure of ingested products can be examined at various length scales:

- *Macroscopic structure* (~>1000 μm). This is the structure of samples that can be observed with the naked eye or sensed by the other human senses (e.g., large air cells in bread or cheese curds in cottage cheese). The propensity and architecture of such structures in the digesta are less described in the scientific literature as digestion is investigated mainly in the prospects of size reduction to the micro- and sub-micro range. Yet macroscopic structures are important to investigate as they affect the physical sensing mechanisms of the mouth and gut and evoke various digestive events, such as neurological responses to intragastric and intraduodenal pressure that affect ghrelin production and gastric emptying. Therefore, it is no surprise that the macroscopic investigation of digestion is heavily focused on the flow and mechanical properties of digesta.
- *Microscopic structure* (~100–1000 μm). This is the structure that can be observed by microscopy (but not by the unaided eye) and consists of molecular associations to form discrete phases, e.g., emulsion droplets, fat crystals, protein aggregates, starch granules, and small air cells. Application of such analyses to digesta mainly rely on direct observations using various microscopy techniques and sample labeling or dying (e.g., fluorescent dying of lipid droplets using Nile Red or dying of protein phases using Rhodamine B) (Matalanis, Lesmes, Decker, & McClements, 2010). Such analyses not only provide insights on the structures in

the digesta but may also shed light into the partitioning of different substances within the digesta. In recent years, there has been a rise in the use of magnetic resonance imaging (MRI) to externally monitor the structure/partitioning of materials during digestion in vitro and in vivo (Kozu et al., 2017; Liu et al., 2016). Yet such analyses show poor resolution at the microscopic range and are better suited for macroscopic structure analysis.

- *Molecular, supramolecular, and nanostructures* (<1–100 nm). Ultimately, the overall physicochemical properties of a food depend on the type of molecules present, their three-dimensional organization, and/or self-assembly into supramolecular and various structured assemblies [e.g., starch inclusion complexes (Obiro, Sinha Ray, & Emmambux, 2012; Zabar, Lesmes, Katz, Shimoni, & Bianco-Peled, 2009) or food-protein nanofibrils (Loveday, Anema, & Singh, 2017; Moayedzadeh, Madadlou, & Khosrowshahi asl, 2015)]. These in turn affect the nature, intensity, and kinetics of molecular interactions that ultimately direct their digestive fate. Thus, in addition to various chemical analysis methods, there are a growing number of studies applying various methods to analyze the nano-attributes of foods (Luykx, Peters, van Ruth, & Bouwmeester, 2008) and their digesta. For example, one study used interfacial tension measurements to demonstrate the ramifications of galactolipids to emulsion droplet interfacial tension and fluidity and subsequently to droplet susceptibility to intestinal lipolysis (Chu et al., 2009).

Physicochemical Properties

Beyond the effect on texture perception in the mouth, the physiochemical properties of digesta (rheological, colloidal, and physical properties) ultimately determine their digestive fate. This is governed by the responsiveness of the digesta to digestive physiology and its interactions with the cells lining the gut. Since food can be regarded in most cases as a soft matter, there are numerous traits that can be measured; these may range from colloidal aspects, like droplet coalescence, to aspects of soft matter physics, like biopolymer gelation and particulate disintegration or self-assembly. Overall, such analyses are more focused on the physical aspects of the digesta. Yet various combinations of methods can be used to bridge the various length scales in which digestive phenomena may occur.

3 What Are the Key Considerations for Method Selection?

Following the definition of research hypothesis and the properties to be analyzed, researchers should make a rationalized selection of the analytical method and the sampling strategy/scheme. In most cases, digesta is an intricate mixture of analytes in a highly complex set of structures and spatial organizations. This analytical challenge is often intensified by the research need to determine the kinetics and loci specificity of digestive events. Many analytical techniques are destructive, time-consuming, expensive, or labor-intensive, so it is not economically feasible to

pursue all analyses that one would desire. It is usually not practical to have large series of samplings during digestion, as these may affect the test subjects or the in vitro experiments. Moreover, many experiments are also limited in the amounts of samples to be digested. Therefore, selection of analytical methods and sampling strategy are critical for the successful physicochemical analysis of digesta. Table 1 offers a practical checklist of key considerations that should be taken into account when considering analytical methods to be applied to the analysis of digesta.

Similarly, there is great interest to adequately select the sampling scheme. This has to take into account the nature of the object of investigation (e.g., liquid, semisolid, or solid food), sample size, sample organization (continuous or compartmentalized

Table 1 Key considerations for selection of analytical methods for analysis of digesta

Analytical consideration	Brief definition
Precision	A measure of the ability to reproduce an answer between determinations performed by the same scientist (or group of scientists) using the same equipment and experimental approach
Accuracy	A measure of how close one can actually measure the *true value* of the parameter being measured (e.g., fat content, or sodium concentration)
Sensitivity	A measure of the lowest concentration of a component that can be detected by a given procedure
Specificity	A measure of the ability to detect and quantify specific components within a food material, even in the presence of other similar components (e.g., fructose in the presence of sucrose or glucose)
Reproducibility	A measure of the ability to reproduce an answer by scientists using the same experimental approach but in different laboratories using different equipment
Nature of Food Matrix	The composition, structure and physical properties of the matrix material surrounding the analyte often influences the type of method that can be used to carry out an analysis (e.g., whether the matrix is solid or liquid, transparent or opaque, polar or non-polar)
Cost and speed	The total cost of the analysis, including the reagents, instrumentation and salary of personnel required to carry it out. The time needed to complete the analysis of a single sample or the number of samples that can be analyzed in a given time
Simplicity of operation	A measure of the ease with which relatively unskilled workers may carry out the analysis
Destructive/ Nondestructive	In some analytical methods the sample is destroyed during the analysis, whereas in others it remains intact for further analyses
Safety	Many reagents and procedures used in food analysis are potentially hazardous (e.g., strong acids or bases, toxic chemicals, or flammable materials)
On-line/Off-line	Some analytical methods can be used to measure the properties of a food during the digestion experiments, whereas others can only be used after the sample has been taken from the production line
Official Approval	Various international bodies have given official approval to methods that have been comprehensively studied by independent analysts and shown to be acceptable to the various organizations involved, e.g., ISO, AOAC, AOCS

nature), sample homogeneity or inhomogeneity, and the sampling plan. The latter is of great importance as collection of samples should have minimal impact on the experiment and the status of the collected sample. For example, there are various inhibition schemes and materials used to inactivate proteolytic enzymes in digesta samples. These may range from snap freezing using liquid nitrogen, through pH elevation using NaOH or irreversible inactivation using protease inhibitors, like PMSF and AEBSF (Egger et al., 2015; Minekus et al., 2014). Another example is the freezing of digested emulsions for structural analysis that will pose a major challenge during the subsequent deforesting of samples for analysis. This is likely to induce a freeze-thaw effect that is independent of the effects induced by the digestion experiments and may cause an erroneous observation.

4 Chemical and Biochemical Analyses of Digesta

A battery of compositional analyses may be applied to samples of digesta, ranging from highly focused analyses, such as HPLC-based quantification of specific compounds, to generalized analyses, like proteomic analyses. These can be best classified according to the purpose of the analysis and its analyte specificity. In respect to the purpose of analysis, one can mine digesta for macronutrients, micronutrients (e.g., vitamins), or other specific analytes (e.g., bioactive compounds such as resveratrol and lycopene). In respect to analyte specificity, one can scour for a highly specific compound (e.g., a specific peptide) or a general class of substances (e.g., short chain fatty acids). The following section reviews the relevant published literature.

4.1 Compositional Analyses

In general, compositional analyses of food digestion are performed in the context of nutrients, nutrition, and health. Overall, it is accepted that small molecular weight species (e.g., water, ions, and alcohol) are taken up in the stomach while higher molecular species released from food (e.g., monosaccharides, monoglycerides, cholesterol, medium and long chain fatty acids, and various vitamins) are absorbed through active and/or passive mechanisms along the intestine. In addition, the roles of the colon and its microbiome are increasingly studied (Albenberg & Wu, 2014; O'Hara & Shanahan, 2006; Payne et al., 2012), beyond the colonic role in adsorption of water, electrolytes, vitamin K and biotin.

Analyses of Macronutrients
Various methods of analysis have been applied in the determination of proteins, carbohydrates, and lipids in digesta samples. In general, these analyses have to carefully balance the complexity of the digestion media (e.g., the use of pancreatic

extracts versus isolated digestive enzymes), the sampling protocol (namely, the inactivation procedure), and the analyte type and size (e.g., whey protein solution versus a real dairy product) in face of the research hypothesis.

Carbohydrates are a staple macronutrient, with starch accounting for up to 50% of the total energy intake in some diets. Yet the physicochemical diversity of carbohydrates in foods challenges scientists until today. In the prospect of digestion, carbohydrates can be classified into digestible and indigestible carbohydrates and are analyzed accordingly, as reviewed elsewhere (Englyst, Liu, & Englyst, 2007a). Thus, digestive amylosis (namely release of simple sugars) and carbohydrate fermentability are the two major digestive activities investigated in the context of carbohydrates.

Digestible carbohydrates are the ones that can be degraded by human physiology, that is enzymes, acidity, and motility, and then taken up along the intestine. Overall, the physiological uptake is mainly restricted to monosaccharides liberated from oligo- and polysaccharides. To this end, the time-lapsed rise and drop in blood glucose levels after the consumption of a carbohydrate-containing food/drink is a widespread clinical measure used to monitor carbohydrate digestion and consumer physiological status. This gave rise to the definition of "Glycemic index" (Jenkins et al., 1981), a parameter defining the relative bioavailability of carbohydrates in a test food compared to an equivalent dose of pure glucose. In fact, this value is defined through Eq. (1)

$$\text{Glycemic index} = 100 * \frac{\text{AUC}_{\text{test meal containing 50g carbs}}}{\text{AUC}_{\text{reference food with 50g of glucose}}} \tag{1}$$

Where AUC is the respective area under the curve of the blood glucose levels measured for 1 h after the ingestion of the denoted oral dose. The expensive, time-consuming and ethically challenging nature of such experiments has fueled efforts to find viable alternatives to predict glycemic index values of foods or at least shed light into the gastrointestinal bioaccessibility of carbohydrates from foods. In fact, such studies have shown tight correlation between in vivo and in vitro findings (Bohn et al., 2017). Practically, most analyses monitor either the dissipation of the target high molecular weight carbohydrate (e.g., amylose and amylopectin) or the formation of the absorbable monosaccharides. Due to the nutritional importance of starch, numerous studies investigate starch digestion and stratify starch into readily digestible starch (RDS), slowly digested starch (SDS) and resistant starch (RS) using the Englyst method (Englyst et al., 2006; Englyst, Liu, & Englyst, 2007b). For example, a recent study applied this and other methods to investigate the digestibility of gluten-free pasta products (AlHasawi et al., 2017).

Others have monitored changes in molecular weight distribution of carrageenan (a polysaccharide food additive) during gastric digestion using size exclusion chromatography coupled with multi-angle light scattering detection (SEC-MALS) (Capron, Yvon, & Muller, 1996). Similarly, one may apply other analyses to analyze high MW species in digesta, namely thin layer chromatography (TLC) or

high-performance anion exchange chromatography (HPAEC). Additional and more in-depth analyses may even include 1H NMR, MALDI-TOF MS, and X-ray diffraction (e.g., SAXS) to shed light into fine architectures of oligosaccharides and polysaccharides (e.g., degree of polysaccharide branching, side moieties, and nanostructural properties) (Zabar, Lesmes, Katz, Shimoni, & Bianco-Peled, 2010).

Complementary to all these analyses, one can directly monitor the formation of monosaccharides or low MW carbohydrates via various chemical assays. Such methods mandate sample preparation procedures to remove lipids, proteins, starch and fibers from the digesta to minimize bias and analytical error. In practice, 60–80% ethanol solutions are excellent solvents for monosaccharides, oligosaccharides and amino acids but precipitate polysaccharides and fibers. Thus, concentrated ethanol solutions are often used prior to chemical analysis of the low MW carbohydrates in digesta. Once digesta samples have been carefully prepared for analysis, it is possible to determine carbohydrate concentration by a number of ways, which can be categorized into two main groups:

1. Quantification of glucose and other low MW carbohydrates—Colorimetric glucose determination in biological fluids is commonly measured via chemical or enzymatic degradation reactions that generate products with known light absorbance that can be quantified using a spectrophotometer, as reviewed for glucose determination in the blood (Buzanovskii, 2015) and for glucose sensors (Oliver, Toumazou, Cass, & Johnston, 2009). However, the field of glucose analysis in biological fluids is a rapidly changing field with novel technologies constantly emerging (e.g., TLC or microfluidics) (Belyaeva & Beklemishev, 2011; Hu, Lu, Fang, Duan, & Zhu, 2015). Determination of other monosaccharides (e.g., fructose and galactose) and low MW carbohydrates (e.g., lactose) is commonly performed using more specific techniques, such as HPAEC (Monti et al., 2017).
2. Determination via physical methods—molecular interactions, for example with iodine, and other physical methods rely on their being a change in some physicochemical characteristic of a food as its carbohydrate concentration varies. Commonly used methods include polarimetry, refractive index, density, and more advanced methods such as IR and NMR.

Indigestible carbohydrates are those that resist digestion and uptake in the upper GI and may then be fermented by the gut microbiome (e.g., resistant starch or inulin) or secreted in the feces (e.g., cellulose and sulfated galactans) (Macfarlane, Macfarlane, & Cummings, 2006; Michel & Macfarlane, 1996; Rastall, 2010; Van Loo et al., 1999). Out of the fermentable carbohydrates, 90% are converted into short chain fatty acids via bacterial biotransformations and/or cross-feeding between bacteria (Belenguer et al., 2006; Louis & Flint, 2009). These are commonly analyzed by GC or GC-MS analyses. Further, the health benefits associated with some short chain fatty acids (e.g., butyric acid) have given rise to the field of prebiotics and the mining of foods for prebiotic disaccharides, trisaccharides, oligosaccharides, and polysaccharides (Flamm, Glinsmann, Kritchevsky, Prosky, & Roberfroid, 2001; Gibson & Roberfroid, 1995; Rastall, 2010; Roberfroid, 2007; Torres, Gonçalves, Teixeira, & Rodrigues, 2010).

Proteins are an important macronutrient, due to nutritional and economic considerations. Thus, analysis of digestive proteolysis may interrogate the digestive dissipation of proteins or the formation of their breakdown products (i.e., peptides and amino acids (AAs)). Due to the low sample size of digesta, bulk methods like Kjeldahl or Dumas are scarcely used for quantification of proteins in digesta. Instead, reducing or non-reducing polyacrylamide gel electrophoresis (PAGE), which require very low sample quantities, are used to gain qualitative information on the breakdown of known proteins, that are identified either against isolates or by their mere size, against relevant MW ladders that are commercially available, as reported in various studies (Egger et al., 2015; Dupont et al., 2010; Kopf-Bolanz et al., 2012; Mandalari, Mackie, Rigby, Wickham, & Mills, 2009). To this end, it is important to ensure proper protease inactivation and proper identification of digestive proteins (e.g., pepsin and trypsin) to avoid erroneous band denotation. SDS-PAGE images can be further processed to provide quantitative information based on band color intensity using image analysis software, as done in the past (Dupont et al., 2010). SDS-PAGE gels can also be used as an isolation method where a selected band or bands can be cut out and subjected to subsequent analyses, such as MS identification. Additionally, direct and in-direct ELISA as well as immunoblotting has been used to monitor the dissipation of specific test proteins in digesta (Dupont et al., 2010). In-depth analyses of proteins, peptides, and amino acids present in digesta samples have been reported to be feasible using HPLC-based amino acid analyzers, NMR, and LC-MS methods. Such methods have been successfully applied to resolve and identify peptides and AAs in digesta of processed dairy, meat, fish, and even human breast milk (de Oliveira et al., 2016; Ferranti et al., 2014; Hernández-Ledesma, Quirós, Amigo, & Recio, 2007; Kopf-Bolanz et al., 2014; Marcolini et al., 2015; Vidal et al., 2016; Wada & Lönnerdal, 2015). The analyses may be targeted at a specific analyte (e.g., carnosine) (Marcolini et al., 2015), or generalized and untargeted proteomic analyses, such as peptide and AA profiling (Ferranti et al., 2014; Kopf-Bolanz et al., 2014; Moscovici et al., 2014). In respect to in vivo or human trials, studies commonly seek to determine officially approved determinants, such as PDCAAS (protein digestibility-corrected amino acid score) or DIAAS (digestible indispensable amino acids score). These scoring methods have a holistic approach to the nutritional quality of food proteins and, although analytical in nature, lack to provide in-depth or mechanistic information on digestive proteolysis.

Lipids are important for their high caloric value and various beneficial effects on health and eating behaviors (i.e., satiety and satiation); however, their diverse chemical compositions and physical states make their analysis in digesta a formidable challenge. In this respect, digestive lipolysis of emulsified lipids has been extensively studied (Marze & Choimet, 2012; McClements, 2010; Singh, Ye, & Horne, 2009; Wilde & Chu, 2011). Such studies commonly focus on the colloidal properties of the oil droplets or the general lipid breakdown into free fatty acids. The mild physiological contribution of gastric lipase to lipolysis as well as its low commercial accessibility account for the low numbers of studies interrogating gastric lipolysis. This has led to recent efforts to elucidate the role of gastric lipase and find viable

alternatives for lab use (Capolino et al., 2011; Sams, Paume, Giallo, & Carrière, 2016; Sassene et al., 2016). To date, intestinal lipase-induced release of free fatty acids from triglycerides is the most commonly studied reaction of digestive lipolysis. This is increasingly performed using an intestinal pH-stat method (Li, Hu, & McClements, 2011) in which FFA release kinetics and/or extent are measured by titrating the fatty acids liberated by pancreatic lipases against a known solution of sodium hydroxide. The most common method is based on maintaining a reaction vessel at a static/constant pH of 7.0. Percentage of free fatty acids (FFA) released during this pH stat lipolysis is determined through Eq. (2):

$$\%\mathrm{FFA} = 100 \times \left(\frac{V_{\mathrm{NaOH}} \times M_{\mathrm{NaOH}} \times \mathrm{MW}_{\mathrm{lipid}}}{2 \times W_{\mathrm{lipid}}} \right) \tag{2}$$

Where V_{NaOH} is the volume of NaOH required for neutralizing the released FFA (in mL), M_{NaOH} is the molarity of NaOH (mole/liter), $\mathrm{MW}_{\mathrm{lipid}}$ is the molecular weight of the oil, and W_{lipid} is the initial weight of oil in the reactor. Concomitantly, various in vivo studies have investigated digestive lipolysis, as reviewed recently (Bohn et al., 2017). However, this analysis should carefully consider the fact that experimental conditions should permit ionization of all the FFAs released. Otherwise the released FFA is not titratable and would generate an experimental error. Additional studies rely on the unique physical properties of oils to conduct spatiotemporal MRI experiments that shed light into the macroscopic and microscopic level of lipid breakdown. Other studies use medical devices (e.g., nasogastric tube) or medical patients (e.g., ileostomy patients) to gain access to bio-relevant digesta of lipid formulations and gain insights into the micro, nano, and molecular levels of lipid breakdown.

Analyses of Micronutrients and Other Bioactive Compounds

A recent review of the scientific literature (Bohn et al., 2017) has exposed some of the limitations of our understanding into the digestion of micronutrients (e.g., vitamin D) and other non-nutritive bioactive compounds (e.g., polyphenols and carotenoids) (Reboul et al., 2006; Schweiggert et al., 2014). These limitations do not stem only from analytical challenges of isolating and identifying the analytes but also from their intricate release from food matrices, absorption (i.e., uptake and cellular metabolism) and colonic fermentability. One such example is the application of HPLC to monitor the release and degradation of vitamin D from casein micelles during in vitro digestion of yogurt (Cohen et al., 2017). Others report the use of HPLC and/or NMR to track the bioactives such as genistein from soy or carnosine during digestion (Cohen, Schwartz, Peri, & Shimoni, 2011; Marcolini et al., 2015). Overall, the need for high sensitivity and specificity for such trace substances limits researchers to utilize advanced analytical methods, ranging from analysis by HPLC to high-tech tandem MS and NMR analyses.

4.2 Functional Analyses

Positioning consumer health and well-being at the heart of research, quantifying the functionality of luminal contents can be classified into two main domains: analysis of biochemical activity, such as antioxidants, and analyses of bioactivity, namely cell viability/proliferation, allergenicity, and toxicity. Antioxidant activity is a vivid field of biochemical research with numerous possible mechanisms of action and analytical methods to determine those (Huan, 2005; Moon & Shibamoto, 2009). In this respect, ferric reducing antioxidant power (FRAP), oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and metal chelation assays are the most commonly used methods. When seeking to quantify antioxidant power in digesta it is important to bear in mind the diversity of chemical methods that monitor electron donation capacity, proton absorption capacity, or metal chelation abilities. Also, it is important to address the hydrophobicity or hydrophilicity of the antioxidative analytes, as some methods may be more suitable for one or the other. For example, FRAP and DPPH methods have been selectively applied to monitor the antioxidant capacity of Maillard reaction products before and during digestion, respectively. Moreover, it is important to note that differential antioxidant partitioning in foods (Alamed, Chaiyasit, McClements, & Decker, 2009) and in digesta may pose a challenge in adequate collection of digesta samples and the subsequent analyses.

Although bioactivity of materials and colloids in digesta are of great importance to the analysis of digesta functionality, these are separate fields of research that are covered by others. In general, with regard to the bioactivity of luminal contents, analyses focus on two major aspects: immunogenicity and impact on gut functions. Digesta immunogenicity is mostly analyzed in the prospect of allergenicity, although, it may have other interactions with the immune system (e.g., impact on inflammation). Thus, the literature is strewn with a multitude of relevant immunoassays described in the medical literature, such as ELISA, cell culture tests up to skin prick tests. In general, it is common to believe that allergenic elicitors are liberated during digestion but there are also numerous allergenic proteins that are resistant to processing and digestive conditions. Since this immense field of research is outside the scope of this chapter, readers are advised to consult with some scientific publications (Apostolovic et al., 2016; Bogh & Madsen, 2016; Clare Mills, Sancho, Rigby, Jenkins, & Mackie, 2009; Gamez et al., 2015; Jiménez-Saiz, Benedé, Molina, & López-Expósito, 2015; Moreno, 2007; Rahaman, Vasiljevic, & Ramchandran, 2016; Verhoeckx et al., 2015). Epithelia responsiveness to digesta is another major aspect of bioactivity that is investigated. In this respect, Caco-2 monolayers and various cocultures are extensively used to study the impact of substances in digesta on epithelia integrity, cell viability, apoptosis, uptake/transport of specific substances (Vors et al., 2012), and even the migration of substances to the epithelia (Macierzanka et al., 2014). Overall, it is important to rationally apply adequate analyses to study the bioactivity of digesta, yet these are not within the reach of this chapter.

5 Physical Analyses of Digesta

The viscoelastic and colloidal properties of foods determine to a large extent the physical properties of the luminal contents. In turn, these may affect digestion by modulating various transport phenomena (e.g., transport of acid into solid foods during gastric digestion (Kong & Singh, 2011), nutrient diffusion to the gut lining (Tharakan, Norton, Fryer, & Bakalis, 2010), or the diffusion of pepsin into the digested food (Luo, Borst, Westphal, Boom, & Janssen, 2017)). In fact, food's physical properties may activate physical sensing modalities of the gut that are involved in gut–brain communication and neuroendocrine pathways involved in hunger, satiety, and satiation. Moreover, during the course of digestion food undergoes numerous structural changes, spatial reorganizations, and destabilization events. Such physicochemical phenomena are some of the underlying determinants of digestion rate and extent, as shown extensively for various food emulsions (Marze, 2015a; McClements, Decker, Park, & Weiss, 2008; McClements & Li, 2010; Singh et al., 2009; Singh & Ye, 2013). However, the dynamic nature of digestion sets a significant technical challenge to adequately sample and handle the digesta aliquots prior to analysis. For example, protein aggregation in the stomach may be disrupted when elevating the pH of digesta aliquots, a common procedure used for the inactivation of gastric pepsin activity. In general, the various physical analyses applied to digesta samples can be classified into analyses of structure (e.g., ingredient partitioning observed by confocal microscopy) and analyses of soft matter properties, such as texture, viscosity, and other rheological traits.

5.1 Analyses of Structure and Organization

Direct observations of digesta are not uncommon and may provide coarse information on the physical stability of the chyme and macroscopic structural attributes. For example, the pH profiles occurring during gastric digestion may induce the formation of dairy and whey protein cruds that can be noted by the naked eye. Such observations can be collected using digital cameras, as reported by various researchers (Cohen et al., 2017; Ye, Cui, Dalgleish, & Singh, 2016; Ye, Cui, Dalgleish, & Singh, 2017). However, this enables rather qualitative analysis that can be strengthened by monitoring sample transmission/absorption profiles over time or via optical microscopy analyses.

In respect to physical stability, monitoring the time and space resolved optical transmission of digesta filled into designated test tubes may give information that can be transcribed into quantifiable parameters of instability mechanisms, such as sedimentation rate, creaming index and instability index. To date, two key technologies have been applied: Turbiscan™ and LUMifuge/LUMisizer™. Both rely on monitoring the timed transmission/absorption extinction profiles throughout the test tubes under controlled conditions. Turbiscan enables measurements of a single

sample under natural gravitation and controlled conditions, therefore providing insight into the realistic conditions of the digesta. Contrary, LUM technology enables accelerated testing under centrifugation (up to 2000 g), controlled temperatures and higher throughput of up to 12 samples simultaneously. For example, LUM technology has been used to study the oral and gastric stability of emulsions stabilized by protein nanoparticles complexed with anionic polysaccharides (shimoni, Shani Levi, & Levi Tal, 2016). However, such analytical centrifugation enables comparative analysis between samples and has limited predictability to real conditions of natural gravitation.

Although laborious, microscopies offer the opportunity to gain both qualitative and quantitative information down to a resolution of 1000–500 nm. Qualitative analysis can provide microstructural and mechanistic insights (e.g., open versus closed flocs as well as differentiation between emulsion flocculation and coalescence). Qunatitative measurements can be obtained through either operator or computerized image analysis (e.g., sizing of flocs). The use of specific dyes may enable improved resolution of the inspected organization of the substances in the digesta. Thus, the use of Nile red to dye lipids and rhodamine B to dye proteins may facilitate tracking the spatial organization of these macronutrients in digesta samples. For example, confocal microscopy has been successfully used to track the microstructural organization and permeability of the intestinal mucus to probe latex beads (Macierzanka et al., 2014).

Improving the resolution of analyses to the micro- and nano-range is commonly pursued through various advanced analyses (Luykx et al., 2008). In respect to digesta, the most common methodologies are laser-based particle sizing and advanced microscopies. Particle sizing technologies are diverse but are generally used to monitor the size and/or electrophoretic mobility (presented as zeta potentials) of particles in the digesta. It is important to note the differences between static and dynamic laser based techniques that are suited to monitor particles in the 2000–0.4 μm and 6000–0.5 nm range, respectively. Such methods have been extensively used to monitor digestion-induced changes in the colloidal properties of various systems, such as liposomes (Liu et al., 2017; Liu, Ye, Liu, Liu, & Singh, 2013), emulsions (Marze & Choimet, 2012; Singh & Ye, 2013), starch supramolecular assemblies (Lesmes, Barchechath, & Shimoni, 2008), protein self-assemblies (Haham et al., 2012), nanofibrils/aggregates (Humblet-Hua, Scheltens, van der Linden, & Sagis, 2011; Moayedzadeh et al., 2015), and coacervates (Matalanis, Jones, & McClements, 2011; McClements & Li, 2010). Studies have linked nullification of droplet zeta-potentials to flocculation and coalescence of droplets due to nullification of electrostatic repulsion between droplets. For example, laser-scattering based droplet sizing and fluorescent microscopy of emulsions can be followed during in vitro gastrointestinal digestion (Lesmes & McClements, 2012; Marze & Choimet, 2012; Singh & Ye, 2013), as seen in Fig. 2.

Yet such analyses should carefully consider data processing and presentation; since means, medians and size distribution curves can be extracted from experimental data in different ways (e.g., mean size by volume ($d_{4,3}$) versus mean size by number ($d_{1,0}$)). Moreover, the limitations of each methodology should be kept in

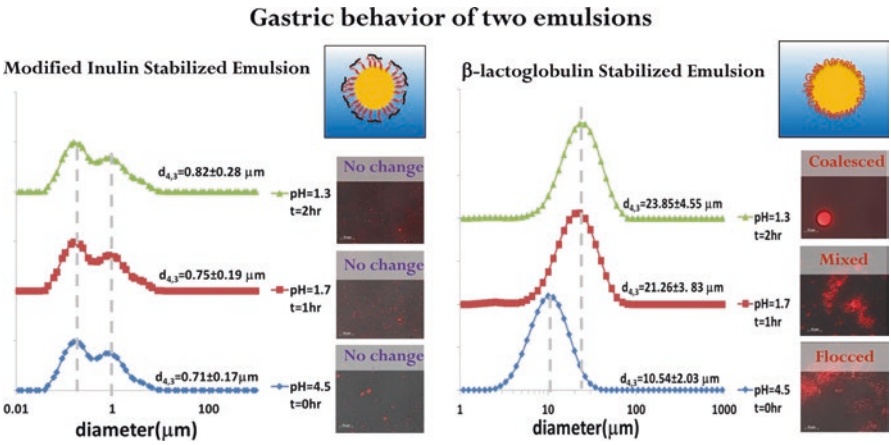

Fig. 2 Some physical characterizations of emulsions during simulated gastric digestion, adapted from Meshulam, Slavuter, and Lesmes (2014)

mind and verified by additional supporting analyses. For example, particles that can be measured by DLS are different than those detectable by SLS and such findings can be verified through various microscopic techniques.

Overcoming the resolution limits of light microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), and atomic force microscopy (AFM) have been applied to digesta samples. On one hand, such methods enable very high resolution, even below 1 nm, for example for the observation of micelles, fibrils, and nanoparticulates. For example, AFM enabled linking nanoparticle surface roughness measurements (in the 100–1000 pm range) to the rate of gastric proteolysis of protein–polysaccharide nanoparticles (David-Birman, Mackie, & Lesmes, 2013). Similarly, TEM can be used to monitor the structural changes in nanoparticles and liposomes during digestion (Liu et al., 2017, 2013). On the other hand, advanced microscopies are expensive, labor-intensive, require sample preparation (e.g., fixation and coating for TEM imaging), and mandate high professionalism to avoid artifacts and erroneous interpretations.

Additional methods that have been used to shed light on the digestibility of ingested formulations address interfacial and supramolecular properties. Changes in interfacial properties (e.g., surface tension) may be studied by drop shape analysis and/or dilatational rheology. One such analysis has enabled researchers to establish the role of galactolipids in modulating water–lipid interfacial tension that consequently delays digestive lipolysis (Chu et al., 2010, 2009). Small angle X-ray scattering (SAXS) and X-ray diffraction (XRD) have also been used to study the fine architectures of starch–lipid complexes or resistant starch that are linked to their intestinal breakdown or colonic fermentability, respectively (Lesmes, Beards, Gibson, Tuohy, & Shimoni, 2008; Zabar et al., 2009, 2010). In addition, there are reports that use circular dichroism (CD), ATR-FTIR and fluorescence correlation spectroscopy (FCS) to study the structure–digestion relationships for edible proteins (David-Birman et al., 2013; Joubran, Mackie, & Lesmes, 2013; Luo et al., 2017; Moscovici et al., 2014).

5.2 *Flow and Soft Matter Analyses*

Various studies have established that food structure and viscoelastic properties affect food's digestive fate. In this respect, when studying the digestion of food one can categorize foods into three classes: liquids, semisolids, and solids (Bornhorst et al., 2015). All classes exhibit distinct soft matter properties and flow behaviors (e.g., Newtonian and non-Newtonian liquids) as well as the possibility of harboring different physical states (e.g., the mixture of gas and liquid in whipped cream). To date, much of the research has focused on three main characteristics: texture, viscosity, and various rheological properties (e.g., gelation and storage modulus).

In the case of liquids and semisolids, viscosity during digestion is the most common fluid characteristic studied. Overall, five key rheological tests of digesta viscoelasticity can be applied: constant shear test, creep test, relaxation test, oscillation test, and ramping test. Reports on measurements of shear thinning behavior, apparent viscosity, or zero-shear viscosity that are related to digestion and glycemic responses can be found in recent literature (AlHasawi et al., 2017; Kong & Singh, 2011; Kozu et al., 2010; Logan, Wright, & Goff, 2015; Ruiz-Rodriguez, Meshulam, & Lesmes, 2014; Tharakan et al., 2010; Vingerhoeds, Silletti, de Groot, Schipper, & van Aken, 2009). For example, one study has shown that the shear thinning behavior of an emulsion stabilized by silica nanoparticles does not significantly alter emulsion intestinal lipolysis, as shown in Fig. 3.

Yet many of these studies do not monitor viscosity during digestion but after collection of digesta samples. This emphasizes the importance of sampling and inactivation protocols for the adequate representation of digestion and minimization of artifacts.

In respect to the digestion of solids, texture profile analysis (TPA) is the most common analysis applied. This analysis targets the oral phase of digestion with the main aim of understanding or modulating the sensorial perception of food during mastication. This is commonly determined by compression tests in which samples are compressed and the compression force or stress is recorded. Typically, samples

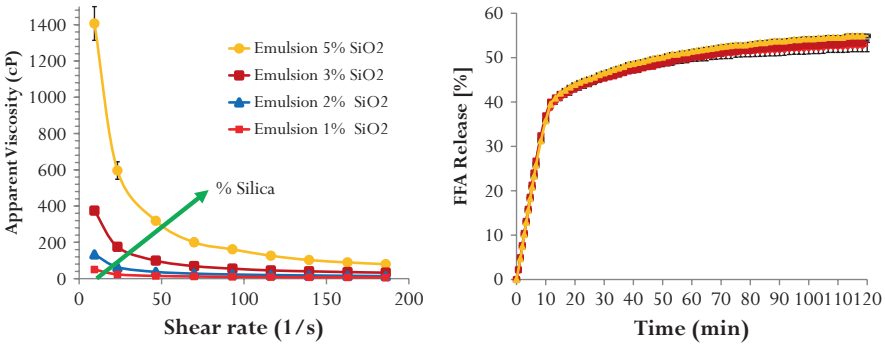

Fig. 3 Experimental findings demonstrating that differences in shear thinning behavior of an emulsion stabilized by silica (SiO_2) (on the left) may not affect emulsion intestinal lipolysis (on the right)

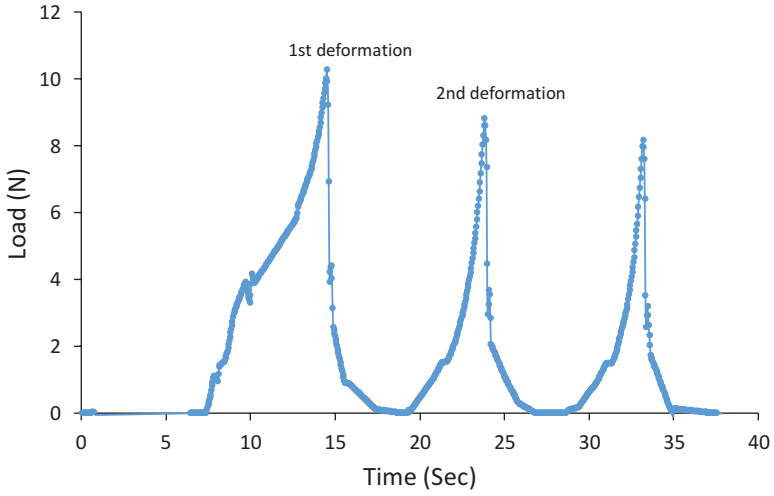

Fig. 4 Example of TPA measurements performed for whole wheat bread

are compressed for two consecutive times with an intermediate pause to generate a curve of the measured force (or stress) versus time (see Fig. 4).

In accordance, researchers can determine parameters such as hardness, cohesiveness, gumminess, springiness and chewiness. These are defined as follows:

Hardness
The peak force during the first deformation.

Cohesiveness
The area of work (area under the curve) during the second deformation divided by the area of work (area under the curve) during the first deformation.

Gumminess
The maceration energy needed before ingestion which is the multiplication of sample hardness times its cohesiveness.

Springiness
The time to reach 50% strain during the second deformation divided by the time to reach 50% strain during the first deformation.

Chewiness
Applicable to solid foods and defined as the multiplication of gumminess and springiness.

However, many foodstuffs fail under compression tests, and hence fracture force and other fracture characteristics are applied in their analysis. Obviously, solubility and insolubility in the physiological juices present obstacles in the adequate rheological characterization of digesta samples. Thus, practical measurements should also monitor the uptake of liquids by solid foods or "solid loss," as performed for

digested carrots (Kong & Singh, 2011). Yet scant reports can be found on the gelling, gel strength or Young's modulus of digesta. In this respect, it seems that our understanding of the flow and soft matter behaviors of foods during digestion is still evolving.

6 Conclusion

There are numerous challenges to secure a safe, sustainable, and efficient food sector. One of these challenges is generating the technological and scientific insights that will enable deliberate engineering of food's digestive fate. For this purpose, food, nutrition, and health professionals should systematically investigate and quantify the physicochemical aspects of digested materials. Such analyses should rely on clear hypotheses that are laid out under the notion that food should be nourishing, pleasurable, cost-effective, and as sustainable as possible. Careful attention should be paid to sample collection, handling and analysis to cope with the complexity of chemistries and structures found in digesta. In addition, a rigorous design of experiments should seek to apply more than one analyses in order to avoid method-associated bias and error.

This chapter scoured a wealth of physicochemical analyses that have been applied to digesta as well as some guiding principles that should be applied during method selection and application. Altogether, the physiological and spatiotemporal complexity of digestion maintain a need to further develop and refine relevant methodologies. In fact, adapting a holistic foodomics approach, coupling it with state-of-the-art analytics and computerization tools (e.g., bioinformatics and big-data handling) seem highly promising. Yet the future success of such endeavors requires enthusiastic, dedicated researchers and a considerable interdisciplinary work.

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An Engineering Perspective on Human Digestion

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1 Introduction

Studying digestive processes has become a major topic in the field of Food Engineering. However, while there have been significant advancements in studying nutrient reaction mechanisms in the body, the flow and mixing of food through the gastrointestinal (GI) tract is still relatively poorly understood. Adopting an engineering approach to characterise digestion can benefit from previous experience of characterising and quantifying industrial processes, many of which are analogous to digestive processes.

Food digestion is a multiscale process, as phenomena occur at a range of length scales (Bornhorst, Gouseti, Wickham, & Bakalis, 2016). For example, mastication and bolus formation happen on the centimetre length scale, while food breakdown and transport in chyme occurs on a millimetre length scale and the biochemical action of enzyme hydrolysis and mass transfer of nutrients to cells happen in the submicron to molecular level.

Current anatomical and biochemical descriptions of digestive processes include how the organs work as well as mechanisms explaining cellular and biomolecular

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functions. However, these descriptions are typically less quantitative and provide more descriptive information on the digestion process. The value of taking an engineering approach to digestion is that it may form a basis for a quantitative description of the digestion processes that can benefit from existing knowledge of industrial unit operations combined with physiology and biochemistry.

2 An Engineering Approach to Food Digestion

Food digestion is a multi-step process that strongly influences our health and well-being. It involves many interlinked biological and engineering phenomena including enzymatic reactions, fluid mechanics, diffusion, and solid deformation, acting over multiple length scales. As a result, understanding the behaviour of food during digestion is challenging and an active area of research. An engineering approach to digestion, where the physiological processes involved in digestion are related to analogous processes that occur in food and chemical process plants may provide a useful tool to quantitatively understand and describe digestive processes.

Chemical and biological engineering studies the conversion of raw materials to end products via a (series of) process(es), during which materials undergo chemical and/or physical changes. A wealth of knowledge and problem-solving approaches have been developed for describing and modelling complex, non-ideal systems. As food digestion can be viewed as one such complex system of chemical and biochemical reactions, multiphase fluid-flow and mass transfer, similar engineering approaches may be useful to apply to digestion processes.

2.1 Unit Operations

Unit operations represent a fundamental step in a process that includes one or more of either: fluid flow, heat/mass transfer, mechanical processes, and chemical reaction(s). A complex process may be split into one or more unit operations. By applying this approach to food digestion, it is possible to take advantage of the many well-characterised unit operations to help build an understanding and quantitative description of these physiological processes.

For example, applying this concept to digestion, the gastrointestinal tract may be viewed as the “system”, which in turn may be split into discrete “processes”, corresponding to the mouth, the stomach, the small intestine and the large intestine. The particular actions carried out by these “processes” may then be linked with corresponding unit operations (Table 1).

Unit operations are described by equations, which can be solved. Thus, a model of the whole-system (food digestion), can be pieced together from the unit operation component parts (Fig. 1). To set up equations describing a unit operation, parame-

Table 1 Summary of food digestion processes and their parallel unit operations from the food and chemical processing industries [from Bornhorst et al. (2016)]

Body part	Process	Unit operation(s)
Mouth	Mastication	Crushing, grinding
	Bolus formation	Mixing of solid and liquid (dough formation)
Oesophagus	Bolus transport	Peristaltic pumping
Stomach	Particle breakdown	Grinding, milling
	Mixing	Agitated tank/mixing vessel
	pH regulation	Feedback control system
	Acid/enzymatic hydrolysis	Bioreactor
	Gastric emptying	Sieving, size separation
Small intestine	Chyme transport	Peristaltic pumping
	Mixing	Agitated tank, mixing vessel
	Enzymatic hydrolysis	Bioreactor
	Absorption	Membrane filtration
Large intestine	Chyme transport	Peristaltic pumping
	Mixing	Agitated tank, mixing vessel
	Fermentation	Fermentation reactor
	Absorption	Membrane filtration

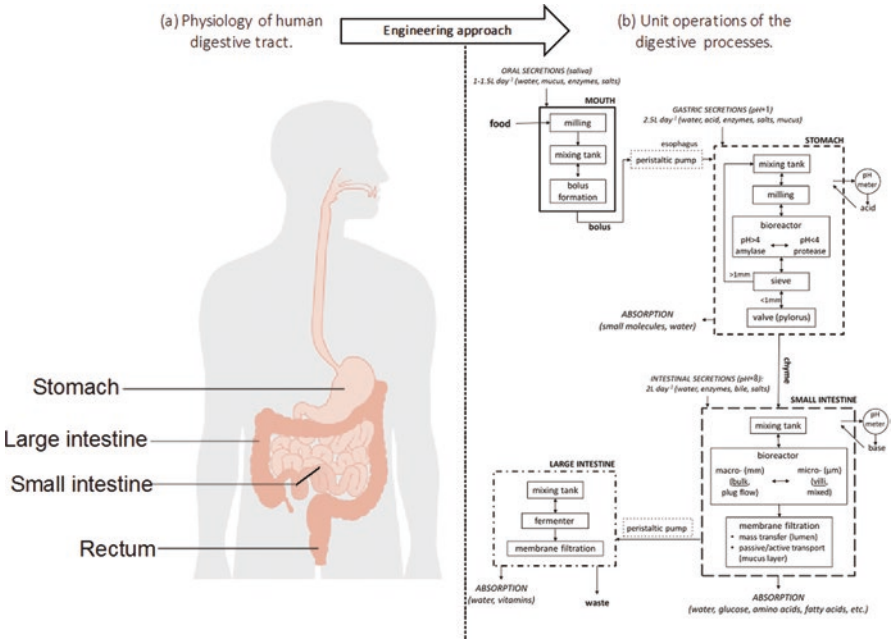

Fig. 1 Schematic of an engineering approach applied to the human digestive tract. (**a**) Shows the traditional physiological model of the digestive tract (adapted from Cancer Research UK). (**b**) Shows the engineering interpretation of the human digestive tract as processes made up of unit operations [adapted from Bornhorst et al. (2016)]

ters that describe the system are identified and related to one another using relevant criteria, such as mass and energy balances, physical equilibria, kinetics and properties of matter, as discussed in the following section.

2.2 Characteristic Engineering Parameters and Dimensional Analysis

Characteristic parameters can be used to describe the actions of a unit operation. These parameters can then be used to construct equations that quantitatively define the unit operation. Quantitative descriptions then allow one to predict the performance of a system for a given set of conditions.

A physical quantity can be described in terms of basic physical dimensions raised to a rational power. The SI standard recommends the use of the following dimensions; length (L), mass (M), time (T), absolute temperature (Θ), electric current (I), amount of substance (N), and luminous intensity (J). Hence, a quantity Q has dimension given by Eq. (1);

$$\dim Q = L^a \cdot M^b \cdot T^c \cdot \Theta^d \cdot I^e \cdot N^f \cdot J^g \tag{1}$$

where a, b, c, d, e, f, g are dimensional exponents. Dimension is a qualitative description of a system, but we also need a quantitative description, which requires the use of units. For example, while length (L), is the dimension that defines distance, units such as metre, kilometre, millimetre etc. are used to give value to the distance being described (Système international d'unités (SI), 1960).

Equation (1) indicates that parameters are used to construct equations that describe a unit operation. However, engineering problems can be too complex for these equations to be solved solely by theoretical, or mathematical rigour. These problems are prevalent in the areas of fluid mechanics, diffusion and kinetics, which are particularly relevant to digestion.

One method of tackling these problems is by empirical experimentation. This requires recording the result of experiments where each variable of interest is systematically varied while ensuring the others remain constant. The results may allow a useful equation to be derived that would otherwise not have been possible to obtain via theoretical deduction. However, this method is time consuming, it may require specialised test equipment, and outcomes may still be difficult to combine into a useful correlation.

A technique exists that bridges the gap between theoretical and empirical analysis, termed *dimensional analysis*. The basis of dimensional analysis derives from the fact that theoretical equations require dimensional consistency, which allows many factors to be gathered into a smaller set of dimensionless groups of parameters. These dimensionless groups can then be linked by empirical equations, which simplifies the task of fitting experimental data to design equations. Dimensional analysis is also useful for checking the consistency of units in equations, unit con-

version and scaling data obtained in test environments to full scale equipment. Commonly used dimensionless groups that are applicable for the analysis of digestive processes are summarised in Table 2.

2.3 Relating Digestion Processes to Chemical Processes

The digestion of food can be divided into four main 'process plants'; (1) mouth, (2) stomach, (3) small intestine and (4) large intestine. These four plants can then be further described by a set of unit operations that parallel the physiological mechanisms present within each process. The key variables and characteristic parameters of these unit operations may then be used to model and thereby help understand digestion.

The mouth begins the digestion process by breaking food down into smaller pieces. Chewing food serves several functions, namely particle breakdown, lubrication, mixing and bolus transport. Comparing digestion processes of the mouth with analogous industrial processes provides insight into potential important variables and parameters (Table 3).

Food breakdown through mastication is comparable to industrial size reduction operations, such as jaw crushers for breaking down large particles. Similarly, size reduction during mastication can be related to grinders like hammer mills and attrition mills for making smaller particles, as used in the food processing and mining industries, for example grinding coffee beans or milling ore (Geankoplis, 1993; McCabe et al., 2005). Mixing food particles with saliva, achieved by the combined action of the tongue and palette (Hutchings & Lillford, 1988; Shama, Parkinson, & Sherman, 1973), can be interpreted as equivalent to the action of industrial mixers for cohesive solids, such as paste mixers and kneading machines (McCabe et al., 2005). As the food particles are mixed with saliva, enzymes start acting on digestible matter, which converts complex biopolymers into simpler molecules like industrial fermenters (Almeida, Grégio, Machado, Lima, & Azevedo, 2008).

Input variables and characterisation parameters for these operations have been adapted with some success to mastication and bolus formation. For example, the Rosin–Rammler distribution function, which was originally used for describing cement (Rosin & Rammler, 1934), has been shown to provide a good description of particle size distribution of food, [e.g. Optosil (artificial test food), peanuts in a gel matrix and peanuts in chocolate], during mastication (Hutchings et al., 2011; Olthoff, Van Der Bilt, Bosman, & Kleizen, 1984). The degree of mixing in human mastication has been investigated using coloured chewing gum and has shown there are significant variations in the mixing ability of individuals (Liedberg & Öwall, 1995; Van Der Bilt, Mojet, Tekamp, & Abbink, 2010).

In the stomach, food boluses are mixed with gastric secretions, which include electrolytes, enzymes, mucus and HCl (Guyton & Hall, 2006). The stomach serves several roles; it mechanically breaks down food boluses by peristaltic contractions of the gastric wall; it breaks down food by chemical reactions, including acidic and

Table 2 Dimensionless groups (Catchpole & Fulford, 1966; Geankoplis, 1993; Green & Perry, 2008; McCabe, Smith, & Harriott, 2005)

Dimensionless no.	Equation		Explanation	Description
Reynolds	$\mathrm{Re} = \frac{\rho u L}{\mu}$	ρ = density (kg m^{-3}) u = velocity (m s^{-1}) L = length (m) μ = viscosity (Pa s^{-1})	$\mathrm{Re} = \frac{\text{inertial forces}}{\text{viscous forces}}$	Fluid mechanics, predict laminar to turbulent flow
Sherwood	$Sh = \frac{hL}{D}$	D = mass diffusivity (m^2 s^{-1}) h = mass transfer coefficient (m s^{-1}) L = length (m)	$Sh = \frac{\text{convective mass transfer rate}}{\text{diffusion rate}}$	Mass transfer
Schmidt	$Sc = \frac{\mu}{\rho D}$	D = mass diffusivity (m^2 s^{-1}) ρ = density (kg m^{-3}) μ = viscosity (Pa s^{-1})	$Sc = \frac{\text{momentum diffusivity}}{\text{mass diffusivity}}$	Fluid mechanics, simultaneous momentum and mass diffusion
Prandtl	$\mathrm{Pr} = \frac{c_p \mu}{k}$	c_p = specific heat capacity (J kg^{-1}K^{-1}) k = thermal conductivity (J m^{-1} K^{-1} s^{-1}) μ = viscosity (Pa s^{-1})	$\mathrm{Pr} = \frac{\text{viscous diffusion rate}}{\text{thermal diffusion rate}}$	Mass transfer
Peclet	$Pe = \frac{uL}{D}$	D = mass diffusivity (m^2 s^{-1}) u = velocity (m s^{-1}) L = length (m)	$Pe = \frac{\text{advective transport rate}}{\text{diffusive transport rate}}$	Transport phenomena
Damköhler	$Da = k_r C_o^{n-1} \tau$ $Da_{II} = \frac{k_r C_o^{n-1}}{k_g a}$	C_o = initial concentration (mol m^{-3}) k_r = reaction rate constant (mol^{1-n} m$^{3(1-n)}$ s^{-1}) k_g = global mass transport coefficient (m^{-2}s^{-1}) a = interfacial area (m^2) τ = mean residence time (s)	$Da = \frac{\text{reaction rate}}{\text{convective mass transport rate}}$ $Da_{II} = \frac{\text{reaction rate}}{\text{diffusive mass transport rate}}$	Reaction engineering, relate rate of chemical reaction to mass transport phenomena

Dean	$De = \frac{\rho \upsilon d}{\mu}\sqrt{\frac{d}{2R_c}}$	ρ = density (kg m^{-3}) υ = axial velocity (m s^{-1}) d = diameter (m) μ = viscosity (Pa s^{-1}) R_c = radius of curvature (m)	$De = \frac{\sqrt{\frac{1}{2}(\text{inertial forces})(\text{centripetal forces})}}{\text{viscous forces}}$	Fluid mechanics – fluid flow through curved pipes
Grashof	$Gr = \frac{g\rho^2\beta^*(C_{i,s} - C_{i,a})L^3}{\mu^2}$	ρ = density (kg m^{-3}) L = characteristic length (m) g = gravititational acceleration (m s^2) $C_{i,s}$ = concentration at surface (moles m^{-3}) $C_{i,a}$ = concentration in ambient (moles m^{-3}) μ = viscosity (Pa s^{-1}) β^* = density change with concentration (m^3 mole^{-1})	$Gr = \frac{\text{buoyancy term}}{\text{viscous term}}$	Quantify the effect of natural convection
Galilei	$Ga = \frac{g\rho^2 L^3}{\mu^2}$	ρ = density (kg m^{-3}) L = characteristic length (m) g = gravititational acceleration (m s^2) μ = viscosity (Pa s^{-1})	$Gr = \frac{\text{gravitational term}}{\text{viscous term}}$	Describe viscous flow

Table 3 Processes, related unit operations, key variables, and characteristic parameters for food digestion in the mouth and oesophagus [adapted from Bornhorst et al. (2016)]

Digestion process	Unit operation	Key variables	Characteristic parameters
Mastication (size reduction)	Crushing, grinding, milling	Degree of size reduction, particle sizes, mass flow rate of particles	Grinding rate function, breakage function, particle size distribution, power required
Mixing food with saliva	Paste mixer, kneader	Saliva viscosity, particle size, solid to liquid ratio	Degree of mixing, residence time, power required
Bolus transport	Peristaltic pump	Viscosity, bolus size, impeller shape and velocity	Reynolds number

Table 4 Digestion processes and their associated unit operations, including key variables and characteristic parameters in the stomach [adapted from Bornhorst et al. (2016)]

Digestion process	Unit operation	Key variables	Characteristic parameters
Mixing	Agitated tank, mixing vessel	Viscosity, particulates, impeller shape and velocity, residence time	Mixing power number, mixing index, concentration standard deviation
Acid/enzyme hydrolysis	Bioreactor	Enzyme concentration, degree of mixing, particle size, pH	Hydrolysis kinetics, fractional conversion (dimensionless), pH
Gastric emptying	Size separation	Particle size, sieve capacity, flow rate	Particle size distribution, efficiency, mass flow rate
Mechanical bolus breakdown	Crusher, grinder	Degree of size reduction, particle mechanical properties	Grinding rate function, breakage function, power required

enzymatic hydrolysis; and it controls the release of food via the pylorus. These functions can be paired with analogous industrial unit operations: mixer, bioreactor, and storage vessel (Table 4).

Gastric mixing is carried out with peristaltic contractions, while industrial mixers use impellers to mix fluid. However, despite these differences, the result of mixing in the gut is similar to what happens in an industrial mixing tank. Characteristic parameters for mixing are the pH distributions and rates of emptying and bolus breakdown. The gastric mixing process has been described using a mixing index for four different types of soft and stiff food particles (Bornhorst et al., 2014). The mixing indexes were calculated based on statistical variations in the standard deviation of marker concentrations at specified sampling locations in the stomach of pigs. It was concluded that the mechanical properties, as well as the gastric emptying rate, affect the degree of mixing (Bornhorst et al., 2014).

The extent of chemical and enzymatic reaction in the stomach is analogous to the operation of an industrial bioreactor. The residence time is a characteristic parameter used in describing reactor operation. Gastric emptying has been characterised by reporting emptying half-time, which is defined as the time it takes to empty half of

Table 5 Digestion processes and their associated unit operations, including key variables and characteristic parameters in the small intestine [adapted from Bornhorst et al. (2016)]

Digestion process	Unit operation	Key variables	Characteristic parameters
Chyme transport	Peristaltic pump	Velocity, viscosity, tube diameter	Reynolds number
Chyme mixing	Agitated tank, mixing vessel	Viscosity, particulates, impeller shape and velocity	Mixing power number, mixing index, concentration standard deviation
Enzymatic hydrolysis	Bioreactor	Enzyme concentration, degree of mixing, particle size	Hydrolysis kinetics, fractional conversion (dimensionless)
Water absorption	Membrane filtration	Pore size, concentration, flow rate, transmembrane pressure	Permeate flux, solute rejection, Sherwood number, permeability

the stomach contents. This parameter is, in effect, a measure of residence time for content in the stomach and has been measured in vivo using Scintagraphy, MRI and plasma analysis (Marciani et al., 2001; Marciani et al., 2013; Whitehead, Fell, Collett, Sharma, & Smith, 1998).

The small intestine receives chyme (the mixture of food particles, food liquid and gastric secretions) from the stomach via the pylorus and functions of the small intestine can also be paired to parallel industrial unit operations, such as bioreactors, membrane separators and peristaltic pumps (Table 5). The chyme is mixed with intestinal secretions and travels down the small intestine, driven by peristaltic contractions. The chyme mixes with pancreatic secretions in the duodenum, which increases pH to around 7 and the chyme is further broken down by pancreatic enzymes, such as lipase, phospholipase, α-amylase, trypsin and others (Barrett, 2014; Bornhorst & Singh, 2013).

The mixing and flow of chyme is related to industrial processes using mixing tanks and peristaltic pumping. Also, the enzymatic hydrolysis of chyme is similar to processes used in industrial bioreactors, such as fermenters (Riedlberger & Weuster-Botz, 2012). The majority of nutrient absorption occurs in the small intestine (roughly 80%), nutrients and water diffuse through the mucus wall to the lumen, from which they are transported through the cell membrane of the epithelium, by enterocyte cells, that pass nutrients to the blood stream (Barrett, 2014). This separation process is analogous to industrial membrane separation processes, such as reverse osmosis units. Mass transfer through the membrane is controlled by the fluid flow, as well as the chemical and physical interaction of the nutrients with the membrane (intestinal wall).

The large intestine, also known as the colon, or large bowel, starts at the end of the small intestine and ends at the rectum, and its functions can also be related to industrial unit operations (Table 6). Release of small intestinal chyme into the large intestine is controlled by the *ileocæcal sphincter*. Main functions of the large intestine include (re)absorbing water from the digestive chyme, fermentation, and

Table 6 Digestion processes and their associated unit operations, including key variables and characteristic parameters in the large intestine [adapted from Bornhorst et al. (2016)]

Digestion process	Unit operation	Key variables	Characteristic parameters
Chyme transport	Peristaltic pump	Velocity, viscosity, tube diameter	Reynolds number
Chyme mixing	Agitated tank, mixing vessel	Viscosity, particulates, impeller shape and velocity	Mixing power number, mixing index, concentration standard deviation
Fermentation	Bioreactor	Microbial population, degree of mixing	Fermentation kinetics
Water absorption	Membrane filtration	Pore size, concentration, flow rate, transmembrane pressure	Permeate flux, solute rejection, Sherwood number, permeability

formation of solid wastes from unabsorbed material that are excreted from the body (Barrett, 2014).

Like the small intestine, movement through the large intestine is controlled by peristaltic contractions, comparable to industrial peristaltic pumps. Absorption of water and some vitamins occurs in the large intestine, which are similar to industrial membrane separation units. The large intestine also breaks down remaining food by fermentation, carried out by microbial colonies, in a similar way to industrial anaerobic digesters and fermenters (Chen et al., 2012).

2.4 Concluding Remarks

The process of digestion is complex, operating over multiple length scales and involving many mechanical, chemical and biological phenomena (Bornhorst et al., 2016). Using an engineering approach, one can describe the gastrointestinal tract as a series of unit operations, which can provide the basis for a method of quantitative analysis. The advantage of this approach is that the knowledge and experience of characterising industrial unit operations that involve two-phase flow, absorption, and mixing can be transferred to the analysis of analogous digestion processes. This will allow a quantitative description of the digestion processes that can utilise the knowledge from physiology and biochemistry to refine the operation of analogous industrial unit operations.

3 Mixing and Flow in Digestion

Fluid flow and mixing are critical in determining the rate and extent of digestion. The interaction of liquids (drinks, GI secretions etc.) and solid food boluses influence and potentially dictate the breakdown and absorption of nutrients and calories

into our bodies. Hence it is important to attempt to quantify and describe the mechanisms of flow and mixing in the gut.

Typically, gastric emptying models are built around exponential decay-based equations to describe the flow of the stomach contents into the small intestine. To model nutrient absorption in the small intestine, the advection-diffusion-reaction partial differential equation has been used in the literature (Logan, Joern, & Wolesensky, 2002; Ni, Ho, Fox, Leuenberger, & Higuchi, 1980). While these methods can describe the overall process and provide good fits to data when parameter estimation techniques are used, they are unable to provide a detailed account of the interaction and mixing of multiphase chyme–food particle mixtures.

3.1 Computational Fluid Dynamics

Computational fluid dynamics (CFD) is a branch of fluid mechanics that uses numerical techniques to solve the Navier–Stokes and continuity equations for fluid flow in physical systems. The design space is set up to reflect the desired geometry, which allows the problem boundaries to be defined. Some examples include flows in pipes, turbines, combustion engines, reactors, blood vessels and intestines.

Software packages available for engineers couple Multiphysics packages with CFD packages and provide a computer aided design (CAD) interface that allows engineers to model more sophisticated, real-life physical systems. Two examples of these software tools are the ANSYS software package and the COMSOL Multiphysics tools (ANSYS; COMSOL Multiphysics Reference Manual).

Magnetic resonance imaging (MRI) is used to image the stomach and intestines of patients. These data can be used in the set-up of CFD model geometries to maximise the physiological relevance of the models.

3.2 Gastric Mixing

The stomach is split into three sections; the fundus, the corpus and the antrum. The fundus refers to the very top section of the stomach, the corpus refers to the central body of the stomach and the antrum refers to the bottom of the stomach just before the pyloric canal, where chyme empties into the small intestine. Gastric mixing defines how the food is distributed in the stomach, but also how the gastric juice is distributed within the stomach and the ingested meal. Food enters the stomach via the oesophagus, where it will initially come in contact with gastric secretions. These secretions are produced via various glands located in the gastric wall.

Mixing in the stomach is generated by the gastric wall contractions. The amount and properties (e.g. liquid-like, solid-like) of the food, the amount and properties (e.g. pH) of the gastric juice, and the location of the gastric juice producing glands combined with the contraction behaviour of the stomach will define how the food is

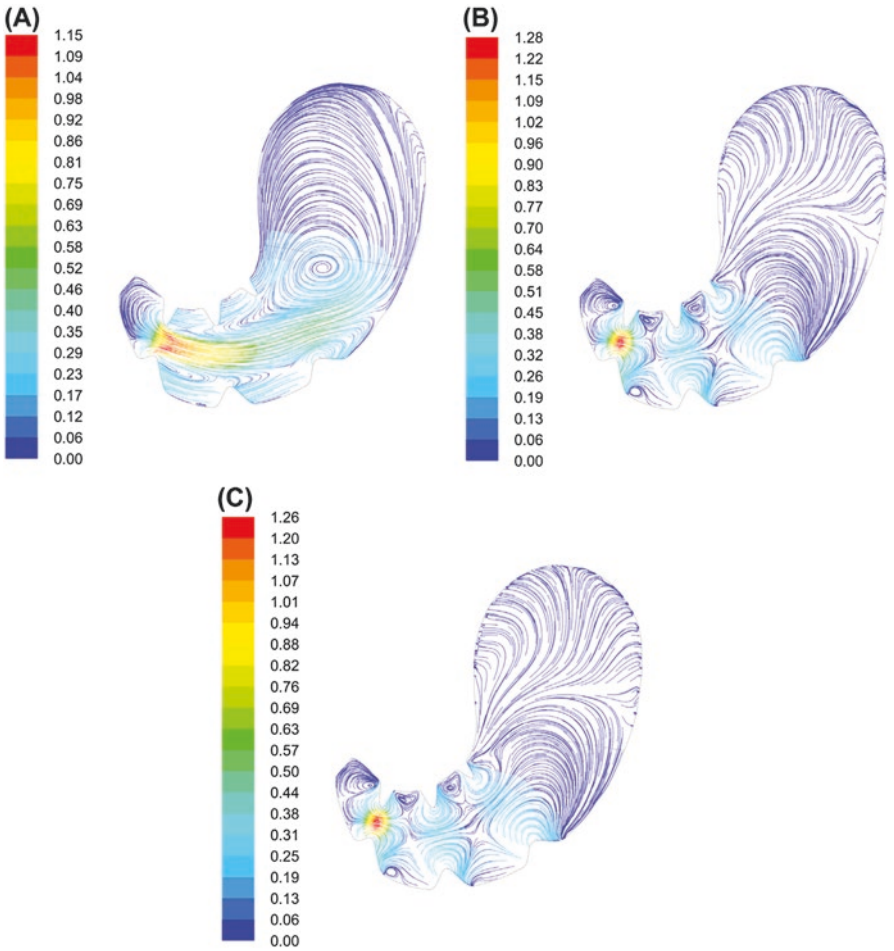

Fig. 2 Instantaneous streamlines at 57% luminal occlusion (15 s out of 20 s dynamic ACW dynamics), coloured by velocity magnitude (cm/s). (**a**) Water (Newtonian 1×10^{-3} Pa s). (**b**) Honey-like fluid (Newtonian 1 Pa s). (**c**) Tomato juice (0.02–0.17 Pa s) (from Ferrua, Xue, and Singh (2014)]

distributed in the stomach. A 3D CFD model has been used to study contractions of the stomach and characterise the fluid dynamics of gastric contents at different viscosities (Fig. 2). Results indicated that most mixing occurs in the antrum, while minimal mixing occurs in the fundus and corpus sections of the stomach (Ferrua, Kong, & Singh, 2011; Ferrua & Singh, 2010).

Gastric mixing further determines the dynamic pH changes in the stomach. The gastric pH profile is important because it determines enzyme activity, which relates to rate and extent of nutrient hydrolysis: α-amylase, present in the bolus from oral digestion, becomes inactive at pH below ≈3 while pepsin becomes active at low pH

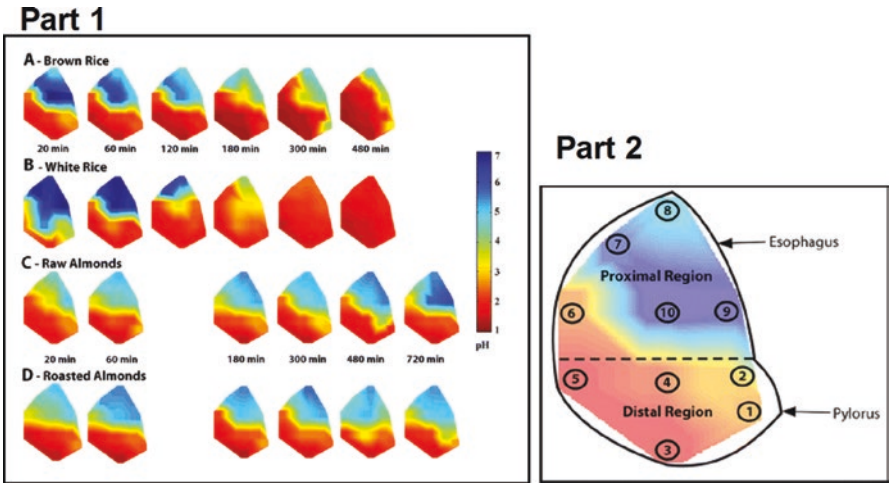

Fig. 3 Part 1—Colour maps of the gastric pH distribution in pigs, after ingesting (**a**) brown rice, (**b**) white rice, (**c**) raw almonds and (**d**) roasted almonds. Part 2—shows the 10 sampling regions in the stomach that were used to generate the plots in part 1. Used with permission from Bornhorst et al. (2014)

(of ≈1–4). Research on the digestion of rice and almonds in pigs nicely shows the pH distribution in the stomach for the different meals during an 8–12 h digestion period (Fig. 3). This study showed limited intragastric mixing at shorter digestion times, but as digesta viscosity decreased at longer digestion times, increased mixing was observed (Bornhorst et al., 2014). In addition to gastric secretions and mixing conditions, the gastric pH distribution is dependent on the composition and structure of the ingested food, especially the food buffering capacity.

Due to the large variations in pH, gastrointestinal enzymes may have reduced activity depending on their residence time in certain regions of the stomach. For example, pepsin is an aspartic protease that has an optimum activity at pH of ~2. The enzyme is not very active at pH above 3. Figure 3 indicates the locations where pepsin can be active, which is mostly in the antrum section of the stomach. Predictions on the activity of pepsin as function of pH can be made based on the acid–base equilibrium equation (Cornish-Bowden, 1995; Kondjoyan, Daudin, & Santé-Lhoutellier, 2015) (Fig. 4). This suggests that if a high-protein meal were consumed, only limited protein hydrolysis would occur in the meal while it was in the proximal region of the stomach at earlier digestion time points, due to limited pepsin activity.

The pH profile in the stomach is also dependent on the type of food that is ingested. Protein-rich foods have typically high buffering capacities. The buffering capacity of protein comes from the ionisable groups on the polypeptide chains, including the side chains of the amino acids, the terminal α-amino groups, and the terminal α-carboxyl groups. Titration experiments show that about 1.5 mmol HCl per gram protein is required to bring an ovalbumin solution from neutral pH to

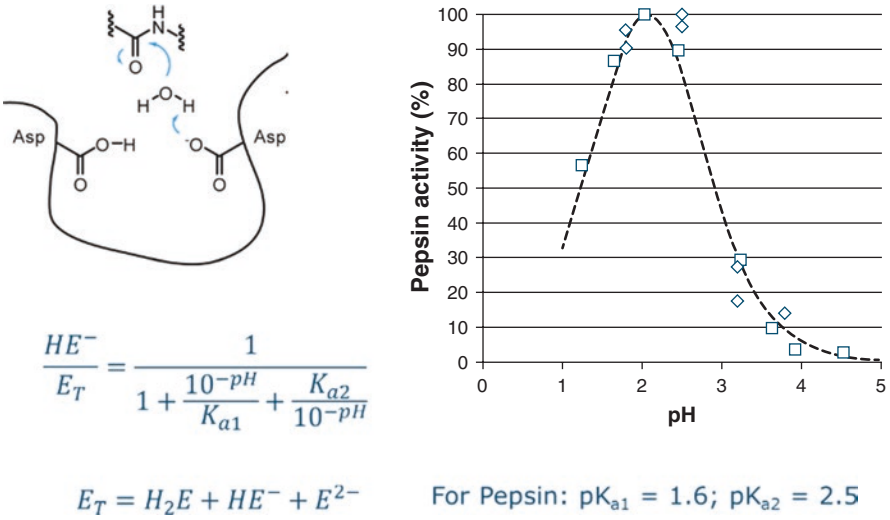

Fig. 4 Mechanism of pepsin and activity profile as function of pH [adapted from Kondjoyan et al. (2015) and Cornish-Bowden (1995)]

pH = 2 (Luo, 2018). Assuming gastric juice has pH = 1.8 (meaning 0.016 M HCl) it can be calculated that 95 ml gastric juice per gram protein is needed to lower the pH to 2. Although this is only an estimation, it shows that the protein-containing food has a large effect on the pH in the stomach and therewith affect the rate of their hydrolysis.

By developing a quantitative understanding of the processes in the stomach such as gastric juice production, enzyme kinetics, buffering capacity, and mixing behaviour, an overall model can be built that describes the breakdown of food in the stomach. Building such a model will help to increase our understanding, to make predictions on the digestion of food and to better design food products for specific groups of consumers.

3.3 Intestinal Mixing

Flow of chyme through the intestines is controlled by muscular contractions. Two main types of contractions have been observed. The first is called peristaltic contractions, whose main function is to move food through the intestine. This occurs through the sequential contraction of muscle rings around the intestine to push digesta along. The second type of contractions is referred to as segmental contractions, whose main purpose is to promote mixing and motility of digesta. These occur when singular muscle rings contract, breaking up the digesta and forcing both backwards and forwards movement facilitating mixing of digesta.

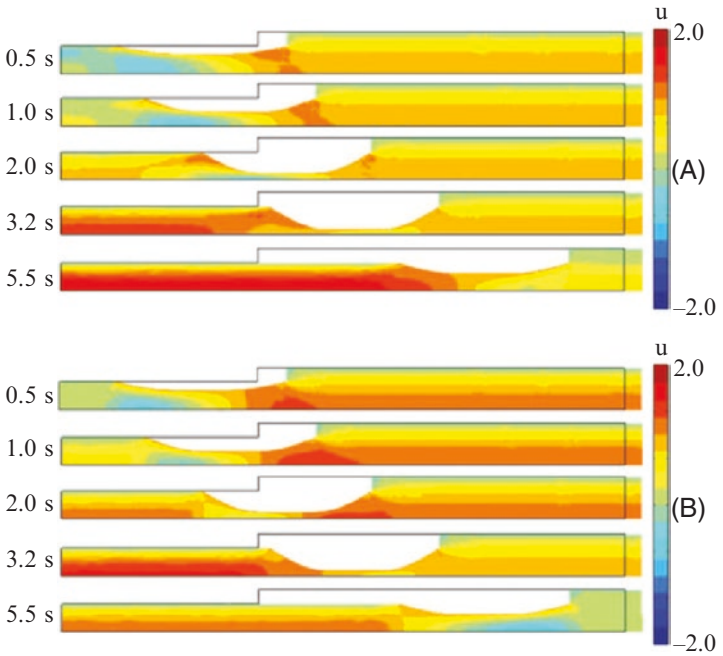

Fig. 5 Horizontal velocity profiles (centimetres per second) from simulations of flow of fluids of different Newtonian kinematic viscosities (υ) through a tube with an enlarged abroad profile (50% greater than orad profile); travelling in advance of the zone of coaptation; (**a**) $\upsilon = 10^{-6}$ m^2 s^{-1} and (**b**) $\upsilon = 10^{-3}$ m^2 s^{-2} [from Love et al. (2013)]

Peristaltic and segmentation contractions in the intestines have been simulated with an expanded finite element model for digesta of varying viscosities (Love, Lentle, Asvarujanon, Hemar, & Stafford, 2013) (Fig. 5). It was found that none of the simulations returned Reynolds numbers higher than 200, which indicates that conditions in the intestines will favour laminar rather than turbulent flow. Another implication of the model was that insoluble dietary fibre may physically inhibit the absorption of nutrients, because of its effect on the flow properties of the digesta. Increased fibre may increase the apparent viscosity, which reduces the vorticial flow around the lumen and promotes creep flow (Love et al., 2013).

In the intestines, both macro and micro mixing have been studied (De Loubens et al., 2014; Love et al., 2013; Wang et al., 2010). A micro-scale CFD model was used to predict the effect of apical villous crowding on fluid flow and mixing (Lentle et al., 2013) (Fig. 6).

The crowding of villous tips is affected by muscle contractions and the simulations concluded that the increase in villous tip density in muscle contraction folds and decrease in villous tip density on crests caused liquid digesta to be expelled from and draw into intervillous spaces, which significantly augmented the local mixing around the villi (Lentle et al., 2013) (Fig. 7). However, these actions did not significantly impact bulk luminal mixing.

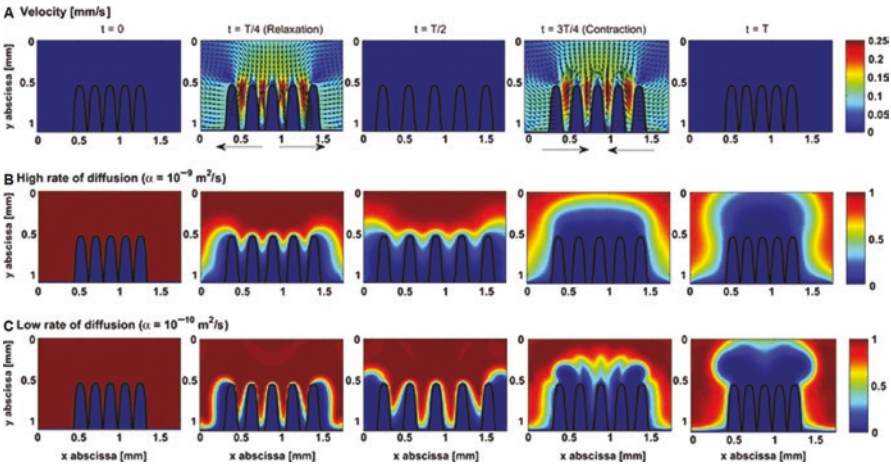

Fig. 6 Micro-scale simulation of the effect of apical villous crowding on velocity and mixing adjacent to the mucosa during a complete pendular contraction cycle. (**a**) Velocity plots show alternating flow between the intervillous spaces and the surrounding fluid. Concentration plots show that greater quantities of nutrient molecules of high diffusivity (**b**) are absorbed at the villous tips than are those of lower diffusivity (**c**) [from Lentle et al. (2013)]

These insights support the conclusions drawn by Wang et al., who postulated that the traditional role of villi to "simply" increase available surface area is probably incorrect, or at least too simplistic so as to trivialise the actual role played by villi. The 2D multiscale CFD model led Wang et al. to postulate that villi motion plays an important role in generating a "micro-mixing layer" (MML), which enhances the mixing and consequently the mass transfer between digesta and epithelium cells (Wang et al., 2010).

In another study, a 2D CFD study analysed macro-mixing in the duodenum of rats (De Loubens et al., 2014). They found the CFD model accurately simulated experimental results for central luminal mixing, however peripheral mixing was underestimated. It was concluded that multiscale strategies to incorporate phenomena at varying length scales are needed to accurately model mixing and mass transfer at the periphery of the lumen (De Loubens et al., 2014).

These studies imply that the mastication (i.e. bolus formation) as well as the mechanical properties of food will affect physiological processes, such as nutrient absorption. This opens the possibility of developing characterised foodstuffs that may be used to help manage weight by controlled calorie release. Another potential application could be to investigate abnormalities in a patient's gut operation by using foods with a fully characterised flow and breakdown profile for a "healthy" gut. Discrepancies in the flow and breakdown of food may help identify problems that would not otherwise be as readily diagnosed if one was to only measure physiological responses, such as nutrient absorption and hormonal responses.

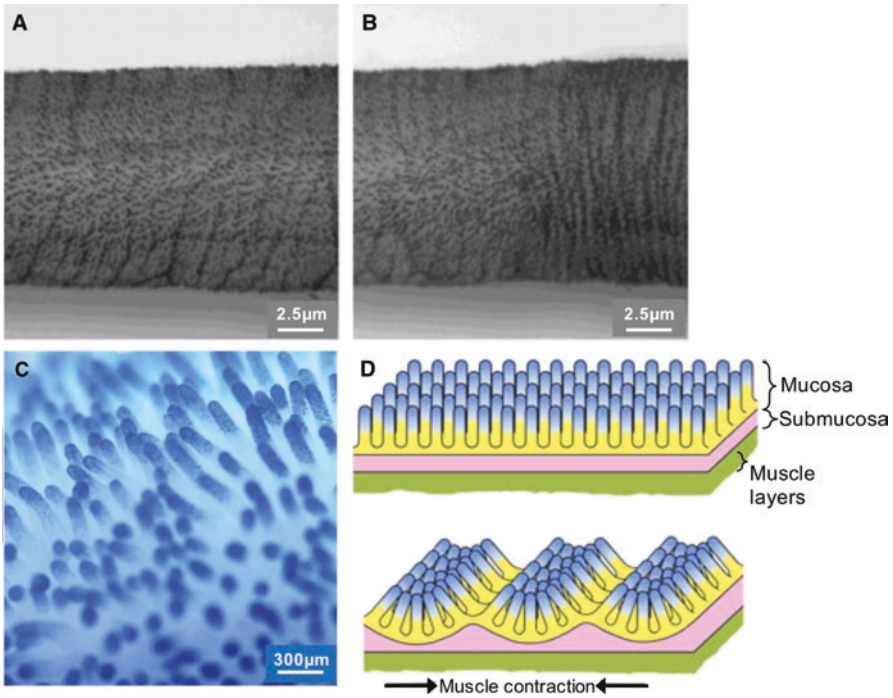

Fig. 7 Everted mucosal surface of ileum at rest (**a**), and during a longitudinal (**b**), and a simultaneous longitudinal and circular contraction. Staining of villi tips following application and elution of methylene blue (**c**). Radially orientated areas of apical crowding can be seen to develop between lighter areas on the crests of microfolds at the site (right) of longitudinal shortening (**b**). (**d**) Schematic showing development of microfolds and associated apical crowding [adapted from Lentle et al. (2013)]

4 Conclusions

Studying digestion from an engineering perspective offers the advantage of utilising a wealth of knowledge and experience of relevant industrial processes that can be used to quantitatively describe analogous processes involved in digestion. This engineering approach divides the GI tract into a sequence of unit operations that are described by characteristic parameters. Dimensionless groups can be used both to derive empirical relationships, as well as to define particular phenomenological behaviours (e.g. laminar to turbulent transition), which can then be applied to systems at different scales. Modelling the mixing and flow of digesta can also be tackled using CFD packages, coupled with design and multiphysics software.

The idea of studying digestion from an engineering perspective is still relatively novel (Bornhorst et al., 2016). Building a quantitative description of digestive processes through combining engineering unit operations for fluid flow, mixing and reactors, with the detailed catalogue of physiological processes and bio-molecular

mechanisms offers the potential for furthering our understanding of food consumption patterns and diseases, such as diabetes, cardio vascular disease and certain cancers. However, there are still many challenges in implementing models for the complex systems involved in the food digestion process.

This chapter has given an overview of an engineering approach to food digestion and highlighted the flow and mixing of digesta and chyme as a particular area where engineering can add quantitative description of digestive mechanisms in the future.

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Part IV
Case Study: Starch Digestion, Real-World Relevance

Starchy Foods: Human Nutrition and Public Health

Cathrina Hanse Edwards and Frederick James Warren

1 Starch in Human Foods, and Its Role in Human Nutrition

Starch is one of the most important components in the human diet, forming the main source of exogenous glucose, and therefore energy, in the modern western diet (Butterworth, Warren, & Ellis, 2011). A number of chronic diseases such as type II diabetes mellitus (T2DM), have been associated with high levels of starch consumption, particularly where the starch is readily digested and elicits a rapid rise in blood glucose concentrations (Liu et al., 2000). The human body is highly adapted to a starch rich diet, which we have consumed throughout our evolution (Hardy, Brand-Miller, Brown, Thomas, & Copeland, 2015). Humans have copy number variations in the genes encoding both the salivary (AMY1) and the pancreatic (AMY2) amylase, which lead to elevated levels of amylase proteins, and have been linked to high levels of starch in our diet during evolution (Carpenter et al., 2015; Hardy et al., 2015). There is emerging evidence that low levels of salivary amylase are linked with increased rates of obesity (Carpenter et al., 2015; Falchi et al., 2014; Mandel, des Gachons, Plank, Alarcon, & Breslin, 2010). In order to ensure that the starch rich diet we consume does not cause deleterious health problems, we need to understand how consuming starch can cause health problems, and what the key differences are between different starchy foods. This will allow for individuals to make more informed, healthier food choices, and also inform the design of novel healthy foods. Achieving these aims requires in vivo studies of human physiological responses to food in combination with in vitro modelling of digestive processes to develop a mechanistic understanding of the factors underlying differences in how foods are digested.

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Ingested starch is digested in a sequential process, starting in the oral cavity where the food is masticated, its particle size reduced, and it begins to mix with digestive fluids (Mandel et al., 2010). Salivary α-amylase begins the digestive process in the mouth, acting to cleave the α(1 → 4) linkages in the starch molecules to generate maltose and maltooligosaccharides (Bijttebier, Goesaert, & Delcour, 2008). Salivary amylase may continue its action in the stomach, where the bolus can act to effectively buffer the low pH of the gastric secretions, allowing the enzyme to continue its action (Bornhorst, 2017). Once in the small intestine, the starch is further digested by the action of pancreatic α-amylase to produce further maltose, maltooligosaccharides and also limit dextrins. These are then further digested by brush border enzymes (maltase–glucoamylase and sucrase–isomaltase), which are capable of hydrolysing maltose and α(1 → 6) linked glucans to glucose (Diaz-Sotomayor et al., 2013; Lee et al., 2016), which can then be absorbed across the epithelium and into the bloodstream by SGLT-1 and GLUT2 (Röder et al., 2014). Humans have a complex range of sensing and signalling systems which measure and respond to the amount of exogenous glucose that is absorbed in order to maintain blood glucose levels within the normal physiological range. Sweet taste receptors in the mouth and intestinal wall, in combination with SGLT-1 and GLUT2, act as a signalling mechanism for intake of glycaemic carbohydrate (Dyer, Salmon, Zibrik, & Shirazi-Beechey, 2005; Mace, Affleck, Patel, & Kellett, 2007; Nelson et al., 2001; Röder et al., 2014) Detection of glucose in the intestine triggers release of glucagon-like peptide 1 and gastric inhibitory polypeptide by the L cells and the K cells in the gut, respectively. This acts in concert with intracellular glucose sensing via glucokinase (Efeyan, Comb, & Sabatini, 2015) to detect glucose from both exogenous and endogenous sources, and activates the insulin–glucagon system (Aronoff, Berkowitz, Shreiner, & Want, 2004) that regulates glucose and glycogen metabolism in the liver to maintain blood glucose homeostasis whether in the fed or the starved state. T2DM results from a progressive dysregulation of glucose homeostasis, in particular reduced insulin sensitivity, which leads to a loss of control of blood glucose levels. The resultant hyperglycaemia can lead to both microvascular complications (such as retinopathy, nephropathy and neuropathy) and cardiovascular disease (DeFronzo et al., 2015). A number of factors have been implicated in the development of T2DM, including genetic predisposition as well as environmental factors such as a poor diet and obesity (DeFronzo et al., 2015). Thus, there is significant scope to improve public health by reducing the incidence of T2DM through altering diet. One of the most compelling routes is through the development of starchy foods with the property of slow digestion, but measuring and quantifying the digestion of starch in vitro in a manner that is meaningful and can be reliably related to in vivo measures of starch digestion has proven to be a major challenge. In this book chapter, we will first give an overview of the mechanism of starch digestion, and then take a look at the development of in vitro approaches to measuring starch digestion. Finally, we assess in vivo approaches to understanding differences in starch digestion, and how we can relate in vitro and in vivo findings.

2 Mechanism of Starch Digestion

Starch is digested in the human body in a stepwise manner starting with salivary and pancreatic α-amylases. The genes encoding these two enzymes are situated in the same gene cluster, and share a high degree of sequence similarity, arising from gene duplication events (Carpenter et al., 2015). These enzymes are part of the glycoside hydrolase (GH) family 13. They contain the characteristic $(\beta/\alpha)_8$ or TIM barrel domain of this GH family, where the active site is located (Henrissat, 1991). They have a nucleophilic attack mechanism, with active site carboxylic acid D197 believed to be the nucleophile (Rydberg et al., 2002), and possess a retaining mechanism, retaining the α configuration of the sugar residues in starch to produce maltose as the primary product (Van Der Maarel, Van Der Veen, Uitdehaag, Leemhuis, & Dijkhuizen, 2002). Mammalian α-amylase enzymes are unusual in comparison to their bacterial equivalents in that they lack the characteristic carbohydrate binding modules (CBMs) which typically form a domain of bacterial amylases, and result in the very high substrate affinity of these enzymes (Janeček, Svensson, & MacGregor, 2003). In amylases which lack CBMs, substrate binding primarily occurs through the active site, but recent research has uncovered the importance of surface sites on the enzyme distant from the active site. Secondary binding sites on pancreatic amylase were tentatively identified in the early crystal structures (Qian, Haser, & Payan, 1995), and through mutational analysis have subsequently been shown to be important in binding of pancreatic amylase to granular starch (Zhang et al., 2016). As an insoluble substrate, starch poses particular challenges to digestive enzymes, as it requires a soluble enzyme to bind to and degrade an insoluble surface (Warren, Royall, Gaisford, Butterworth, & Ellis, 2011). In its native form the crystalline structure of starch represents a challenge to the mammalian amylase to digest, and there is evidence that the presence of crystalline domains at the surface of the starch granule reduces the available surface area for amylase to adsorb onto in order to degrade starch (Warren, Butterworth, & Ellis, 2013). The majority of starch in the human diet is in the cooked form. Hydrothermal treatment of starch results in gelatinisation of the starch granule, and a dramatic reduction of crystallinity in the granule [a discussion of gelatinisation is beyond the scope of this chapter, and is reviewed in depth elsewhere (Cooke & Gidley, 1992; Waigh, Gidley, Komanshek, & Donald, 2000)]. This dramatically increases the mobility of glucan chains within the granule, and hence their availability for digestion by α-amylase (Baldwin et al., 2015). Thus, the structuring of the starch substrate is an important determinant of the rate and extent of digestion, depending on the availability of glucan chains for hydrolysis. This has important implications for starch digestion, as it implies that certain starch structures will be digested at different rates, and that links may be drawn between structure, digestibility and physiological responses (Edwards et al., 2015). The role of structure in digestibility has been incorporated into the concept of resistant starches (RS). That is, starches which are not digested within the time during which they are resident in the small intestine, and which reach the colon intact (Dhital, Warren, Butterworth, Ellis, & Gidley, 2017). Once in the colon, the

combination of the extended residence time and the multimodular amylases with high affinities for crystalline starch lead to the majority of starch being fermented to produce short chain fatty acids (SCFA) and other metabolites (Walker et al., 2011; Ze, Duncan, Louis, & Flint, 2012; Ze et al., 2015). The identification of RS as a nutritionally important component of foods led to one of the earliest attempts at classifying starch digestibility on the basis of structure, attempting to define RS on the basis of structures which limit digestibility in the small intestine (Englyst & Cummings, 1987; Englyst, Kingman, & Cummings, 1992; Ring, Gee, Whittam, Orford, & Johnson, 1988). Although widely adopted, this approach has recently come in for some criticism as it fails to fully take into account the kinetic aspects of starch digestion in the small intestine and does not take into account effects of starch structure on the microbiome (Dhital et al., 2017).

As discussed above, the digestion products from α-amylase activity in the small intestine are subsequently degraded by brush border enzymes. The two major enzymes involved are maltase–glucoamylase and sucrase–isomaltase. These are both double-headed membrane bound enzymes, with two active sites possessing differing enzyme activities. They are capable of cleaving maltose, α(1 → 6) linked glucans and a range of other sugars (Diaz-Sotomayor et al., 2013). Recently, some attempts have been made to incorporate some of these enzyme activities into starch digestion models (Diaz-Sotomayor et al., 2013; Lin, Hamaker, & Nichols, 2012), but the majority of starch digestion models either ignore the activity of these enzymes, or replace them with fungal amyloglucosidase (Warren, Zhang, Waltzer, Gidley, & Dhital, 2015), so for the purposes of this chapter we will focus on models of starch digestion which primarily use α-amylase as the main saccharolytic enzyme, on the assumption that it represents the rate-limiting step in starch digestion in the small intestine (Butterworth et al., 2011; Dhital et al., 2017).

3 In Vitro Model Based Approaches

An important step on the road to understanding the digestion of starchy foods in humans is the use of in vitro model approaches, coupled with detailed structural characterisation of the starchy foods, to understand the mechanistic basis of differences in digestion rate. A full understanding of the digestion behaviour of starchy foods requires a combination of analytical approaches to measure nutrient content of food, physical chemistry and imaging methods to understand how these nutrients are built up into the structure of the whole food matrix, and enzyme kinetic methods to determine digestion rates in vitro. All this information can then be combined to make predictions regarding the in vivo physiological response of different foods.

A wide range of different models of varying complexity have been used to model in vitro digestion of starch over the past 30–40 years (Dhital et al., 2017). These vary from simple biochemical models, which aim to uncover the underlying kinetics governing starch digestion (Butterworth, Warren, Grassby, Patel, & Ellis, 2012; Slaughter, Ellis, & Butterworth, 2001), to highly complex, computer controlled

dynamic models, which aim to accurately mimic the dynamic processes that occur during passage through the digestive tract (Ballance et al., 2013; Fässler et al., 2006; Minekus, Marteau, & Havenaar, 1995). The correct choice of model is an important factor in generating useful, informative data, and will depend on a number of factors. The complexity of the food matrix being investigated will have a bearing on the choice of model, both in terms of biochemical (e.g. Are multiple components such as protein, fat and starch present in the food that will require multiple digestive enzymes, or are purified starches being investigated?) and physical (e.g. Does the food have structures at the mm to cm scale which will be disrupted by mechanical forces, and will therefore require accurate modelling of shear forces?) properties. It is also important to consider the analytical methods and data treatment that will be used to interpret the results of the digestion model, and whether these are appropriate to the substrate and experimental question.

Starch digestion experiments have been carried out dating back to the 1940s in order to understand the basic mechanism of starch digestion, and differences between different amylolytic enzyme activities in the digestion tract (Sandstedt & UEDA, 1969; Schwimmer, 1945). Rate comparisons between different foods, in humans at least, were not routinely conducted due to the incorrect belief that the digestion of starch in the duodenum was completed so rapidly that the rate limiting step of starch digestion and product adsorption was the transport of sugars across the gut lumen (Gray, 1970).

With the realisation in the 1980s that different starchy foods were in fact digested at different rates, and this had important physiological implications (Crapo, Reaven, & Olefsky, 1977; Jenkins et al., 1981; Otto & Niklas, 1980), and that there was a fraction of starch which may be resistant to enzyme hydrolysis (Englyst, Wiggins, & Cummings, 1982), it became an important research question to quantify and classify the starch digestion rates of different foods in vitro. One of the first successful methods was the so-called "Englyst" method of classification (Englyst & Cummings, 1987; Englyst et al., 1992). Developed from methods of classifying dietary fibre, this method involves digesting starch under controlled conditions using a combination of porcine pancreatic α-amylase and a fungal amyloglucosidase to mimic the action of brush border enzymes. Carrying out a digestion of 120 min, the proportion of starch that is digested at 20 min (termed rapidly digestible starch, or RDS), between 20 and 120 min (termed slowly digestible starch, or SDS) and residual remaining starch (termed RS) is quantified (Fig. 1). The relative proportions of these three fractions in starchy foods can then be correlated to in vivo measurements such as glycaemic response and starch entering the large bowel (Englyst & Cummings, 1987). While it was an important advance for its time, the Englyst approach has led to some unfortunate errors to creep into the literature. Most notably is the tendency to classify RDS, SDS and RS as chemically or kinetically distinct fractions within starch, which are digested at different rates. It is important to note that this was not the intention of the original method as described by its authors (Englyst et al., 1992). This is illustrated by Fig. 1, which shows an example digestion curve for a native maize starch being digested under Englyst conditions, but with continuous monitoring of digestion products. What can clearly be seen is that,

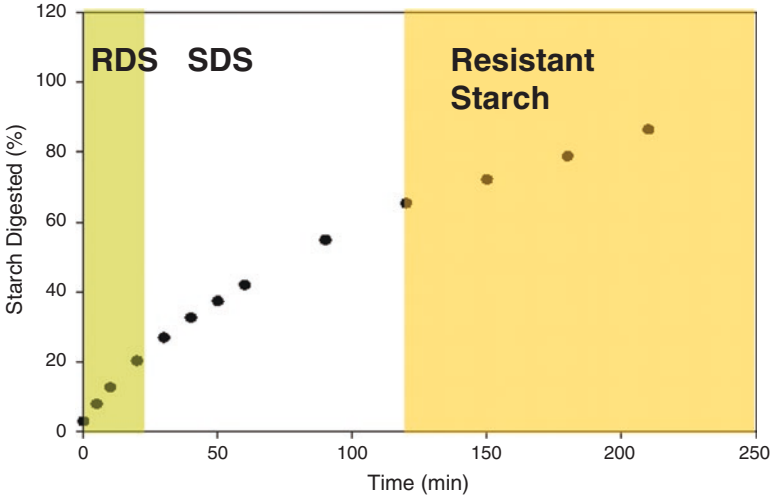

Fig. 1 A continuously monitored digestion curve carried out under Englyst conditions with Englyst fractions shown

as an enzyme catalysed reaction, starch digestion proceeds at a continuously decaying rate, as the substrate (starch) is exhausted and converted to product. This observation was formalised by Goñi and co-workers (Goñi, Garcia-Alonso, & Saura-Calixto, 1997), who devised a first order kinetic model of starch digestion. In common with many enzyme-catalysed reactions, starch digestion is a pseudo first order process (Slaughter et al., 2001), which can be adequately modelled by a first order equation. This has the significant advantage that it reduces the number of variables needed to describe a starch digestion curve from three (RDS, SDS and RS) in the Englyst classification to two [rate (k) and reaction endpoint (C_∞)]. Building on this work, classical Michaelis–Menten kinetics, and modifications of this model (Baldwin et al., 2015; Slaughter et al., 2001; Tahir, Ellis, Bogracheva, Meares-Taylor, & Butterworth, 2010; Warren et al., 2013) have been applied to the digestion of purified starches to unravel the structural factors of the starch granule that limit different aspects of their degradation by α-amylase, including chain mobility (Baldwin et al., 2015), enzyme binding (Warren et al., 2011) and surface crystallinity (Warren et al., 2013).

Michaelis–Menten kinetics, are only applicable to purified starches as substrates, so for whole foods a different kinetic approach is required. Building on the work of Goñi, the Logarithm of Slope method (Butterworth et al., 2012) has been introduced for the analysis of starch digestion kinetics. This method applies the same first order equation as the Goñi method, but instead of direct curve fitting, uses the logarithm of the first derivative of the digestion curve to calculate the kinetic parameters k and C_∞. This allows for a more accurate determination of C_∞, but has the added advantage that it permits identification of starch fractions within complex foods which are digested at different kinetic rates (Edwards, Warren, Milligan, Butterworth, & Ellis,

2014; Zou, Sissons, Gidley, Gilbert, & Warren, 2015). In this way, in complex foods containing different starch fractions which digest at different rates, genuinely slowly and rapidly digestible starch fractions can be identified, and quantified, which can then be used to improve in vivo predictions of starch digestibility, as well as uncovering structural heterogeneity in food starches (Edwards et al., 2015).

The choice of digestion model used will also depend on the complexity of the foods or materials being used in the study. The kinetics based methods discussed above involve only amylolytic enzymes (α-amylase and in some cases amyloglucosidase), and primarily aim to model duodenal digestion. The breakdown of foods through the gastrointestinal tract is a far more complex process than this and occurs in several steps, through oral processing with chewing and starch digestion; stomach with high and low shear mixing, acidification and protein digestion; small intestinal digestion of all major nutrients, adsorption processes and bile acid emulsification; and finally, colonic fermentation of undigested residues. The relevance of this complexity will depend on the research question being asked, and the material being studied. For a kinetic study of a purified starch a simple duodenal model with α-amylase as the only digestive enzyme will provide the required information. For a study which investigates the digestion of a complex food containing starch, non-starch polysaccharide, protein and lipid, which is structured on the micrometre to millimetre scale, many factors other than starch structure will influence the rate of glucose release as a result of the foods digestion. One of the first, and most ambitious, models to attempt to address this was the TIM model (Minekus et al., 1995), a computer controlled, compartmentalised model of the entire upper human GI tract which attempts to model the full range of mixing and biochemical regimes in the human GI tract, in order to make predictions of the bioaccessibility and bioavailability of diet components. Dynamic models are also available that mimic only specific compartments within the GI tract, for example the dynamic gastric model (Wickham, Faulks, Mann, & Mandalari, 2012), as well as models which include simulations of the colon, such as the widely used simulator of the human microbial ecosystem model (Van de Wiele, Van den Abbeele, Ossieur, Possemiers, & Marzorati, 2015). The variety of dynamic models available has been well reviewed elsewhere (Guerra et al., 2012), but they all have in common that they are very costly, complex machines which are only available in specialised labs, and they require complex statistical methods to analyse the output data due to the continuously changing biochemical and physical environments as foods pass through the models. While these models are essential for understanding the digestion of complex foods, the cost and complexity of running the models and interpreting the data has limited their use. The recently developed INFOGEST protocol (Minekus et al., 2014) aims to form a half-way house between these two approaches by introducing a standardised protocol for mimicking the biochemical environment of each of the digestive compartments, without the complex mixing regimes of dynamic models. This approach has shown some success in studies of protein digestion (Egger et al., 2017), but its usefulness for digestion studies in starch rich foods remains to be seen.

4 In Vivo Methods

The glycaemic and insulinaemic response to digestion of starch-rich foods is a subject of great interest with regard to studying the role of carbohydrates in human health. It is well-established that foods containing the same amount of starch can elicit vastly different glycaemic responses. The rate, extent and site at which starch-rich foods are digested in the gastrointestinal tract are major determinants of the duration and magnitude of the glycaemic response evoked. In general, starch-rich foods that are digested rapidly in the upper gut elicit rapid increases in blood glucose concentrations, while starch sources that are digested more slowly may provide a more gradual release of glucose into the blood, providing a gradual and sustained blood glucose response. According to this principle, in vitro methods for measurement of starch digestion in foods (Sect. 3) can provide some indication (particularly of relative differences) of glycaemic responses to food in vivo. The literature does contain examples of studies showing agreement between in vitro and in vivo measurements; however, the in vitro systems are limited to describing events in the intestinal lumen, often failing to represent the heterogeneity of digesta resulting from the diverse food structures in the human diet, and do not yet reliably account for physiological processes that are controlled by neuroendocrine signalling. Thus, for the purpose of studying the role of starch-rich foods in dietary management of health and disease, human studies are typically preferred.

5 Glycaemic Index

The glycaemic index (GI) is a well-known methodology which is used for the measurement and classification of starch rich foods according to their acute effects on glycaemia. This concept was first proposed by David Jenkins in the early 1980s, who proposed this as a system to help people with T2DM to select foods with a low GI (Jenkins et al., 1981) (*see later section on public health effects*). For determination of GI, the acute blood glucose response to a test food is measured as an area under the curve (AUC) and expressed as a percentage of the AUC of a reference food consumed by the same person on a different day under standard conditions. In brief, The Glycaemic Load (GL) is the product of the amount of available carbohydrate per serving and the GI of the food, divided by a 100, and is often listed to provide an indication of the glycaemic and insulinaemic impact of a realistic portion of food. Some foods contain very little carbohydrate and are unlikely to be eaten in quantities where they generate a significant increase in glycaemic and insulinaemic responses. The reference food is preferably glucose/dextrose (but some workers still use white bread) and is assigned a GI value of 100. Blood samples for determination of glucose concentrations are collected at regular time intervals (i.e. every 10–15 min) following the meal. For further details about the GI testing protocol, readers are strongly advised to consult the review by Brouns et al. (2008), which

discusses important methodological aspects in great detail and highlights recommendations for best practice (Brouns et al., 2008).

The GI of a broad range of carbohydrate foods has now been tested, and the results are available in published tables or databases (Atkinson, Foster-Powell, & Brand-Miller, 2008; Foster-Powell, Holt, & Brand-Miller, 2002) and from commercial entities providing a "GI-testing" service.

The usefulness and reliability of glycaemic index testing has been the subject of scrutiny, with concerns raised regarding interindividual variations in blood glucose levels, failure to consider the insulinaemic response, applicability to mixed meals, and lack of consideration for nutritional quality of the food product (Venn & Green, 2007). It is important to be aware of potential limitations, some of which may be overcome by ensuring compliance with a standardised and appropriately powered test protocol. Assuming that appropriate procedures are followed, the greatest sources of error/misinterpretation from GI values from reputed laboratories are likely to be improper determination of total carbohydrate content of test foods and the lack of detail about how test meals are prepared. Although the GI of a food is a function of the availability of starch-digestion products in the intestinal lumen, there are a number of other food properties (e.g. structure, composition, bioactives) that can be responsible for the observed health effects (Venn & Green, 2007). Thus, mechanistic understanding of such food properties is encouraged.

6 Chronic Effects and Public Health

Although the Glycaemic Index of food is an acute measurement, it can be linked to longer term health outcomes. Immediately after a meal, the low glycaemic potency of low GI foods stimulates less insulin secretion and a smaller incretin response than a high GI food. A diet rich in high GI foods is associated with higher insulin levels and seems to trigger overstimulation of insulin secretion and pancreatic β-cell dysfunction, leading to reductions in insulin sensitivity and impaired glucose tolerance which can contribute to the development of T2DM (Jenkins et al., 2002; Thondre & Henry, 2011; Wolever et al., 1992). Diets rich in low-glycaemic carbohydrate, on the other hand, have been shown to improve T2DM status, offering improvements in HbA1C and glycated proteins in people with T2DM. A recent meta-analysis concluded that low GI dietary advice delivers clinically useful improvements in glycaemic control in patients with T2DM, at a level that is comparable to other pharmacological interventions (Brand-Miller, Hayne, Petocz, & Colagiuri, 2003). Diabetes is projected to become the seventh leading cause of death by 2030. In 2014, 8.5% of adults had diabetes (predominantly type 2), and prevalence is rising rapidly, particularly in middle- and low-income countries (Mathers & Loncar, 2006). Given the alarming increase in T2DM prevalence worldwide, dietary prevention and management of this disease is an important strategy to protect public health, particularly in countries where access to medical care is

limited. Thus, there is a need to improve understanding of how diet can be used to counteract this disease.

A diet rich in low GI foods has also been suggested to be of benefit to cardiovascular health and weight management. A systematic review found an association between high GI and coronary heart disease outcomes (Mente, de Koning, Shannon, & Anand, 2009), and it seems that a low GI diet may reduce cardiovascular risk factors such as total and LDL cholesterol (Goff, Cowland, Hooper, & Frost, 2013), though the exact mechanisms are unclear. It has also been proposed that low GI diets may be beneficial in the prevention/management of obesity and overweight. This notion is based on the expectation that low GI foods being digested more slowly provide prolonged stimulation of nutrient-sensing receptors in the gastrointestinal tract, thereby stimulating sustained satiety signalling (Murphy & Bloom, 2006). Indeed, a systematic review (Bornet, Jardy-Gennetier, Jacquet, & Stowell, 2007) revealed that a number of short-term studies have demonstrated an increase in satiety (typically measured after 2–6 h) with low GI but not high GI foods or meals. An increase in satiety may contribute to reduced energy intakes, which, if sustained over a longer time period, could support weight loss. A number of chronic studies have examined effects of glycaemic index on energy intakes [these are reviewed in (Bornet et al., 2007)]; however, the results are variable, and it has not yet been convincingly demonstrated that the satietogenic effects of low GI meals translate to effects on weight loss in the longer term.

One challenge with considering the effect of high or low GI diets on health outcomes is that there are a number of factors that influence the glycaemic response to food. In particular, the low glycaemic interventions often contain more dietary fibre and less available energy, which can influence gastrointestinal transit, satiety and nutrient bioaccessibility. Thus, it is important to recognise that the health benefits associated with a low GI diet may not necessarily be directly attributed to the carbohydrate component. Nevertheless, following a diet rich in low GI sources of carbohydrate (particularly minimally processed cereals and pulses) is unlikely to be detrimental to the health of a general consumer, and therefore, governing bodies advocate the intake of starch from foods that are digested more slowly, have a low glycaemic response and offer gradual energy release (FAO, 1998).

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Kinetics of α-Amylase Action on Starch

Peter J. Butterworth and Peter R. Ellis

1 Introduction

Starch is a major source of carbohydrate in human diets and its over-consumption can contribute to development of obesity. Increased incidence of obesity is a worldwide phenomenon and raises the risk of cardiovascular disease and metabolic conditions such as type 2 diabetes (Brand-Miller, Holt, Pawlak, & McMillan, 2002). Better understanding of the reasons for observed differences in the rate and extent to which various starch-rich foods are digested by humans is a necessary step in rational development of functional foods that are digested relatively slowly. Enzyme kinetic studies can be a useful tool in working towards an understanding of how starch containing foods are digested in vivo and can be a sensitive index of how starch structure affects digestibility properties and how ease of digestibility responds to hydrothermal processing. Since starch is consumed in foods such as bread, pasta, rice and potatoes that have usually been hydrothermally processed by commercial and/or domestic cooking, the changes in amylase digestion kinetic properties accompanying starch processing can be monitored in attempts of providing predictive information about the digestive behaviour of the starchy food in vivo.

The earliest information about how starch can be digested seems to stem from work in the1830s in which it was discovered that human saliva and barley extracts contained agents, which were named ptyalin and diastase respectively, that could catalyse the hydrolysis of the polysaccharide (Butterworth, Warren, & Ellis, 2011). Enzymes that can digest starch are widely distributed throughout animal, plant and microbiological kingdoms but one of the most widely studied seems to be

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α-amylase, particularly the salivary and pancreatic α-amylases of humans and other mammals which digest starch (Henrissat & Davies, 2000; McGregor, Janecek, & Svensson, 2011).

The intense blue colour of starch–iodine complexes disappears as the starch is hydrolysed and this property provided a relatively straightforward way of assaying α-amylase activity (Smith & Roe, 1949). In the early studies, the loss of colour with time of incubation was linear for at least 1 h under the assay conditions used. By comparing the rate of decolorisation of blue starch with that of the appearance of reducing power emanating from liberated maltose equivalents, it was established that α-amylase is an endoenzyme with multiple points of attack on α 1–4-linked glycan chains (Kung, Hanrahan, & Caldwell, 1953; Robyt & French, 1967). These studies also revealed noticeable differences in the rates at which starch substrates from different sources were digested by amylases (Kung et al., 1953; Robyt & French, 1967). That raw granular starch was digested much more slowly than so-called soluble starch (formed by the Lintner process which involves acid treatment) puzzled early workers. The results of a systematic study (Walker & Hope, 1963) published in 1963 established that the same α-amylase was active on both soluble and granular starch. Knowledge of starch granule structure was very limited at the time and so molecular explanations for the slow rate of granule hydrolysis were not forthcoming but the 1963 paper did report that α-amylase binds to starch granules, however (Robyt & French, 1967). The article also included examples of digestibility curves. This appears to be the first published example of digestibility curves presenting data derived from measured product release over 100 h of incubation with amylase. Digestibility curves are now ubiquitous of course, and are seen frequently throughout published literature dealing with starch digestion and nutrition. The early studies were mainly directed towards elucidation of the catalytic and mechanistic properties of α-amylases, that is, they were concerned with the enzyme properties of amylases, whereas much of the current interest is concerned with relationships between starch macromolecular structure and its susceptibility to amylolysis (Butterworth et al., 2011; Dhital, Warren, Butterworth, Ellis, & Gidley, 2017).

2 Methods for Determination of Digestion Rates of Starch Catalysed by α-Amylase

The literature contains numerous examples of methods that have been used for assaying amylase activity. The decolouration of the blue iodine complex has already been mentioned (Robyt & French, 1967; Smith & Roe, 1949). The complex absorbs at a wavelength of ~620 nm and the fall in absorbance as starch is hydrolysed may be followed in a spectrophotometer. The use of this method appears to be quite rare now perhaps because assay mixtures containing starch solutions can be opalescent to various degrees and are therefore less than ideal for spectrophotometry.

p-Nitrophenyl glycosides are available commercially for use as artificial substrates, but they tend to be expensive and have poor affinity (i.e. they have high K_m values) for α-amylase unless the polyglucan chain contains at least five glucose residues. The active site of mammalian α-amylases accommodates at least five glucose residues and the accumulated free energy of binding resulting from occupancy of all the sites is important for effective catalysis (Butterworth et al., 2011; Seigner, Proganov, & Marchis-Mouren, 1987). Any *p*-nitrophenylglucose residue released by amylase action is then hydrolysed by α-glucosidase added to the reaction mixture and the liberated *p*-nitrophenol is determined in a spectrophotometer at ~400 nm (McCroskey, Chang, David, & Winn, 1982). The method is suitable for monitoring amylase activity but cannot be used for direct measurements of starch amylolysis, that is, the initial release of maltose and other products prior to α-glucosidase action. If an experimenter wishes to monitor amylase activity directly, however, a commercially available assay kit can be recommended as an improvement on the *p*-nitrophenyl glucoside method. Enzchek(R) produces a substrate consisting of modified maize starch that is extensively labelled with a fluorescent group. The high degree of labelling results in quenching of the fluorescence until amylase action releases fragments that are highly fluorescent and readily detectable in a plate reader. The rate of increase of fluorescence during the initial stages of digestion is directly proportional to the concentration of amylase in the sample. The method is extremely sensitive, relatively easy to perform, suitable for microplate adaptations and is both reliable and cost-effective.

The most widely used assay methods rely on either determining the rate of release of maltose by measurement of its reducing power or after its conversion to glucose by addition of maltoglucosidase. A very popular colorimetric method for measuring reducing sugar and which is very easy to perform employs dinitrosalicylate reagent. The method lacks sensitivity however, and is therefore not ideally suited for monitoring the earliest stages of a digestion reaction where the concentration of product reducing equivalents will be very low. Measurements of initial rates of reaction are important if the investigation involves studies of Michaelis–Menten kinetics. Prussian blue methods (Slaughter, Ellis, & Butterworth, 2001) and reaction with *p*-hydroxybenzoic acid hydrazide (*p*HBAH) (Moretti & Torson, 2008) are very sensitive and readily adaptable for rapid throughput using 96-well microplates. If glucose is measured by a glucose oxidase method subsequent to conversion of the products of amylolysis by maltoglucosidase, it is recommended that the glucosidase is added after cessation of the amylase step because it has been shown that when the two enzymes are both present in a reaction mixture, activation of amylase can occur and initial rates of amylolysis can therefore be overestimated (Warren, Zhang, Waltzer, Gidley, & Dhital, 2015).

Many authors have tried to imitate the in vivo stage of passage through the mouth and stomach taken by food material before exposure to pancreatic amylase in the small intestine, by including a preliminary incubation of the starch or food in simulated saliva. Amylase, often in the form of pancreatin, is added to the artificial saliva. Pancreatin contains protease and lipase activities in addition to amylase. Protein and lipid bound to starch are known to slow rates of amylolysis and so it is believed that

removal of these substances at a preliminary stage means that subsequent measured rates of amylolysis will approximate intestinal digestion of starch (Htoon et al., 2009).

Pure preparations of porcine pancreatic α-amylase are available commercially and the use of a pure source has the advantage that exact concentrations of enzyme protein concentration and catalytic activity can be selected for experimental work.

3 Kinetic Analysis of Digestion of Starch Catalysed by α-Amylase

3.1 Digestibility Curves

The published literature contains reports of a very large number of studies in which starches, both in native granular and in hydrothermally processed (gelatinised) forms from a variety of botanical sources and starch-containing foods, were incubated with α-amylase. The amount of product released at various time points up to several hours was determined and the results plotted as "progress", that is, digestibility curves of concentration of product against time. Such experiments were, and continue to be, designed to give information in vitro of the rate and extent of starch digestion occurring in vivo. In vivo experiments are difficult and costly to perform and therefore predictive data obtained from laboratory digestibility experiments is deemed to be valuable. Obviously, starch digestion during transit from the mouth and through the small intestine is much more complex than in vitro digestibility simulations because of the added physiological actions coming into play including gastric emptying, peristalsis and intestinal transit for example, as well as effects of hormone release and digesta viscosity. Nevertheless, it seems generally accepted that in vitro studies make valuable contributions to the understanding of the intestinal digestion of starch.

Many authors continue to analyse digestibility curves in terms of a scheme introduced by Englyst, Kingman and Cummings (1992). The curves are divided into phases of rapidly digested starch (RDS) and slowly digested starch (SDS). The fraction of starch digested in the first 20 min is denoted as RDS and SDS is classed as the fraction that is digested in the later periods of the digestion up to 2 h. The continued popularity of this method is unfortunate given that it was shown in 1997 that digestibility curves obtained for gelatinised starch can be fitted by equations for pseudo-first order kinetics (Goni, Garcia-Alonso, & Saura-Calixto, 1997). A first order reaction means that all fractions of the starch have the same intrinsic reactivity but the rate of digestion is directly related to the concentration of the polysaccharide. The rate of reaction decreases exponentially as the substrate is digested. Starch granules and starchy foods can possess fractions that differ in digestibility because of various structural constraints (see below) but a naïve assumption of amounts of RDS and SDS from 20 min and 2 h digestibility values is not justified and should now be discouraged.

First-order curves can be fitted by a simple equation (Butterworth, Warren, Grassby, Patel, & Ellis, 2012):

$$C_t = C_\infty \left(1 - e^{-kt}\right) \tag{1}$$

where C_t is the concentration of product at time t, C_∞ is the concentration of product at the end point and k is a pseudo-first order rate constant with dimensions of reciprocal time. It is "pseudo-first" order because its value is directly dependent on the enzyme concentration (E_0). In digestion experiments it is assumed that E_0 remains constant throughout and therefore k values determined for a number of different starch sources at the same E_0 can be compared directly.

For ease of fitting, the rate equation is converted to a logarithmic form:

$$\ln\left[\left(C_\infty - C_t\right)/C_\infty\right] = -kt \tag{2}$$

Therefore a plot of $\ln[(C_\infty - C_t)/C_\infty]$ against t is linear with a slope of $-k$. Plotting the data calls for an accurate value of C_∞. Many investigations have assumed that C_∞ can be read from a digestibility curve from the point at which the curve becomes flat at the later stage of the incubation. Amylase is known to be inhibited by maltose produced as an end product of starch digestion and the enzyme may lose activity as a consequence of denaturation during a long incubation at 37 °C and so the value obtained from the flattening of the digestion curve is likely to underestimate C_∞. Differentiation of Eq. (1) gives:

$$\ln\left[dC/dt\right] = \ln\left[C_\infty k\right] - kt \tag{3}$$

dC/dt is the slope of the digestibility curve and if this is determined at a number of different time points t, a linear plot can be made of $\ln[dC/dt]$ against t. This is a log of slope plot (LOS) (Poulsen et al., 2003) and it can provide valuable information. The slope is equal to $-k$ but the intercept on the Y-axis equals $\ln[C_\infty k]$ and therefore C_∞ can be calculated. Clearly a guessed value for C_∞ is not required for the fitting exercise but just as important, the total amount of the starch that can be digested can now be calculated. The fraction of starch resistant to digestion is obtained by the difference between C_∞ and the total starch added to the reaction mixture. Knowledge of the amount of resistant starch is helpful in predictions of the quantity that is likely to reach the lower stages of the GI tract and be digested by colonic bacteria. The products of the bacterial fermentation, which include short chain fatty acids, are known to be important for health of the colon (Canani et al., 2011).

The slope of digestibility plots is a sensitive indicator of changes in k. Therefore if the starch source contains fractions that are digested at different rates, discontinuities will appear in the LOS plots and the relative amounts of the different fractions can be estimated. This has been demonstrated (Edwards, Warren, Milligan, Butterworth, & Ellis, 2014; Zou, Sissons, Gidley, Gilbert, & Warren, 2015) LOS; analysis has been shown to provide an excellent fit to digestibility data obtained

from a range of heterogeneous materials represented by milled fractions of durum wheat and chickpeas (Edwards et al., 2014). Starch granules will usually possess discontinuous LOS plots but gelatinised starches are digested with a single rate constant (Butterworth et al., 2012; Goni et al., 1997). To take full advantage of the benefits that LOS can offer, it is important that the concentration of product is measured in numerous samples taken from the reaction mixture in the early stages of digestion. This is the region of a digestibility curve where the slope is changing rapidly and if data points are close together in time the slope between adjacent points can be regarded as linear and so the slope may be calculated from simple fractions of the form $(C_2-C_1)/(t_2-t_1)$, $(C_3-C_2)/(t_3-t_2)$ etc. (Butterworth et al., 2012).

In some studies, the relative ease of digestibility of different starch sources is expressed by comparing hydrolysis indices (HI) (Goni et al., 1997; Mahasukhonthachat, Sopade, & Gidley, 2010) which are determined from the area under the digestibility curves (AUC). The HI for a particular sample is calculated from the ratio of the AUC value to the AUC obtained for an identical starch concentration of a reference food (e.g. white bread) and expressed as a percentage (Goni et al., 1997). HI values are considered to provide an indication of how well a particular starch source will be digested in vivo. AUC can be obtained from integration of Eq. (1) (Butterworth et al., 2012) between time bounds of t_0 and t_x.

$$\mathrm{AUC} = C_\infty \left(t_x - t_0\right) + \left(C_\infty / k\right)\left[\mathrm{e}^{-kt_x} - \mathrm{e}^{-kt_0}\right] \tag{4}$$

If $t_0 = 0$. The equation simplifies to:

$$\mathrm{AUC} = C_\infty t_x + \left(C_\infty / k\right)\left[\mathrm{e}^{-kt_x} - 1\right] \tag{5}$$

The required parameters for using Eq. (4) are readily available from a LOS plot and so the calculation of HI is relatively straightforward.

An alternative approach to analysis of digestibility curves has been proposed by Kansou, Buleon, Gerard, and Rolland-Sabaté (2015). The authors applied a multivariate model to fit data obtained for amylolysis of a number of mutant maize starches. The model uses an empirical extension of the equation of first order reactions (Weibull function) (Kopelman, 1988) to allow for changes in digestion rate of different fractions of starch within a granule. The equation for first order kinetics takes the form;

$$C_t = C_\infty \left(1 - \mathrm{e}^{-kt-h}\right) \tag{6}$$

where h is a value between 0 and 1 and represents the fall in reaction rate during the period of starch hydrolysis. The authors related the kinetic behaviour to the macromolecular structure and crystallinity of the granules (Kopelman, 1988). The data they used for analysis and test of their model were not ideal however, because the digestibility curves were very prolonged and part of the decrease in reaction rate at

the later stages could have originated from denaturation of the enzyme as well from constraints associated with elements of granule structure. Also the long digestibility time is unrealistic for prediction of digestion behaviour in human subjects although the method may be useful for biotechnical applications such as the production of bioethanol.

3.2 Michaelis–Menten Kinetics

Because amylase attacks the polyglycan chains of starch at multiple sites, it can raise the suspicion that the kinetics of enzyme action could be too complex for analysis by Michaelis–Menten applications. Because of structural constraints at various locations in the starch molecule, the rate of reaction is unlikely to be identical at each point of attack by amylase (Dona, Pages, Gilbert, & Kuchel, 2010). Nevertheless, it has been known for a long time that the relation of initial rates of amylolysis to starch concentration is well fitted by the Michaelis–Menten equation. An added complication however, is that maltose released during digestion is a competitive inhibitor and so when using an integrated form of the Michaelis–Menten equation to account for substrate depletion during lengthy digestibility runs, a term can be introduced to allow for product inhibition (Dona et al., 2010). Therefore data collected over a lengthy period of incubation where substrate depletion comes into play as well as inhibition by product, can be fitted to the integrated form of the Michaelis–Menten equation which takes the form (Dona et al., 2010):

$$V_{\max} t = \left(S_0 - S_t\right)\left(K_{\mathrm{m}} / K_{\mathrm{i}} - 1\right) + \left(K_{\mathrm{m}} + K_{\mathrm{m}} \mathrm{S}_0\right) \ln\left(S_0 / S_t\right) \tag{7}$$

where S_0 and S_t are the starch concentrations at time zero and t, respectively, and K_{i} is the inhibition constant for maltose. Two molecules of maltose can be accommodated in the active site of porcine pancreatic amylase but the sugar is a weak inhibitor (Elodi, Mora, & Krysteva, 1972; Seigner, Prodanov, & Marchis-Mouren, 1995; Warren, Butterworth, & Ellis, 2012) with K_{i} values of ~15 and ~90 mM for binding of the first and second molecules of maltose (Seigner et al., 1995; Warren et al., 2012), respectively. Therefore product inhibition can usually be ignored until late stages of a digestion reaction because the starch substrate will outcompete any binding of maltose. Thus the simple form of the Michaelis–Menten equation

$$v = V_{\max} S_0 / \left(K_{\mathrm{m}} + S_0\right) \tag{8}$$

is able to fit initial rate data (Slaughter et al., 2001).

For native granular starches, the experimentally determined apparent Michaelis constant ($K_{\mathrm{m}}^{\mathrm{app}}$) which is a measure of the substrate concentration needed to support an initial rate of $V_{\max}/2$, is relatively large because only a few α-1,4 linkages of the total starch chains are available for amylase binding and subsequent reaction.

Hydrothermal treatment increases the amount of accessible starch and so the determined K_m^{app} decreases signifying easier binding of amylase. The fraction of accessible polyglycan residues after hydrothermal treatment at temperature T, (x_T), can be denoted by A/S where A is the concentration of accessible substrate and S is the total starch concentration. When the starch has become fully gelatinised and thus all the α-glucan chains are potentially available for digestion by amylase, a limiting low K_m value is reached which can be considered an absolute value (K_m^{amy}). Apparent K_m values can be determined after hydrothermal processing at particular temperatures (T) and from the ratio of K_m^{app} / K_m^{amy} the fraction of the starch susceptible to hydrolysis after hydrothermal pretreatments can be estimated (Slaughter, Ellis, Jackson, & Butterworth, 2002). Solution-state NMR studies carried out in parallel with enzyme kinetic work demonstrated an increase in flexible polyglycan chains produced by hydrothermal processing of starch granules and the increase in flexibility exactly mirrored the raised availability of enzyme attack predicted from the changes in apparent K_m values (Slaughter et al., 2002). Furthermore, it was demonstrated that the early stages of amylase action on starch granules is restricted to hydrolysis of polyglycan chains that are exposed to solvent. Such chains are readily available for reaction with the enzyme in the bulk solution (Baldwin et al., 2015).

The catalytic efficiency (or specificity constant) (Fersht, 1999) of an enzyme acting on its substrate is given by the k_{cat}/K_m ratio. This is an apparent second-order rate constant that relates the reaction rate to the concentration of free enzyme. For an enzyme that reacts upon a number of different substrates, the ratio is useful for comparing the relative activities of the various substrates. Because of variations in starch structure arising from botanical source and/or hydrothermal processing, CE values can provide information that allows for comparison of the relative reactivity of different starch forms. CE values obtained from in vitro kinetic studies can add to explanations for the known variations of in vivo digestion rates observed for different starch containing foods.

V_{max} equals k_{cat}/E_0 and the rate constant can be calculated from the kinetic data and the enzyme concentration. The use of pure preparations of α-amylase of known molecular weight makes for easy estimation of E_0. If impure preparations such as pancreatin are used where E_0 is unknown, relative CE values can be obtained from V_{max}/K_m determined for each starch provided that the amount of pancreatin in reaction mixtures is known and carefully controlled.

Studies of inhibition of amylase activity can also be usefully analysed by Michaelis–Menten kinetics. Guar galactomannan (a water-soluble form of dietary fibre) which is known to decrease postprandial glycaemia and insulinaemia following a starch rich meal (Ellis, Apling, Leeds, & Bolster, 1981), has been shown to bind porcine pancreas α-amylase and inhibit catalytic activity by a non-competitive action (Slaughter et al., 2002). Kinetic studies have shown that amylase activity is inhibited by cellulose in a mixed competitive–uncompetitive manner (Dhital, Gidley, & Warren, 2015) and a similar conclusion was reached for direct inhibition of amylase by retrograded starch (Patel et al., 2016).

3.3 Freundlich Kinetics and Direct Binding Studies

The interaction between α-amylase in solution and starch granules is a two phase system. If the absorption of the enzyme onto the solid granule surface is of kinetic significance, the concentration of bound enzyme is related to the concentration of free (i.e. unbound) enzyme in solution and can be expressed by a Freundlich equation (McLaren, 1963) of the form:

$$E / A = K^f E_b^{\ n} \tag{9}$$

where E/A is the enzyme bound per unit of substrate surface area, E_b is the total enzyme concentration, K^f is a Freundlich partition coefficient and n is a coefficient that ranges between 1/3 and 2/3 dependent on the surface properties of the solid phase (McLaren, 1963). If there is absorption onto a perfectly smooth substrate surface, n equals 2/3 but when edges, cracks or fissures are present, the value approaches 1/3 (McLaren, 1963).For enzyme kinetic purposes, E/A can be equated with the enzyme–substrate complex (McLaren, 1963; Slaughter et al., 2001) so that the catalytic rate, v, is given by $k_{cat}\ K^f E_b^{\ n}$.

In a study of a number of different botanical starches it was found that the coefficients ranged from ~0.5 to ~ 0.9 indicative of different surface properties of the various botanical starches. Estimations of rates of diffusibility of amylase into starch granules have been interpreted to indicate that granules of maize starch possess pores and channels (i.e. present "edges" for absorption) whereas potato granules are smooth and lack pores (Dhital, Shrestha, & Gidley, 2010; Dhital et al., 2017). Freundlich coefficients can go some way towards providing information about surface topology of the granules.

UV spectroscopy was used for studying the binding of maltose with amylase (Elodi et al., 1972) but the opalescence of starch suspensions makes visible and UV spectroscopy largely unsuitable for work with starch. Direct binding of amylase to starch granules can be studied under non-catalytic conditions by enzyme-depletion methods in order to obtain information about the first stage of the catalytic process, that is, the collision and binding of amylase to starch (Warren, Butterworth, & Ellis, 2013; Warren, Royall, Gaisford, Butterworth, & Ellis, 2011). Incubations of enzyme and starch at 0 °C (where catalytic digestion of starch will be minimal), are sampled at various times and the starch with bound enzyme is separated from the reaction mixture by centrifugation or rapid filtration. Determination of the enzyme activity in the supernatant allows for the amount bound to starch to be calculated. Dissociation constants (K_d values) determined at 0 °C for a number of botanical starches ranged from 0.87 to 2.05 mg/ml and were shown to be directly related to the volume surface area of the granules and to K_m values obtained in catalytic assays performed at 37 °C (Warren et al., 2013). Also there is a relationship between K_d and $1/K^f$ for the various starch granules (Warren et al., 2011) (K^f is an association constant and hence the

reciprocal relationship). The proportionality of K_d with K_m and $1/K^f$ values is particularly interesting because it emphasises the kinetic importance of the initial binding step in determining the rate of catalysis and indicates that the complex formed from enzyme and starch granule interaction is close to being at equilibrium with free enzyme and starch. The rate constant for binding at 0 °C was estimated to be ~1.4×10^6 $M^{-1}s^{-1}$.

A recent publication describes the properties of starch binding sites on the surface of human pancreatic α-amylase deduced from site directed mutagenesis (Zhang et al., 2016). The authors of this study point out that measured binding affinities of amylase–starch interaction are likely to represent a combination of binding to the active site together with other binding events on the surface of the enzyme.

4 Conclusions

Many, perhaps the majority, of kinetic investigations of enzyme action have been largely focussed on defining individual catalytic properties and are for help in establishing mechanisms of reaction. Determinations of 3D molecular structures of enzyme proteins in combination with sound kinetic data have enabled identification of catalytic sites and interaction with substrates to be described at a molecular level. Studies of amylases have followed this general pattern and the early work established the general properties of the enzymes. Because starch is a major source of dietary energy in the form of a glucose polysaccharide, interest in α-amylase extends beyond the realm of pure enzymology into animal and human nutrition, medicine and other health sciences and human evolution. The development by hominins of controlled use of fire and cooking meant that starch present in gathered plants and roots could become gelatinised and therefore digested more fully. The increase in brain size during human development would have demanded greater consumption of glucose since this sugar is the main source of fuel for the nervous system. Starch would have been the principal source of glucose.

Observed differences in the susceptibility of various starches to amylolysis and to intestinal digestion have continued to stimulate research. A full understanding of the amylolysis step in starch foods in combination with structural information on the starch itself and/or the food matrix is sought to provide predictions of likely glycaemic responses following ingestion of a starch-containing foods such as bread, pasta, rice and potatoes. This presents a formidable challenge in that future studies will need to investigate more complex starch-containing foods at different scale levels, that is, macrostructure at mm scale of plant tissue and of food composites down to the nm scale of α-glucan chains. Hence, appropriate analyses of digestibility curves and kinetic studies performed in vitro will continue to be of considerable importance for prediction of in vivo digestion of starch.

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Influence of Physical and Structural Aspects of Food on Starch Digestion

Ingrid Contardo and Pedro Bouchon

1 Mass Transfer and Starch Digestion Kinetics

High consumption of foods rich in starch can be linked with some conditions and diseases such as obesity and type II diabetes (Zimmet, Alberti, & Shaw, 2001). The physical state of starch granules influences how they are digested while simple sugars are absorbed in the gastrointestinal system. Even though the field of food design is seeking to generate starchy foods with beneficial properties for health, focusing on reducing or slowing the absorption of glucose in the blood, it is important to understand how starch is hydrolyzed and absorbed in the body, and how the design of novel foods can provide them with functionality through their structure and disintegration.

1.1 General Aspects and Approaches to Modeling

The kinetics of starch digestion are influenced by the physical and chemical characteristics of the starch granules and the interactions with physiological events occurring within the gastrointestinal tract (Bjorck, Granfeldt, Liljeberg, Tovar, & Asp, 1994). The features of starch are also influenced by food composition and structure, and food processing conditions. Extrinsic factors that influence starch digestion include the nature of starch; its physical form; interactions with proteins, lipids, or sugars; the presence of enzyme inhibitors; food processing; food structure (initial hardness or porosity); and bolus hydration/disintegration, while intrinsic factors

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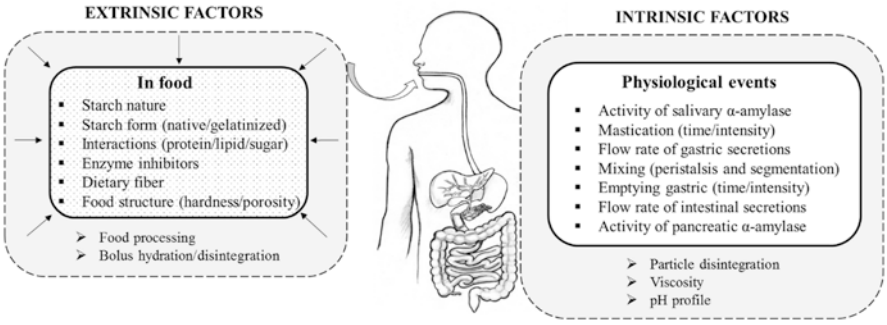

Fig. 1 Extrinsic and intrinsic factors that influence the kinetics of starch digestion [based on Bornhorst and Singh (2013), Englyst, Englyst, Hudson, Cole, and Cummings (1999), and Guyton and Hall (2006)]

consist of particle disintegration, activity/concentration/flow rate/viscosity of secretions, mastication time, and mixing (peristalsis and segmentation). These factors are represented schematically in Fig. 1. In addition, the amount of glucose available for absorption is a critical factor in the reduction of the blood sugar level, thus starch hydrolysis could be limited or modulated in order to obtain the desired effects on luminal glucose absorption.

Static in vitro models have been established to study starch digestibility, which mimic some physiological conditions for digestion, including physical transformations and enzymatic hydrolysis, as well as measuring the amount of glucose released from a food during timed incubation under well determined conditions. Static models provide technical simplification under rapid, simple, and controlled conditions; and they allow for preliminary results to be obtained. Englyst, Kingman, and Cummings (1992) developed an enzymatic in vitro method to classify starchy foods based on their digestibility for nutritional purposes, where rapidly available glucose (RAG) was defined as the fraction of glucose that was obtained after 20 min of hydrolysis. Slowly available glucose (SAG) was said to be the fraction obtained between 20 and 120 min of hydrolysis. These amounts of glucose are likely to be available for rapid and slow absorption, respectively, in the human small intestine. Finally, unavailable glucose (UG) was defined as the fraction of glucose that is present in the food but could not be released after 120 min of hydrolysis. Furthermore, Goñi, Garcia-Alonso, and Saura-Calixto (1997) developed a static in vitro procedure to measure the rate of starch digestion in starchy common foodstuffs. The possible estimation of glycemic index (GI) from the percentage of total hydrolyzed starch, depending on the sampling time, was also analyzed. The authors concluded that this in vitro procedure could be useful in the estimation of GI. More recently, a standardized static method was proposed by the COST action INFOGEST network (Minekus et al., 2014) to harmonize the variety of in vitro protocols found in the literature as variations in factors such as the amount and type of enzymes, incubation time, pH, and specified temperature generate different results from the same

foods. The proposal involves the simulation of oral, gastric, and intestinal digestion; and includes a standardized assay for enzymatic activity determination of the enzymes that are added at each step.

During testing using static models, the oral, gastric, or intestinal phases are reproduced by a mono-compartmental step and the specific test conditions are adapted for each specific application. Typically, in experiments with static models, the foods are mixed with simulated fluids during a pre-determined time of digestion while controlling the environment in temperature and pH and fixing the concentration of enzymes. Static models lack the simulation of realistic enzyme to substrate ratios, continued changes in pH, transit times or removal of digested products, in time and place. In contrast, dynamic in vitro models involve increased complexity, with the addition of digestive fluids with control of flow rate and composition of their secretions, realistic gastrointestinal profiles of pH, complex peristaltic movements or mixing segmentation, diffusion, and gastric emptying cycles. One dynamic model is the TNO Gastrointestinal Model (TIM), developed at The Netherlands Organization for Applied Scientific Research (TNO) of the Nutrition and Food Research Centre. This is a multicompartmental model designed to realistically simulate conditions in the lumen of the gastrointestinal tract. TIM combines a reproducible and accurate simulation of digestion processes with detailed kinetic elements. This model has been developed to study the fraction of a compound that is available for absorption through the gut wall. Under this approach, the model has been used to predict the glycemic response after intake of carbohydrates (Nalin et al., 2015). The TIM-Carbo technology was validated against 21 different in vivo plasma glucose response curves after the intake of carbohydrate food products ($R = 0.91$). Another model is the Dynamic Gastric Model (DGM) developed by the Institute of Food Research (IFR) (Norwich, UK), which can simulate both the biochemical and mechanical aspects of gastric digestion (stomach and antrum) in a realistic, time-dependent manner. Secretion rates can be adapted dynamically to the changing conditions of acidification or fill state and the system allows for the use of complex food matrices (comparable to in vivo studies) and emulates the peristaltic movements of the stomach in amplitude, intensity, and frequency (Wickham, Faulks, Mann, & Mandalari, 2012). Similarly, the Human Gastric Simulation (HGS) is another dynamic in vitro stomach model, which consists of a latex vessel and a series of rollers secured on belts that are driven by motor and pulleys to create a continuous contraction of the latex wall in order to simulate the peristaltic movements of stomach walls, with similar amplitude and frequency of contraction forces as reported in vivo. It also incorporates gastric secretions, emptying systems, and temperature control that enable accurate simulation of dynamic digestion processes for detailed investigation of the changes in the physical chemical properties of ingested foods. Kong and Singh (2010) used HGS during the digestion of rice. They demonstrated that the amount of acid became a limiting factor for the acid hydrolysis as the solids content of emptied digesta was affected by the amount of rice available for digestion relative to the amount of acid present (see Fig. 2).

Some differences between in vivo and in vitro methods used to study starch digestibility from solid foods exist, as in vitro methods cannot emulate all the com-

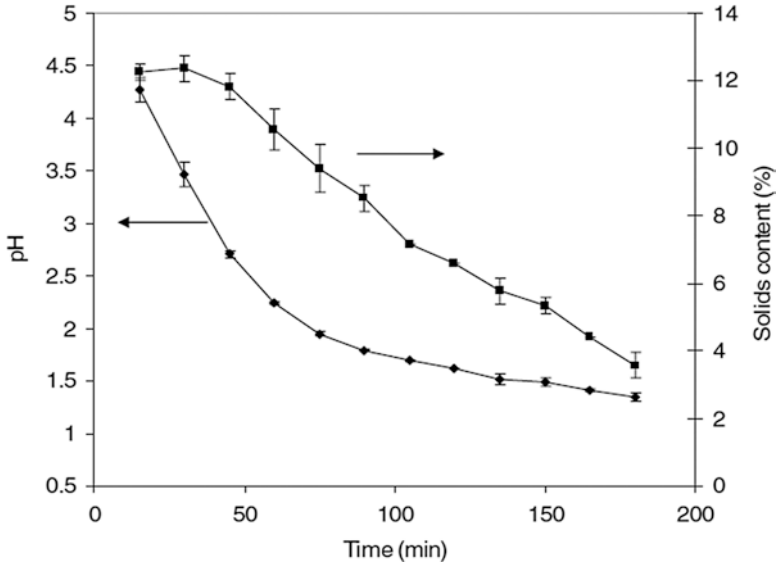

Fig. 2 Profile of pH and change of solids content in emptied digesta during in vitro digestion of rice using Human Gastric Simulation (HGS) (mean of $n = 3$) [extracted from Kong and Singh (2010)]

plex interactions among extrinsic (food characteristics) and intrinsic (body physiological) aspects involved in the digestion process (e.g., the nature of the exposure to salivary α-amylase prior to simulated gastric phase digestion, simultaneous peristaltic movements, gradual pH changes of the food digesta, or origin of α-amylase). Generally, in vitro approaches to quantify starch digestibility are performed in a two-stage (gastric digestion and intestinal absorption) simulated digestion system. However, the human digestive tract is a complex system of organs which can be segmented into at least four main units: the mouth, the stomach, the small intestine, and the large intestine. Starch is affected in different ways in these steps. Figure 3 shows a representative summary of hydrolysis of starch and absorption of glucose in the gastrointestinal tract focusing on the main processes (into the mouth, stomach, small intestine, and large intestine) and highlighting the key variables that may influence starch digestion kinetics.

Various mathematical models have been used to study the kinetics of starch digestion after the ingestion of solid starchy foods. As discussed, starch digestion is a complex process involving a simultaneous mass transfer related to bolus formation, bolus disintegration, hydrolysis of starch, and glucose release and absorption. Likewise, digestibility of starch-containing solid foods is widely affected by the physical–chemical characteristics of starch granules and interactions with the food matrix (component interactions or food structure). The kinetics of starch digestion frequently show simple decay curves with apparent first-order behavior; this can include the rate of starch degradation, the rate of glucose release, or the appearance

Organ/unit (secretions)	Physical aspects		Chemical aspects	
	Process (function)	Variables	Process (function)	Variables
Mouth (salivary α-amylases, water, mucin, salts)	**Mastication** (milling, lubrication, mixing, bolus formation) *Output: bolus*	Mastication time Mixing time Salivary secretions flow rate/mass Hardness/porosity of solid food Particle hydration Viscosity	**Enzymatic hydrolysis** (initial starch breakdown by salivary α-amylase) *Output: starch, maltose, maltotriose, and α-limit dextrin*	Salivary secretions flow rate/mass Activity of enzyme Residence time
Stomach (acid, gastric enzymes, water, salt, mucus)	**Peristalsis and mixing waves** (bolus transport, particle disintegration, mixing, pyloric pump) **Gastric emptying** (propel of chyme) *Output: Chyme*	Degree of mixing Secretions flow rate/mass Bolus volume Particle size Rate of bolus disintegration Viscosity	**Enzymatic hydrolysis** Protein and lipid breakdown **Acid hydrolysis** pH regulation (pH>4 to amylase) Starch breakdown *Output: starch, maltose, maltotriose, and α-limit dextrin*	Gastric secretions flow rate/mass Activity of enzymes Residence time Enzyme inhibitors pH profile
Small intestine (alkaline mucus, enterocytes, pancreatic α-amylases, water, bile, salt)	**Peristalsis** (chyme transport) **Segmentation** (mixing to facilitate absorption) **Convection - Diffusion (active/passive)** (absorption of glucose) *Output: absorption of glucose to bloodstream*	Chyme flow rate/mass Residence time Adsorption of enzymes onto solid substrates Available glucose Viscosity	**Enzymatic hydrolysis** (main starch, maltose, maltotriose, and α-limit dextrin breakdown by pancreatic α-amylases) pH regulation *Output: glucose*	Activity/secretion of enzymes Residence time Secretions flow rate/mass Enzyme inhibitors Digestible starch pH profile
Large intestine (alkaline mucus, bicarbonate ions)	**Haustrations** (mixing to facilitate fermentation) **Mass movements** (Resistant starch transport) *Output: absorption of vitamins and water*	Degree of mixing Particle size (substrate) Flow rate Pressure	**Fermentation** *Output: production of short-chain fatty acids (SCFA) (acetate, propionate, butyrate), oligosaccharides, and lactate*	Microbial population Bacterial growth Concentration of resistant starch

Fig. 3 Representative overview of the processes during starch hydrolysis and absorption in the gastrointestinal tract (mouth, stomach, small intestine, and large intestine), highlighting physical and chemical aspects influencing starch digestion kinetics from solid food, and the key variables in an engineering approach [adapted from Bornhorst and Singh (2014)]

of oligosaccharides over time. Thus, empirical exponential models (e.g., first-order equation and Weibull function) and Michaelis–Menten models have been used to fit the kinetics of starch hydrolysis or glucose release from starch. Accordingly, using empirical exponential models, the end-point product concentration, the pseudo first-order digestibility rate constant and the fraction of digested starch can be fitted by linear fits to logarithmic plots (Log of Slope (LOS)); through which it has been shown that starch amylolysis in solid foods occurred via a two-phase system (rapid and slower phase) reflecting differences in substrate accessibility; also, glucose was found to inhibit amyloglucosidase after a "grace" period at low glucose concentrations; and small starch particle sizes increase the rate of enzymatic action.

Likewise, amylolysis progress-curves can be well described by Weibull function (Dona, Pages, Gilbert, & Kuchel, 2010). For instance, Goñi et al. (1997) used a first-order equation that governs the hydrolytic process:

$$C = C_{\infty}\left(1 - e^{-kt}\right) \tag{1}$$

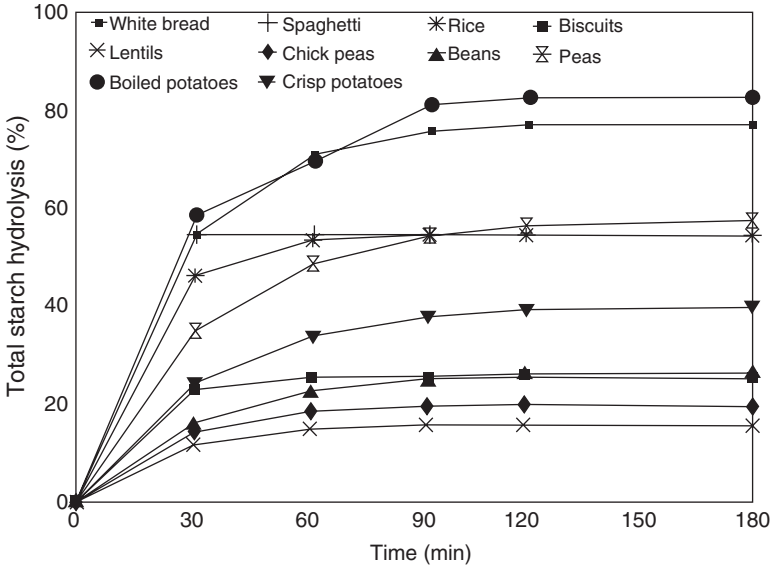

Fig. 4 Total starch hydrolysis rate, using a first-order equation [extracted from Goñi et al. (1997)]

Where C is the concentration of hydrolyzed starch (kg/kg) at t time (min), C_∞ is the equilibrium concentration (concentration of digestible starch in the stage where no more product is formed), k is the kinetic constant (min^{-1}) and t is the chosen time (min). Experimental values for each investigated starchy food were adjusted to the equation to calculate k and C_∞, using LOS plots. In general, the k values reflect the susceptibility of starch or starchy foods towards hydrolysis by amylase, and typically range from 10^{-5} to 10^{-3} min^{-1} but are dependent on enzyme concentration. The decay equation suggests that the rate of reaction decreases with time due to substrate depletion and it is related to the diffusion of amylase onto the surface of the granule particle and the physical nature of substrate. The curves of starch digestion show a first part, where the starch hydrolysis rate increases, and a second, where a maximal plateau level is slowly reached (see Fig. 4). Additionally, the hydrolysis indices (HI) from the determination of the area under the curves (AUC) obtained by integration of the first order rate equation is a simple alternative to the glycemic index (GI) values for starchy foods. Moreover, amylolysis progress-curves can be well described by Weibull function (Dona et al., 2010). Likewise, Wang, Zeng, Liu, and Yuan (2006) used an exponential model considering taking into account product inhibition to study the hydrolysis of corn starch by glucoamylase:

$$\frac{ds}{dt} = \left(\frac{ds}{dt}\right)_{p=0} \exp\left(-k_i\left(p - p_0\right)\right)\left(1 - \frac{p}{p_m}\right)^n \tag{2}$$

Where S is substrate concentration ($kg \cdot L^{-1}$), t is time (h), k_i is the product inhibition constant ($L \cdot kg^{-1}$), p is product concentration ($kg \cdot L^{-1}$), p_0 is the minimum product concentration ($kg \cdot L^{-1}$) below which product inhibition is not observed, p_m is the product concentration obtained when all the starch in the system is hydrolyzed into sugars ($kg \cdot L^{-1}$), and n is an empirical constant (non-dimensional). The experiments showed that a product inhibition effect appeared when product concentration was above p_0.

Furthermore, another empirical model used to explain starch digestion is the Michaelis–Menten model, which describes reactions where the enzyme concentration is small relative to the substrate concentration:

$$v = \frac{V_{\max S}}{K_m + S} \tag{3}$$

Where v is the reaction velocity ($kg \cdot L^{-1} \cdot min^{-1}$), v_{max} is the maximum reaction velocity ($kg \cdot L^{-1} \cdot min^{-1}$), K_m is the Michaelis–Menten constant which gives an idea of the affinity between enzyme and substrate and represents the concentration of digestible (available) starch ($kg \cdot L^{-1}$), and S is total starch concentration ($kg \cdot L^{-1}$). In addition, $v_{max} = k_{cat} * E_0$, where k_{cat} is the catalytic constant for the enzyme and E_0 is the total enzyme concentration ($kg \cdot L^{-1}$); k_{cat} involves the number of molecules of product formed per molecule of enzyme in unit time (min^{-1}). This model can be employed to describe the release of glucose in the initial stages (within the first 10–15 min) as a function of starch concentration before product inhibition and substrate exhaustion (Dona et al., 2010). In hydrothermally processed starches (e.g., boiling process at 100 °C), the reduction in K_m occurs as a result of the increased substrate availability due to the loss of the semi-crystalline native starch granule structure. Additionally, the gelatinization process affects the catalytic efficiency (k_{cat}/K_m), increasing its values after the hydrothermal process (e.g., values increased by 13-fold (waxy rice) to 239-fold (potato) after the boiling process). Similarly, this phenomenon increases the k_{cat} values in comparison to native starch (Slaughter, Butterworth, & Ellis, 2001). The modifications of this model are related to deviations from Michaelis–Menten kinetics in the early stages of hydrolysis of starch affected by the starch structure (e.g., the degree to which amylose leaching occurs during gelatinization), the complexity of the food (bioaccessibility of starch), or when product inhibition and substrate exhaustion start to happen. Thus, the competitive-inhibition model from the Michaelis–Menten framework has been used to describe product/substrate inhibition of glucoamylase, incorporating high coefficients of variation on parameter estimates. An increase in starch concentration involved in the digestion profile means that the system no longer adheres to Michaelis–Menten behavior. Also, an inhibitory effect from this type of substrate, which may cause differences in the mass transfer of the molecules (e.g., by a higher apparent viscosity, and different rheological or structural features of starch), may further increase mass-transfer resistance. Under this approach, the use of the linearized graphical method such as Lineweaver–Burk plots obtained from inhibition experiments has not been able to distinguish between the two mechanisms, promoting the use of multiple models to describe the kinetics of starch hydrolysis.

1.2 Oral Processing

Breakdown behavior in the mouth and stomach of solid starchy products can determine the accessibility of starch granules during the hydrolysis process. In the mouth, the physical and chemical transformations of starch-based foods by simultaneous processes take place; food mastication, lubrication, mixing, and bolus formation. The main role of mastication is the reduction of food in terms of particle size. Usually, breakdown behavior is described in terms of the number of particles of different sizes present in the chewed/digested food, typically characterized by bimodal behavior. For instance, such data are used to fit the cumulative distribution of particles in terms of either their numbers or surface areas to a Rosin–Rammler model (unimodal Weibull distribution), and quantify their extent of breakdown based on median and spread values; or use of the mixed Weibull distribution function (Drechsler & Ferrua, 2016).

Oral digestion involves the enzymatic hydrolysis of starch, where salivary α-amylases lightly hydrolyzes starch to generate fractions of maltose, maltotriose, and α-limit dextrin (group of low-molecular-weight carbohydrates, 3–9 glucose polymers) (Guyton & Hall, 2006). In addition, the mucin (glycoprotein) present in the saliva acts as a lubricant in bolus formation, contributing to the hydration of the fragmented food (Bornhorst & Singh, 2012). In this first step of digestion, it is to be expected that starch hydrolysis is limited due to the fact that granules remain in the mouth for a short period. However, studies of oral digestion show the importance of the time/intensity of mastication, viscosity, the flow rate/concentration of enzyme and pH on the salivary α-amylase activity on starch digestibility (Butterworth, Warren, & Ellis, 2011). Moreover, in solid starchy foods that require significant physical breakdown during digestion, breakdown behavior is affected by the initial hardness and the rate of softening. The rate of softening is a function of the food structure (e.g., porosity, density) and the amount of acid and enzyme secretions that have entered the food matrix (e.g., hydration in physiological gastric conditions) (Bornhorst, Ferrua, & Singh, 2015). Texture changes fit the Weibull model:

$$\frac{H_t}{H_0} = e^{(-kt)\beta} \tag{4}$$

Where H_t (N) is the hardness at time t (min), H_0 is the initial hardness (N), k is the scale parameter (min^{-1}); t is the digestion time (min); and β is the distribution shape factor (non-dimensional).

Although the salivary enzymatic degradation of starch has been considered insignificant (not more than 5% of all starches) in comparison to pancreatic amylase in the small intestine, it may influence the final digestive process by affecting starch granules and the food structure (Hoebler et al., 1998). Individuals with high salivary amylase concentrations may be better adapted to ingest starches, whereas those individuals with low amylase activity may be at greater risk of insulin resistance and diabetes if chronically ingesting starch-rich diets (Mandel & Breslin, 2012). The

salivary flow rate and its composition can be stimulated by differences in starch-based foods. Engelen, Fontijn-Tekamp, and Van Der Bilt (2005) studied the influence of the characteristics of various starchy products on the swallowing threshold, under the hypothesis that the urge to swallow food could be triggered by a threshold level in both food particle size and lubrication of the food bolus. The influence of oral physiology on the swallowing threshold was determined by measuring the salivary flow rate, maximum bite force and masticatory performance. They used about 10 cm^3 of bread, toast, Melba toast, breakfast cake, peanuts or cheese to determine the influence on the swallowing threshold of various food characteristics (e.g., hardness, moisture, and fat) and showed that salivary flow rates were significantly and negatively correlated with the number of mastication (chewing) cycles of melba toast and breakfast cake. Hence, subjects with more saliva needed less chewing cycles for these dry foods.

Likewise, inactivation of salivary α-amylase may be influenced by low pH environment or food composition, affecting the amount of hydrolyzed starch in the mouth. Thus, the inhibition capacity to α-amylase by certain food components can be used for its potential ability to modify the postprandial glycemic response. Some α-amylase inhibitors are naturally present in cereals or legumes, or they can be incorporated by formulation or food processing. Phenolic compounds, phytochemicals in grains or crops, and bioactive compounds in seaweed have α-amylase inhibitory properties. Heo et al. (2009) indicated that diphlorethohydroxycarmalol (DPHC) isolated from a brown alga might be a potent inhibitor for α-glucosidase and α-amylase. The increase of postprandial blood glucose levels was significantly suppressed in the DPHC-administered group compared to the streptozotocin-induced diabetic or normal mice.

After the food bolus is formed, it is swallowed and it travels through the esophagus by muscle contractions (peristalsis) down to the stomach. The bolus transport along the esophagus is influenced by the rheological behavior of starch-rich bolus linked to viscosity, the swallowing rate, and the esophagus radius (Mackley et al., 2013; Moritaka & Nakazawa, 2009).

1.3 Stomach Digestion

In the stomach, gastric secretions (acids, enzymes, and electrolytes) are secreted from the stomach wall. With the help of weak peristaltic contraction waves, they come into contact with the bolus to generate the chyme (semifluid mixture), and they inactivate the salivary α-amylase due to their low pH. Not all amylase is inactivated at the same time as the profile of pH inactivation is affected by chyme characteristics (e.g., flow rate or viscosity), food pH and composition, or the amount of bolus. The pH is adjusted to the characteristics of the bolus and its behavior during its gastric passage. As such, the buffering capacity of the food probably prevents its immediate and homogenous acidification in the stomach. At the same time, the gastric pH may influence the rate of bolus hydration/disintegration, uptake of

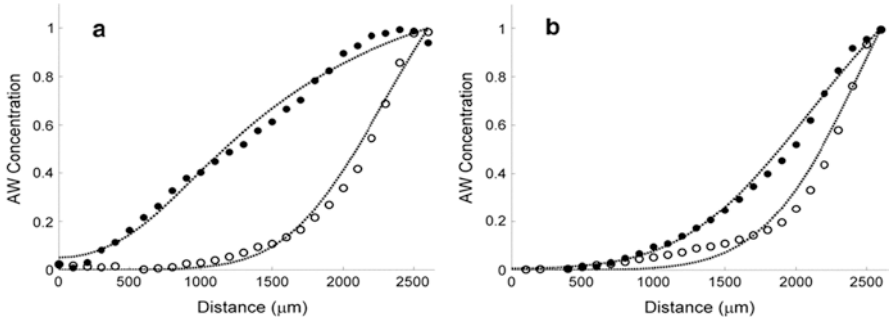

Fig. 5 Acidic water (AW) penetration front profiles as a function of distance (μm) from the carrot center (solid circles) and Edam cheese (white circles) soaked for 10 min at pH 1.5 (**a**) and at pH 7.0 (**b**) [extracted from Van Wey et al. (2014)]

secretions, or food softening during digestion, promoting or limiting the release of nutrients by the relationship between pH and the efficacy of different enzymes in the gastric fluid. Van Wey et al. (2014) showed that the diffusion of acid water into a food particle is pH dependent, and will depend upon the food structure as demonstrated by the different penetration front profiles between a raw carrot core and Edam cheese (see Fig. 5). Mennah-Govela and Bornhorst (2016) showed that the uptake of gastric fluids into sweet potatoes during simulated gastric digestion modifies the food macro- and micro-structures. The pH of the gastric juice increased from 1.8 to 3.1 ± 0.5, and the pH of the food sample decreased (from 7 ± 0.3 to 3.7 ± 0.4) during 240 min of the gastric digestion period. The acidity of the sweet potatoes cubes was significantly influenced by the cooking method and gastric digestion time ($p < 0.0001$) as well as their interaction ($p < 0.0001$). In addition, the mass transfer in sweet potatoes was influenced by cooking methods, demonstrating the importance of the microstructure generated during processing on acid and water diffusion into foods during digestion. Also, Bhattarai, Dhital, and Gidley (2016) showed that increasing pH clearly results in more damage to starch, expressed in terms of pitting (effect supported by SEM in the study) and consequently higher hydrolysis. Wheat flour starch was hydrolyzed using various concentrations of α-amylase ranging from 1×10^3 IU/mL (0.5 U/mg starch) to 12×10^3 IU/mL (6 U/mg starch) at six different pH values (2–7).

Gastric digestion has physical–chemical implications for starchy products. Diffusion of gastric fluids into the bolus and solid loss from the bolus to the gastric site take place. Gastric mixing (muscle contractions), breakdown of the bolus into small fractions, and interactions with gastric secretions damage starch granules, leading to starch hydrolysis (Guyton & Hall, 2006). The flow rate of gastric secretions and mixing are not homogenous during gastric digestion. These parameters can be influenced by food structure, while starch granules that are not accessible may act as physical barriers to the free diffusion of fluids into the bolus or disintegrated food particles. This modifies the contact rate between the active site and enzyme action, and so it may limit the rate and/or extent of starch hydrolysis.

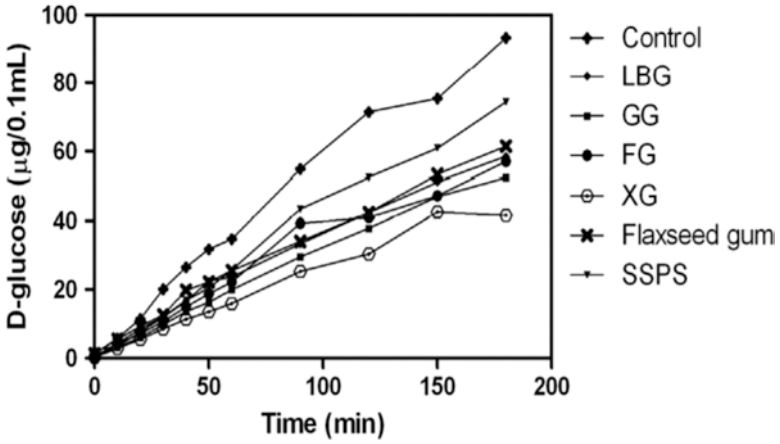

Fig. 6 Available glucose (mg/mL) during 180 min in vitro small intestinal digestion of control (no fibre), locust bean gum, guar gum, fenugreek gum, xanthan gum, flaxseed gum, and soy soluble polysaccharide-fortified solutions [extracted from Fabek et al. (2014)]

Therefore, the mass transfer in gastric starch digestion may be affected by viscosity, food components, and gastric movements.

Viscosity of the bolus can affect the bioaccessibility of starch granules and the quantity of glucose available for absorption, and it may also influence the glucose concentration in the bloodstream. A study developed by AlHasawi et al. (2017) demonstrated that gastric viscosity differed considerably between commercially available oat products, instant oats, steel cut oats, and oat bran, using TNO Intestinal Model (TIM-1). Instant oat and oat bran viscosities were highest at the onset of digestion and decreased over time, whereas the viscosity of steel cut oats at the onset of digestion was the lowest level of viscosity observed, increasing over time, and affecting the rate of starch digestion. Starch content was directly proportional to total bioaccessible sugars and the rate of sugar release was slowest for steel cut oats and quickest for oat bran. Thus, an increase in gastric viscosity may lead to reduction in the diffusion of hydrolyzed glucose. Likewise, cereals that are high in soluble fiber such as β-glucan may induce a high level of viscosity of the digesta once it has reached the small intestine, where the absorption of glucose occurs. The viscosity of soluble fibers depends on their ability to resist changes during gastrointestinal digestion (Würsch & Pi-Sunyer, 1997). Fabek, Messerschmidt, Brulport, and Goff (2014) demonstrated that dietary fiber influences the glucose diffusion of in vitro small intestinal digestion in a simulated food model, which included protein and starch (see Fig. 6). Villemejane, Wahl, Aymard, Denis, and Michin (2015) investigated the effects of fiber in biscuit composition on the viscosity generated during digestion using TIM-1 (stomach, duodenum, jejunum and ileum). The results showed a significant effect of viscous soluble fibers on the chyme viscosity, up to the ileal compartment. In the stomach, during the first hour of the digestion, fibers were progressively liberated from the matrix and solubilized, which allowed main-

tenance of the viscosity of the gastric content. These findings suggest that the inclusion of more resistant to digestion ingredients, such as hydrocolloids (e.g., xanthan gum, guar gum, or soy soluble polysaccharide) or soluble fibers (pectin, mucilages, or β-glucan) in a food might be an effective strategy in lowering postprandial glycemic responses in humans (Würsch & Pi-Sunyer, 1997).

The effect of viscosity on gastric emptying has shown diverse results. Increasing bolus viscosity may delay gastric emptying rate, so it may increase satiety and modulate postprandial glycemic responses (Marciani et al., 2001; Zhu, Hsu, & Hollis, 2013). In contrast, Bornet et al. (1990) concluded that the α-amylase susceptibility of test carbohydrates (25 g starch or equivalent glucose units) is a determining factor in the insulin response of healthy subjects, while viscosity of the test meals and the gastric emptying rate have no effect.

The rate of bolus disintegration has also been shown to play a key role in gastric emptying, as well as possibly influencing postprandial blood glucose levels. This is conditioned by food composition or structure. Bornhorst and Singh (2013) demonstrated that the disintegration rate and profile of bread boluses were significantly influenced by bread composition in both static and shaking conditions. Each bread, almond wheat, barley, rye, sourdough, wheat, or white, was characterized by its moisture content, firmness, and water holding capacity. The initial moisture content of breads also influenced the amount of gastric secretions absorbed. The total amount of fluids absorbed by the bolus seemed to be inversely proportional to the initial bread moisture content, while the firmness of bread and its water holding capacity were found to be complementary food properties that must be considered to explain the differences detected in mass retention profiles. Additionally, the gastric disintegration of a bolus can be affected by the properties of the gastric fluids (composition and rheology) and the gastric movements (stomach motility and antral contractions) (Kong & Singh, 2008). The structural breakdown of food has a significant influence on starch hydrolysis, both in terms of bolus formation and disintegration (Bornhorst & Singh, 2012). Various studies have shown that different foods will produce different particle size distributions in the bolus during oral processing. For example, the bolus produced after mastication of bread demonstrated a bimodal distribution of particle sizes (30 mm, 500 mm) (Hoebler, Devaux, Karinthi, Belleville, & Barry, 2000). Particle sizes in a bolus have been linked to the rate of in vitro enzymatic degradation. Ranawana, Monro, Mishra, and Henry (2010) reported that the degree of particle size, due to mastication, correlated with the rapidly available starch content (RDS) in chewed rice bolus. The quantity of undigested material remaining at the end of the 120-min digestion correlated significantly with the percentage of particles greater than 2000 μm in masticated rice.

After gastric digestion stomach emptying occurs, where the chyme is transported from the stomach into the small intestine by intensive peristaltic contractions in the antrum. The motion of the gastric fluids causes dramatic changes on the bolus structure, affecting both the glucose release and glucose diffusion during the digestion of starch-based products. Kozu et al. (2010) investigated the effect of intra-gastric flow on food digestion using computational fluid dynamics. They analyzed the flow phenomena induced by gastric peristalsis with different fluid viscosities, focusing on

the antral contraction wave (ACW), as the antrum is considered to act as a grinder and mixer of the swallowed foods and a pump for gastric emptying. The study showed that the flow of the model gastric contents greatly changed in and close to the region occluded by ACW. Thus, the gastric fluid motions induced by peristalsis would promote the mixing of digestive enzymes with the gastric contents. Furthermore, the introduction of mixing increases both glucose release and glucose diffusion (Dhital, Dolan, Stokes, & Gidley, 2014).

1.4 Intestinal Absorption

The chyme is transported to the small intestine, where the starch is predominantly hydrolyzed (~80%) to maltose, maltotriose, α-limit dextrins, or small glucose polymers by pancreatic α-amylase from pancreatic secretions. These end-products are further hydrolyzed to glucose by intestinal epithelial enzymes (Dhital, Warren, Butterworth, Ellis, & Gidley, 2017). In addition, the intestinal motility process is generated by three movement patterns: peristalsis, segmentation, and pendular movements, which induce the mechanical digestion of starch. Peristalsis causes propulsions that move the intestinal content in the anal direction, and segmentation contractions cause mixing to promote absorption of nutrients and water. Finally, the starch is absorbed in the form of glucose in the epithelium into the bloodstream, through the brush border of the epithelial cells. In general terms, the absorption of glucose can be represented by convection and diffusion processes across the intestinal wall. Convective transport can be considered as a result of the flow induced by the intestinal movements that transports and mixes chyme along the intestinal lumen. Regarding the rate of movement of material from high to low concentration by diffusion, it can be described mathematically by Fick's laws of diffusion.

The small intestine can be divided into three parts: duodenum, jejunum, and ileum. The entry of chyme into the upper portion of the duodenum triggers a key event in the beginning of the intestinal phase of nutrient digestion. In this part, the pancreatic secretions (enzymes, bicarbonate, and water) are secreted due to the presence of chyme by pancreatic glands and epithelial cells. The characteristics of pancreatic secretions related to viscosity, pH, and the flow rate/activity of enzymes depend on the type and the amount of starch present in the chyme. Hence, again when in vitro gastrointestinal methods are used to study starch digestion it is important to understand mass transfer in the small intestine, as both biological and engineering approaches are determining aspects to control and achieve realistic results correlated with in vivo results. Regarding the enzymatic activity of pancreatic α-amylase, some molecules present in foods have been shown to inhibit α-amylase in the intestine. Studies on humans have shown that natural α-amylase inhibitors isolated from wheat significantly reduced glucose absorption and the peak of postprandial glucose in healthy and type 2 diabetic subjects (Lankisch, Layer, Rizza, & DiMagno, 1998; Slavin, 2004). The plant forming part of the starch-based foods have chemical constituents with the potential to inhibit α-amylase activity. For

example, the chemical structures of flavonoids and polyphenols have been shown to inhibit α-amylase activity and can reduce blood glucose levels after starchy foods have been eaten. This could be potential constituents for controlling type 2 diabetes (De Sales, De Souza, Simeoni, Magalhães, & Silveira, 2012; Lo Piparo et al., 2008; Nyambe-Silavwe & Williamson, 2016). Likewise, the characteristics of intestinal motility have proven to be relevant to modulate starch digestibility (Jaime-Fonseca, Gouseti, Fryer, Wickham, & Bakalis, 2016). Gouseti et al. (2014), developed in vitro intestinal models to study the effect of gut motility on the accessibility of glucose from model solutions, using a range of food hydrocolloids (guar gum, carboxymethyl cellulose, pectin), and showing how mass transfer has an influence on nutrient bioaccessibility. The study showed that the presence of gum guar and pectin have a significant effect in retarding simulated glucose accessibility, and these results appear to be more pronounced at viscosities levels of around 0.01 Pa·s. Systems with lower viscosities showed enhanced mass transfer levels. The data were analyzed using engineering principles and dimensionless numbers that characterize the fluid flow (Reynolds number) and mass transfer (Sherwood number) in the gut. The flow behavior can be determined by the velocity of the peristaltic flow and the physical properties of chyme. Sherwood numbers represent the ratio of convective to diffusive mass transfer coefficient. The absorption of the glucose involved transportation from the lumen (chyme) to the dialysis membrane, passing through the membrane, and transfer to the recipient fluid. This three-stage process was characterized by the luminal mass transfer coefficient, (K_{lumen}, m/s), diffusion (described by coefficient D_{membrane}, m^2/s) through the membrane of thickness Z_{membrane} (m), and the recipient side's mass transfer coefficient (K_{rec}, m/s). The following equation offers the relationship between the local and overall transfer coefficient.

$$\frac{1}{K_{\text{overall}}} = \frac{1}{K_{\text{lumen}}} + \frac{Z_{\text{membrane}}}{D_{\text{membrane}}} + \frac{1}{K_{\text{rec}}} = \frac{1}{K_{\text{lumen}}} + \frac{1}{K_{\text{system}}} \tag{5}$$

In addition, it should not be forgotten that emotional stimuli intrinsic to the individual also influence the flow of gastrointestinal secretions, and can therefore affect starch digestion in different ways.

2 Influence of Food Composition on the Variability of Starch Digestion

The nutritional quality of starch is associated with its rate of digestion and glucose absorption. Starch bioaccessibility and glucose release may differ depending on starch structure and the form in which the food structure is disintegrated, some with starch being rapidly and others slowly digested. Three aspects of the food composition are important to highlight related to variability of starch digestion: source of starch, interaction of starch with other components, and the presence of dietary fiber.

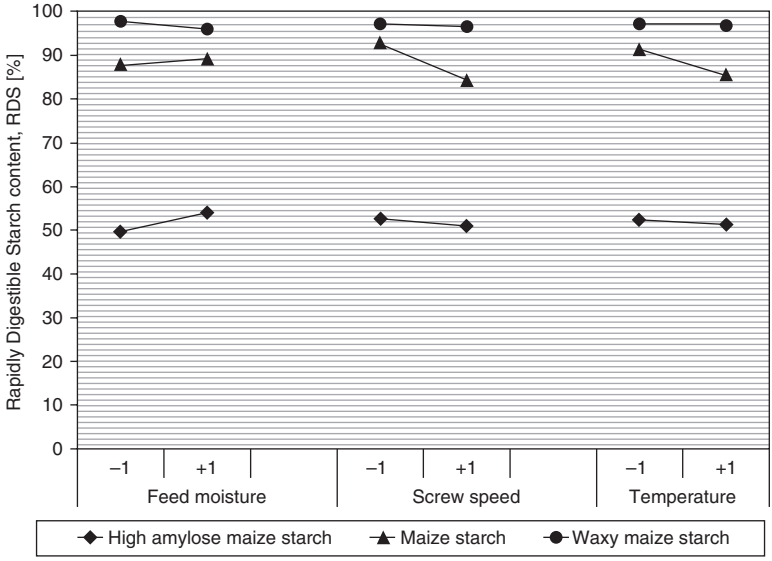

Fig. 7 Rapidly digestible starch, RDS (%) of high amylose maize starch (67% amylose content), maize starch (28% amylose content), and waxy maize starch (6% amylose content). The distance between two grid lines represents the least significant difference (LSD) [extracted from Robin et al. (2016)]

2.1 Source of Starch Granules

Significant differences in the rate of digestibility and metabolic responses of starch-based products have been associated with botanical sources of starch. The amylose and lipid content of the granules varies, forming starches with high amylose or high lipid content. An increase in amylose content has been correlated with slower digestibility (Frei, Siddhuraju, & Becker, 2003; Robin, Heindel, Pineau, Srichuwong, & Lehmann, 2016) (see Fig. 7). In addition, the amylose–lipid complex reduces susceptibility to α-amylase. Thus, complexed amylose may help to decrease the glycemic response of a food product (Hasjim, Ai, & Jane, 2013).

Wheat starch is a major dietary source and widely incorporated in processed products such as snacks that have a high rate of consumption in the human diet. The digestibility of white wheat bread is a typical example; in fact, this processed food is used as a reference during glycemic responses assays in the same way as glucose solutions (Englyst et al., 1999). Chemical characteristics related to amylose content (waxy, normal, or high amylose) can regulate starch digestibility. Chen et al. (2016) concluded that the gastrointestinal digestion rate of waxy wheat starch was higher than that of normal wheat starch in the initial stages, and that a higher degree of crystallisation limited the digestion rate and extent. Thus, the bioaccessibility of wheat starch has critical implications for its digestion.

The particle size of wheat grain is determinant forthe digestion rate of starch and consequent glucose responses. Heaton, Marcus, Emmet, and Bolton (1988) demonstrated that in vitro starch hydrolysis by pancreatic α-amylase was faster with decreasing particle size, and the peak of postprandial plasma glucose was greater for fine-flour wheat than that for cracked or whole grains.

Regarding potato starch, this has a large granular size (<100 μm) and a concentration of covalently bound phosphate in the amylopectin molecules as phosphate monoesters and phospholipids (Singh, Singh, Kaur, Sodhi, & Gill, 2003). Some studies have provided evidence that raw potato starch shows a reduced susceptibility to the action of amylase, due in part to its large granular size, based on the idea that it is only the surface of the granule which is available for initial hydrolysis (Cottrell, Duffus, Paterson, & Mackay, 1995); and the presence of phosphate groups with a high B polymorph content, although these can be modified by processing and storage. Warren, Zhang, Waltzer, Gidley, and Dhital (2015) demonstrated that native potato starch was digested slowly and required more enzymes than maize to achieve complete digestion. Potato starch granules can be completely digested in vitro given enough enzyme and time, demonstrating the likely dependence of in vivo resistant starch levels on endogenous enzyme activity and the small intestinal passage rate, either or both of which may vary between foods and/or between individuals. García-Alonso and Goñi (2000) confirmed that boiled and mashed potatoes showed the highest rate of digestion among raw flakes, oven-baked, French-fries, crisps, and retrograded potato starch.

Legumes have acquired significant nutritional interest due to their rate of starch digestion being lower in both in vitro and in vivo, compared to other starch sources such as cereals. Their reduced bioavailability of starch has been attributed to the presence of high levels of amylose (30–65%), a high content of viscous dietary fiber components, the presence of antinutrients and B-type crystallites (Tharanathan & Mahadevamma, 2003). These differences in their structural characteristics, as well as their content of resistant starch, shows a slight reduction after processing in comparison to their raw form, which could allow them to be used as an alternative source of resistant starch.

2.2 Interactions of Starch with Other Components

Most starch-based foods offer different metabolic responses even when they are processed under similar conditions. These variances have been attributed to interactions between starch with other food components such as proteins, lipids or sugars during processing.

For instance, starch–gluten products such as bread are mainly processed by baking. In these types of product, gluten proteins (gliadins and glutenins) play a key role in determining the baking quality of bread by conferring water absorption capacity, cohesiveness, viscosity, and elasticity on dough (Wieser, 2007). During processing, the proteins may physically become embedded in the starch, and inter-

actions are a consequence of the attraction between positively and negatively charged colloids due to the physical inclusion of starch in the gluten network (Delcour et al., 2000). Hence, starch–protein interactions may impair α-amylase access and modify starch digestibility. The ability of starch–gluten interactions to influence the bioaccessibility of starch depends on the characteristics of the protein matrix and the degree of interaction. Previous studies have shown that in vitro digestibility of starch and its glycemic response increases when the starch–gluten interaction is disrupted by sheeting passes or the mixing of dough (Kim et al., 2008; Parada & Aguilera, 2011a, b).

Other important food components that can interact with starch during processing are lipids, which mainly interact with the amylose molecules that affect the susceptibility of starch to hydrolysis. The formation of amylose–lipid complex is given by the ability of amylose to form inclusion complexes with polar lipids (e.g., monoglycerides, fatty acids, or lineal alcohol) during heat processing. The processing temperature influences the type of complex formed and the time required for complexation. Two types of complexes can be formed: (1) complexes with an amorphous structure (form type I) that melt at a lower temperature in a differential scanning calorimetry (DSC) (10–30 °C) and (2) complexes with a crystalline structure (form type II) giving rise to the V-pattern in X-ray diffraction, although structure type I is not detectable using this technique. In addition, the formation of amylose–lipid complex modifies the starch properties and functionality. Solubility in water and swelling capacity are reduced, retrogradation is retarded, and susceptibility to enzymatic hydrolysis is reduced (Parada & Santos, 2016). Starch digestibility is reduced as complexed amylose becomes more resistant to digestive enzymes than amylose, and decreases the swelling capacity of starch granules. Consequently, there is less opportunity for enzymes to gain access to the granule interior and less amylose leeches from the granules. Therefore, the rate and the extent of hydrolysis of amylose–lipid complexes has been inversely related to the degree of organization of helices into the aggregated structure, and complexes with greater crystallinity are more resistant to enzymatic degradation (Seneviratne & Biliaderis, 1991). Some authors have reported that the complex formation reduced the digestibility of freshly gelatinized starch but increased the enzyme susceptibility of stored starch, by competing with amylose retrogradation (Cui & Oates, 1999). Furthermore, it is important to consider the time factor when making digestibility analysis of lipid-rich products, which might affect the true results.

On the other hand, the presence of sugar influences the gelatinization by competition of available water in sugar–flour water systems, where sugar solubility may be an important factor affecting gelatinization temperature in a limited water system (Pareyt & Delcour, 2008). When the sugar content increases the gelatinization temperature increases and the degree of starch gelatinization decreases (Hesso et al., 2015). Accordingly, previous studies have demonstrated that the lower gelatinization of starch involves a delay in starch digestibility.

Dietary fiber may also have an effect on starch digestibility. The effect of soluble dietary fiber on starch digestibility is mainly attributed to increasing the bolus viscosity once it has reached the small intestine, which is where the absorption of

glucose occurs. This high viscosity delays glucose absorption. Some studies have demonstrated that increased β-glucan intake improves glycemic control, indicating it should be considered as a complementary mechanism in the treatment of patients with type 2 diabetes (Jenkins, Jenkins, Zdravkovic, Würsch, & Vuksan, 2002). In addition, Oh, Bae, and Lee (2014) established that under in vitro starch digestion, decreasing levels of the inulin ratio in cakes resulted in a decrease in rapidly digestible starch values. Interestingly, different types of soluble fiber have varying effects on viscosity, and some studies have shown no correlation in all types of fiber between high fiber content and reduced risk of diabetes, demonstrating that the mechanics by which the hydrolysis of starch can be delayed are influenced by intrinsic and extrinsic factors. Viscous fibers incorporated in food not only increase the viscosity of the lumen but may also protect starch from enzymatic attack (Gouseti et al., 2014). Furthermore, the presence of insoluble dietary fiber in complex food systems has been associated with contributing to the control of diseases such as obesity and diabetes, mainly due to the beneficial nutritional effects on satiety and glycemic responses (Zhang & Hamaker, 2016). Thus, the compositional differences link to white or whole-wheat flour influences the rate of starch digestibility. Comparative studies between refined and whole grains (containing the outer part of the bran) have demonstrated that whole grains of wheat with a high content of dietary fibre helped lower the risk of diabetes mellitus (Liu et al., 2000).

3 Structural Aspects of Food and Starch Digestibility

Microstructural aspects of food can influence the digestion of starch. The bioaccessibility and bioavailability of starch is affected by the food matrix, influencing enzymatic functions and the residence time in the human stomach or intestine. Accordingly, transformations of the food matrix and hormonal regulation mechanisms can dominate the rate and extent of glucose release during gastrointestinal transit (Parada & Aguilera, 2011a, b). Some microstructural aspects in solid starchy foods and interrelated transformations are represented in Fig. 8, which involves multiple reactions, mass transport, and glucose control mechanisms.

The main microstructural characteristics are linked to starch transformations, particle size that can be obtained after the masticatory process, entrapment of starch granules in the matrix, and viscosity and pH provided to the bolus by the components forming the food matrix. Also, the physical texture (associated with the hardness or density of the food) seems to have an impact on the availability of starch for enzymatic digestion. As already discussed, the degree of starch gelatinization drastically modifies the structure of materials in which the granules are entrapped, and the digestion of starch and absorption of glucose within the digestive system. Likewise, physical properties of the food bolus can be altered, providing a more/less accessible structure, and so affecting the motility and susceptibility in the specific activity of amylolytic enzymes. In addition, the hardness of a starchy food influences the particle size of its bolus. Chen, Khandelwal, Liu, and Funami (2013)

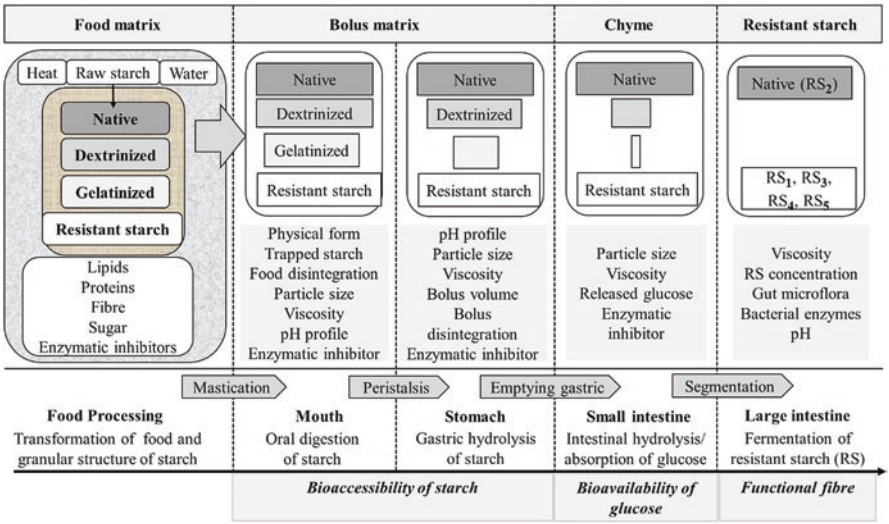

Fig. 8 Microstructural aspects in starchy food and interrelated transformations involving multiples reactions, mass transport, and glucose control mechanisms during digestion of starch [adapted from Parada and Aguilera (2011a, b)]

studied the physical properties of food boluses, in particular the bolus particle size distribution in relation to the hardness of the food. It was observed that bolus particle size decreased with the increase of food hardness (in cheese, peanuts, or cashew nuts). The correlation between these two properties could be described by a power–law relationship. Similarly, Alam et al. (2017) observed that the addition of 10% rye bran had a significant effect on the structural, textural and mastication properties both for puffs and flakes. The addition of rye bran increased hardness, decreased crispiness, and increased the hydrolysis index of puffs and flakes to 89.7 and 94.5, respectively, which was probably attributable to the increased number of particles in the bolus. This was noticeable in the early phase of digestion, i.e. at 30 min, indicating that the disintegration process and consequently the particle size of the bolus had an important role on the starch digestion rate. It is important to mention that particle size is also influenced by inter-individual variability. Le Bleis, Chaunier, Montigaud, and Della Valle (2016) proposed that bolus consistency could be expressed by:

$$K = K_0 \exp\left[-\alpha \beta \left(\frac{Q_s}{W_0} \right)^{n2} t^{(1+n1)^{n2}} \right] \tag{6}$$

This equation provided a basic model to describe the disruption of bread at different stages of mastication, where K_0 is the initial consistency index of the bread (Pa s^n); α is the plasticization coefficient (non-dimensional); Q_s is the stimulated salivary

flow (L min^{-1}); t is the chewing time (min); w_0, represents the median particle size (mm); and the other coefficients [β, adjusted coefficient for salivation (non-dimensional); $n1$ and $n2$, adjusted exponents for fragmentation and salivation, respectively (non-dimensional)] were obtained through fitting of experimental results for breads enriched with fibers and examined in this study. The study showed that bolus consistency decreased with chewing time, and this decrease was linked to bolus moisture by a plasticization coefficient, which varied according to each individual. Thus, the consistency of the bread directly influenced bolus disruption assessed by changes in viscosity.

Starch granules entrapped in the food matrix (e.g., plant cell, gluten network or encapsulation), seem to be another mechanism that hinders the physical accessibility of starch and the diffusion of amylolytic enzymes in the starchy products. Bhattarai, Dhital, Wu, and Gidley (2017) observed that the rate and extent of hydrolysis of starch and protein were greatly increased when the cell wall physical barrier was removed by either mechanical or enzymatic processes. The authors used an in-vitro dynamic model to observe that isolated legume cells have sufficient mechanical strength to survive mixing conditions in a simulated rat stomach–duodenum. Also, the cell wall could limit digestibility by restricting starch gelatinization during cooking, as water transfer (amount of liquid water molecules) into the cell restricts the swelling of starch granules. Therefore, the use of whole grains (e.g., wheat, oat, barley, rye) in starchy products may result in low glycemic responses due to the preservation of food particles in the gastrointestinal tract. This is because hard solid foods are emptied more slowly from the stomach than soft foods.

Pasta products have shown slow and progressive starch breakdown and release of sugar in the body, leading to low postprandial blood glucose and insulin responses (Bjorck, Liljeberg, & Ostman, 2000). These wheat-based products vary in flour variety, shape, type of drying, and proportion of protein added in their formulation, promoting low glycemic responses. Pastas are prepared using durum wheat flour, however it is also possible to incorporate dietary fiber ingredients and hydrocolloids to increase their nutritional value. Sheeting, extrusion, drying, and cooking processes confer the formation of different pasta structures by successive structural changes of two main components, that is, starch and proteins, which provide a potential to regulate the glycemic response of cereal foods. Thus, major structural transformations occur during the cooking stage. Fardet, Hoebler, Bouchet, Gallant, and Barry (1998) established that the presence of a structured and continuous protein network is an important factor in explaining the slow degradation of starch in pasta. The authors proposed that the action of α-amylase may be limited at various levels: (1) by the restricted accessibility and porosity of food structure; (2) by the tortuosity of the protein matrix; (3) by the possible interactions of the α-amylase with the protein matrix; and (4) by the structure of the starch granules in pasta; demonstrating that the physical texture of starch-based food is a determinant factor for bioavailability of starch in human digestion.

Furthermore, the modification of food structures with the addition of hydrocolloids (in order to modify rheological and textural aspects) may also have an effect. Hydrocolloids influence the digestion and absorption of available carbohydrates in

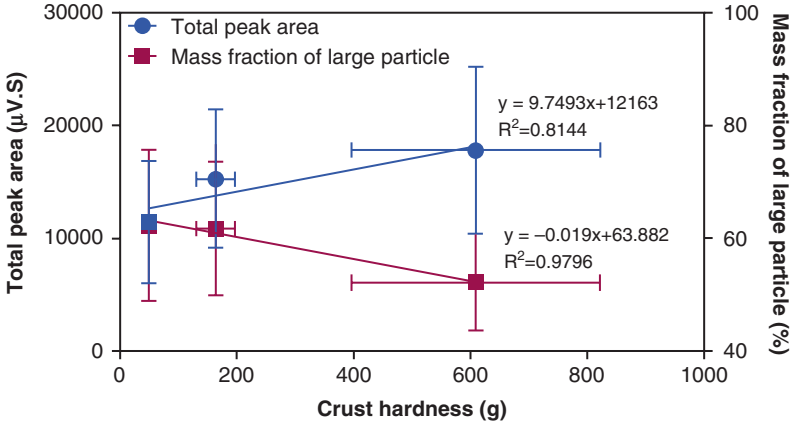

Fig. 9 Correlation plots between total peak area, mass fraction of large particles, and crust hardness [extracted from Gao et al. (2015)]

various ways. For instance, oat β-glucan in breads reduces the glycemic index (GI) and glucose peak by 32–37% compared to a white wheat reference bread, and is suitable for use in the baking of bread products (Ekström, Henningsson Bok, Sjöö, & Östman, 2017). The addition of β-glucan into sugar cookies increased their attribute of hardness, while affecting biscuit texture in turn decreased the carbohydrate degradation and the rate of glucose absorption (Brennan, Samyue, & Abbot, 2004). β-Glucan increased intestinal viscosity and delayed gastric emptying, which resulted in a reduced rate of α-amylase action and reduced intestinal nutrient uptake (Thondre, Shafat, & Clegg, 2013). Likewise, the use of viscous soluble fiber as Psyllium improves glycemic control in patients with type-2 diabetes mellitus (Feinglos, Gibb, Ramsey, Surwit, & McRorie, 2013).

On the other hand, bread with various structures and textures provides different chewing behavior and bolus characteristics, affecting the release of glucose. It has been observed that there is an inverse relationship between food moisture content and saliva secretion. Also, in the case of harder bread, the swallowing threshold of particle size is smaller (see Fig. 9). The larger force and longer time for bread with hard and dry crust during oral processing, resulted in turn in an extensive breakdown of the bread structure, which may contribute to the higher digestibility of bread with a lower moisture content (Gao, Wong, Lim, Henry, & Zhou, 2015).

4 Process Design for the Control of Starch Digestion

Processed starchy foods are subjected to thermal processing to obtain desired properties related to texture, quality, or nutritional aspects. Under processing, the initial structure of the food undergoes physical, mechanical and chemical

transformations, affecting the form and structure of the final food product, and the physical state of the starch granules that form part of it. Starch gelatinization is promoted with the presence of liquid water and high temperature (>65 °C) (Biliaderis, Maurice, & Vose, 1980; Eliasson, 1980). This phenomenon seems to have an important influence on starchy food digestibility. Native starch shows slow hydrolysis in contrast to partially or completely gelatinized starch, which shows a faster rate of hydrolysis. This suggests that during processing native granules lose their crystalline structure and become amorphous, thus facilitating the action of amylolytic enzymes. Therefore, it is interesting to understand the nutritional implications of gelatinization on starch digestibility, and how digestion can be limited by processing conditions.

4.1 Formation of Resistant Starch During Processing and Its Relationship with Slow Starch Digestion

Resistant starch (RS) is a physiological concept that was initially defined as the fraction of starch that was not hydrolyzed after 120 min of incubation with α-amylase (Englyst et al., 1992; Sajilata, Singhal, & Kulkarni, 2006). However, it is now considered to be the fraction of starch and products of starch degradation that escape digestion in the small intestine of healthy individuals. Five types of RS have been established from RS_1 (type I) to RS_5 (type V) (Birt et al., 2013; Fuentes-Zaragoza, Riquelme-Navarrete, Sánchez-Zapata, & Pérez-Álvarez, 2010).

In RS_1, the starch granule is physically inaccessible to digestion due to its entrapment in a matrix (e.g., grains, seeds, or food structure). In RS_2, the resistance to digestion is because the starch granule is in a granular form (e.g., compact structure of granules such as ungelatinized resistant granules with type B- or C-polymorphism of crystallinity). Ungelatinized granules are tightly packed in a radial pattern, which limits the accessibility to digestive enzymes during hydrolysis. RS_3, represents retrograded amylose formed during the cooling of gelatinized starch. It can be formed when starch-based foods are thermally processed with enough water and then cooled. Starch polymer chains begin to reassociate as double helices and can form tightly packed structures stabilized by hydrogen bonding. In RS_4, the resistance to digestion is given by the formation of novel chemical bonds (e.g., cross-linking with chemical agents). This type of RS includes chemically modified starches. The last is RS_5, which represents amylose–lipid complexes. Resistant starches added to food matrices for health benefits are classified as *functional fiber* by AACC (American Association of Cereal Chemists, 2001). In contrast to RS that is naturally found in foods, it is considered *dietary fiber*. The dietary fiber definition committee also reported that RS as a constituent of dietary fiber should be resistant to digestion in humans and this should be assessed using methods that include gelatinization steps to simulate cooking and processing (American Association of Cereal Chemists, 2001).

RS can be naturally found in significant levels in grains, seeds, legumes, tubers, or unripe bananas. Some reasons for higher RS content are the crystallinity pattern of the starch, the content of amylose, and ungelatinized starch. Tubers such as potatoes present a B-type pattern of crystallinity and legumes have a C-type pattern. Bananas are a fruit consumed in a raw form, conserving a high content of ungelatinized starch (RS_2) with a mixture of A-type and B-type patterns of crystallinity depending on the varietal source (Zhang, Whistler, BeMiller, & Hamaker, 2005). The B-type structure is hydrolyzed more slowly than the A-type by α-amylase, β-amylase, and glucoamylase: this is probably due to surface area effects (Williamson et al., 1992). Furthermore, the packing mode of the helices and water content are different in the two polymorphs.

Resistant starches differ in their composition and structure, and the effects of processing and storage on each type need to be analyzed separately. Different RS can be generated during the processing of foodstuffs as such treatments modify the normal starch granules and may promote RS formation or decrease its initial natural content. For instance, thermal treatment (e.g., drying or extrusion) decreases the presence of natural RS in products rich in starch, depending on their botanical source. This is mainly because RS content decreases with increasing starch gelatinization, and therefore depends on the severity of the heat treatment and availability of liquid water. RS formation may be increased due to starch retrogradation during storage. In fact, keeping bread at room temperature for 3 days seems to be the best way to further increase RS content [over 26%, as determined by Amaral, Guerreiro, Gomes, and Cravo (2016)]. The water content in the dough seemed to influence the extent of RS formation. The formed RS can be attributed to highly retrograded amylose fraction (RS_3). Tharanathan and Tharanathan (2001) isolated RS from wheat-based products: purified RS was a linear 1,4-linked α-D-glucan, which is derived from the highly retrograded amylose fraction of starch. Numerous studies have documented that RS provides benefits for health associated with decreasing levels of glycemic response, which can modulate blood-glucose levels (Behall, Scholfield, Hallfrisch, & Liljeberg-Elmstahl, 2006). Also, RS can be fermented by the colonic microflora and produce short-chain fatty acids that provide the same physiological response as functional fiber (Lattimer & Haub, 2010; Topping & Clifton, 2001).

4.2 Processing Conditions Over Starch Gelatinization

In the industry, starch-based products are dependent on the proper gelatinization of starch to produce a desirable texture and mechanical properties. Native starch granules have ordered structures that are semi-crystalline and birefringent. During thermal processing, starch granules suffer severe transformations by high temperature and the presence of water. This order–disorder phase transition (gelatinization) is associated with swelling of the granules (diffusion and water uptake by the amorphous zones), disruption of ordered structures (crystalline and molecular), and solubilization of the micellar network (amylose leaching) (Lelievre & Liu, 1994). The

extent of gelatinization determines the susceptibility of starch to enzymatic digestion, as well as the extent of retrogradation (where the starch returns to the granular state). Incomplete gelatinization permits low starch hydrolysis and slows glycemic responses of starch-based products (e.g., bread, pasta, or potato chips) (Holm, Lundquist, Björck, Eliasson, & Asp, 1988). Understanding how the degree of gelatinization can be limited might allow the starch digestion and consequent glucose absorption to be modulated.

The most drastic effects on starch gelatinization are governed by temperature, although there are also moisture-dependent interactions. In a complex and non-homogenous food system, physical barriers between granules and water molecules can hinder heat transfer and water diffusion, influencing the kinetics of starch gelatinization. Water must pass through the resistance of the matrix (surface or internal), hence gelatinization takes place in parts of the food where the water content and temperature range are high enough (e.g., >30% (w/w) water content, for starch–water systems). In baked foods (biscuits or bread) or pasta with limited water through processing (flour to water ratio about 0.3–0.6), gelatinization occurs at low levels of moisture content and when the range of gelatinization temperature is extended (Schirmer, Zeller, Krause, Jekle, & Becker, 2014).

Likewise, most foods rich in starch are processed by means of boiling, baking, extrusion, or frying, which can promote or limit water conditions, and in which the starch can be partially or totally gelatinized. The low water content during processing limits the degree of gelatinization. In dough matrices, there is competition among the components for the available water, and the degree of starch gelatinization will depend on the distribution of water in the system and the water activity on the colloidal components. De la Hera, Rosell, and Gomez (2014) studied the impact of dough hydration levels on in vitro starch hydrolysis of the rice flour used in gluten-free bread. The results indicated that the estimated glycemic index was higher in breads with higher hydration (90–110% water content). Reduction of dough hydration limited starch gelatinization and hindered in vitro starch digestibility. One explanation is that increased water content during the thermal process promotes regions of amorphous starch, so making an attack by α-amylase more favorable (Roder et al., 2009).

Furthermore, gelatinization is promoted under acidic environmental conditions, although this could be conditioned by starch type and source. For example, Ohishi, Kasai, Shimada, and Hatae (2007) showed that the absorption and gelatinization of rice starch had been enhanced with the addition of acetic acid (0.2 M). An acceleration of water absorption of starch by adding acetic acid promoted the hydration of starch, leading to the enhancement of starch gelatinization. Also, the authors suggested that the gelatinization process might be accelerated by the higher dissolution and degradation of proteins under acidic conditions. This explanation agrees with those studies using the Differential Scanning Calorimetry (DSC) technique, where acid preferentially attacks the amorphous regions in the granule and the transformation of crystalline regions is changed, the crystallites becoming decoupled and no longer destabilized by the amorphous parts. Consequently, starch crystallites of acid modified starches melt at a higher temperature and the transition temperature range

is broader. Furthermore, the addition of an alkali component has been found to significantly enhance the swelling of starch granules and expedites the gelatinization process (Wang et al., 2014).

Therefore, processing can offer alternatives for modifying the final nutritional characteristics of foods. Also, limiting gelatinization of starch appears to be a suitable option to modulate starch hydrolysis and the glycemic responses of starch-based foods. Studies applied to both temperature and pressure have demonstrated that the gelatinization temperature may be lowered by reducing the processing pressure. Thus, the extent of swelling and granule disintegration, as well as leaching of amylose, can be controlled. Various authors have shown that varying the pressure conditions in traditional food processing such as boiling, drying, or frying, allow some specific properties to be maintained such as color, antioxidant capacity, the stability of specific compounds, or incomplete gelatinization. During the process of high-pressure technology, the gelatinization of the starch granules occurs differently from damage by high temperatures, although by applying enough high pressure it is possible to obtain complete starch gelatinization (Baks, Bruins, Janssen, & Boom, 2008). In contrast, in low-pressure processing there is less damage of the granules.

4.3 Low-Pressure Conditions Limiting Starch Digestibility

By means of pressure reduction during processing, it is possible to substantially lower the boiling point of product moisture in a low-oxygen environment, this is the main reason why vacuum technology is a recognized route to protect heat-sensitive foods during dehydration. Applications may range from some traditional ones, including vacuum evaporation in multiple effects, freeze-drying and vacuum drying, as well as some recent ones such as microwave vacuum drying and vacuum frying (Dueik & Bouchon, 2011). Vacuum frying refers to the deep-fat frying process that is carried out under pressures far below atmospheric pressure (Garayo & Moreira, 2002). The processing conditions markedly decrease the boiling point of water, reason why it corresponds to a vacuum dehydration process.

In order to compare vacuum and atmospheric frying, Mariscal and Bouchon (2008) defined the concept of equivalent thermal driving force, which is achieved by keeping a constant difference between oil temperature and the boiling point of water at the working pressure, according to Eq. (7):

$$\text{Thermal driving force} = \Delta T = T_{\text{oil}} - T_{\text{water boiling point at working pressure}} \tag{7}$$

Processing conditions under low pressure may affect the capacity of food building blocks to develop an adequate structure during processing, to provide the required quality attributes. This may be less relevant in raw materials that are already structured by nature, such as tubers, but still important. In formulated products, this may be critical, since specific changes are needed to create a structure. In starchy

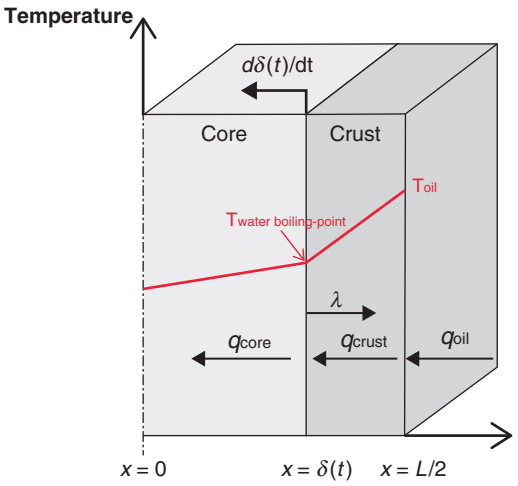

Fig. 10 Semi-slab during frying, showing the heat fluxes and the temperature profile (in red). The water front at $x = \delta(t)$ moves at $d\delta(t)/dt$ and is at $T_{water\ boiling\text{-}point}$ (function of P) [adapted from Bouchon and Pyle (2005)]

matrices, gelatinization is one of these critical steps, which requires the simultaneous presence of liquid water and temperature (above 55–60 °C). Frying is a complex unit operation that involves simultaneous heat and mass transfer, resulting in counter-flows of water vapor (bubbles) and oil at the surface of the piece. After immersion in the hot oil, the surface of the product is heated to the boiling point of water and the crust begins to form. As frying progresses, the evaporation front, which is at the boiling point of the interstitial liquid, will move towards the interior (moving front), delimiting two very well-defined zones: the crust and the core (Ziaiifar, Achir, Courtois, Trezzani, & Trystram, 2008). The crust is the result of several alterations that mainly occur at the cellular and subcellular level, where the temperature exceeds the boiling point of water (Bouchon & Aguilera, 2001). The temperature within the core, on the other hand, cannot exceed the boiling point of water, and thus holds liquid water. A diagram that reflects the aforementioned conditions, showing the temperature profiles in each region (red lines), the heat fluxes and the moving front, is presented in Fig. 10.

Accordingly, during vacuum frying, if the operating pressure defines a water boiling point that is lower to the one required for starch gelatinization (e.g., < 55 °C), starch gelatinization may be impaired. In fact, the crust region will be able to attain temperatures that are higher than the gelatinization temperature, but no liquid water will be left for gelatinization to occur. Conversely, the core region will have enough liquid water, but will be below the required temperature to induce starch gelatinization. Ovalle, Cortés, and Bouchon (2013) demonstrated that when the operational pressure was reduced up to 6.5 kPa, at a water boiling point of 38 °C, no starch gelatinization was observed during heating in water and oil, in situ and in real time, using vacuum hot-stage microscopy (see Fig. 11). This result was attrib-

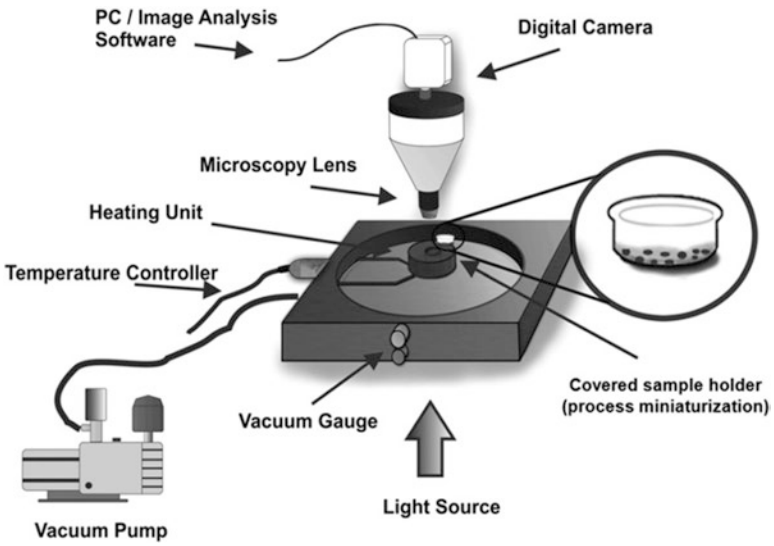

Fig. 11 Representation of vacuum hot-stage microscopy used for vacuum and atmospheric heating miniaturization [extracted from Ovalle et al. (2013)]

uted to the rapid evaporation of water before gelatinization was reached. In addition, when the amount of water was reduced the gelatinization process occurred in a broader range of temperatures.

Contardo, Parada, Leiva, and Bouchon (2016) assessed the effect of vacuum frying on starch gelatinization and in vitro digestibility of starch, in terms of the fractions of rapidly available glucose (RAG), slowly available glucose (SAG), and unavailable glucose (UG) fractions. The authors demonstrated that dough samples in the form of sheets, made of wheat starch (88% d.b.), gluten (12% d.b.), and water, and fried under vacuum (6.5 kPa, $T_{\text{water-boiling-point}} = 38$ °C), showed less starch gelatinization (28%), less rapidly available glucose (27%), and more unavailable glucose (70%) than their atmospheric counterparts (which presented 99% starch gelatinization, 40% rapidly available glucose, and 46% unavailable glucose, respectively), and that the values were close to those of the raw dough. Recently, they complemented their study, by assessing in vivo starch digestibility, after feeding Sprague-Dawley rats (Contardo, Villalón, & Bouchon, 2018). Results showed that vacuum-fried dough had a maximal blood glucose level at 60 min, indicating a slower glycemic response than that of samples fried under atmospheric counterparts (maximal blood glucose level at 30 min), as shown in Fig. 12.

On the whole, both in vivo and in vitro studies were consistent and suggest that starch digestibility can be altered through processing conditions by reducing the operating pressure.

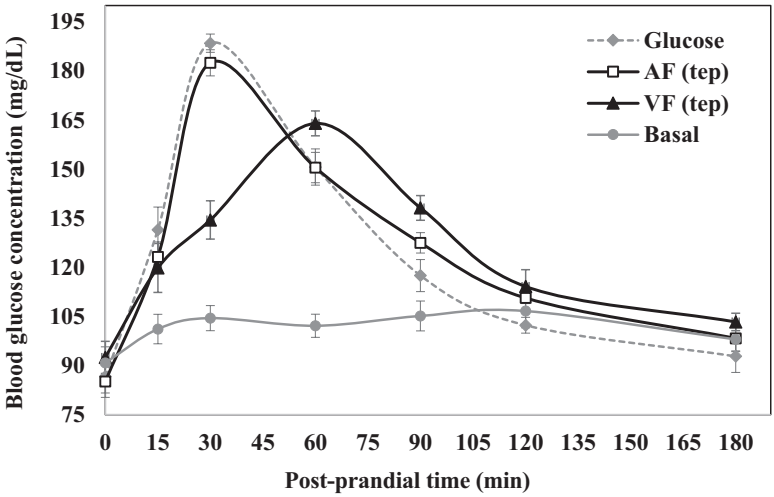

Fig. 12 In vivo starch digestibility expressed in terms of blood glucose concentration at various postprandial times in rats given matrices with different degree of starch gelatinization, from vacuum-fried (VF, 9.9 kPa) and atmospheric-fried (AF) dough after frying up to bubble-end point, as well as Glucose solution (1.2 g/Kg animal) as Control, and physiological serum as Basal [extracted from Contardo et al. (2018)]

5 Conclusions

The food composition and the structure of processed starchy products, as discussed in this chapter, may influence starch digestibility. The physical–chemical characteristics of starch ingested (interactions with other components of the food matrix, as well as transformations during processing) have a relevant impact on starch digestion, and the associated glycemic response. Starch interactions with other components may induce changes in the starch molecule (e.g., interactions between starch and lipids), reducing starch digestibility. Also, limitations of free water availability during processing can hinder the gelatinization process, precluding starch digestibility. Overall, the principles highlighted here may help in the development of strategies to modify starch-rich foods so as to reduce glycemic impact and improve the impact of consuming such foods on health.

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Part V
Concluding Remarks

Future Perspectives and Opportunities for Interdisciplinary Research on Food Digestion

Gail M. Bornhorst

Over the past several decades, research on the processes and mechanisms of food digestion has increased exponentially, although food digestion processes have been of general interest to human society for thousands of years, as discussed in chapter "A short history of digestion studies" A keyword search in Web of Science for "food digestion" yields only 11 publications in 1987, increasing to 206 publications in 1997, 422 publications in 2007, and 1513 publications in 2017. This increase in the literature exemplifies the growth of research on food digestion, yet interest in this area is still increasing as the links between food and health or disease have been recently demonstrated across both epidemiological and clinical studies (Gunter et al., 2017; Marco et al., 2017; Medina-Remón, Kirwan, Lamuela-Raventós, & Estruch, 2018; Pan, Lin, Hemler, & Hu, 2018; Tosti, Bertozzi, & Fontana, 2017). These human studies often result in correlations, but not causational relationships. Such correlations have led to mechanistic questions as to the fate of foods during digestion, and the beneficial or detrimental consequences derived through their consumption. These questions have prompted the growing body of work on food digestion using in vitro, in vivo, and in silico approaches, such as those discussed in chapter "In vivo, In vitro, and In silico Studies of the GI Tract".

Although our knowledge on food digestion has increased in recent years, the rapid growth of this area without corresponding development of standard terminology, digestion methods, and analytical procedures both between research groups and across the diverse fields tackling this multidisciplinary challenge have limited the comparison of results and discovery of novel insights that are widely applicable. Some of these challenges are discussed in more detail in chapter "Challenges in Quantifying Digestion". Due to advancements in analytical techniques and computational power, there are many opportunities for the future of research in food digestion, but there is still information necessary to advance the field and develop

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consistent standard methods across the research community, which can ultimately lead to development of transformative solutions to the questions arising around the contributions of food to health and disease.

1 In Vivo Models

Although the use of invasive in vivo studies on humans and animals may be declining due to ethical and resource constraints, there are great opportunities to utilize noninvasive techniques to gain new insights into the food digestion process. As discussed in chapter "Tools/Methods for Quantifying Digestion: Medical Imaging Aspect", new medical imaging technologies have transformed the way physiological processes can be observed in humans. These technologies include radiographic (e.g., X-ray), nuclear (e.g., positron emission tomography, magnetic resonance imaging), and ultrasound.

In the future, it will be critical to utilize these noninvasive medical technologies to help understand not only the processes of food digestion for various meals, but also the impact of the meal properties on physiological parameters, such as gastric secretions and gastric emptying. There are considerable opportunities for integration of multi-length scale imaging into both in vivo and in vitro digestion studies. One example is micro-computed tomography, where X-ray images are generated at a resolution of <100 μm compared to ~300 μm for conventional computed tomography methods (Schoeman, Williams, du Plessis, & Manley, 2016). Similarly, microscopic magnetic resonance imaging methods have been developed that allow for resolutions down to ~3–4 μm in length (Ciobanu, Seeber, & Pennington, 2002). Combination of these micro-scale imaging techniques with conventional imaging and property measurements will enable a more comprehensive understanding of the multi-scale aspect of the food digestion process, such as those properties discussed in chapters "Exploring and Exploiting the Role of Food Structure in Digestion", "From Bite to Nutrient: The Importance of Length Scales", and "Quantifying Digestion Products: Physicochemical Aspects".

In addition to these imaging technologies, it is likely that the use of ingestible, wireless sensors will increase, as their size decreases and the technical capabilities of the sensors increase. In addition to the use of wireless motility capsules, as discussed in chapter "In vivo, In vitro, and In silico Studies of the GI Tract", novel uses of ingestible sensors are an area of current research in the biomedical and electrical engineering fields. Previously, wireless motility capsules, such as the commercially available SmartPill® have been utilized to monitor the intra-gastrointestinal pH, pressure, and temperature profiles in subjects for up to the sensor's 5-day battery life (Maqbool, Parkman, & Friedenberg, 2009). Such capsules have also been equipped with cameras that can capture images of the entire gastrointestinal tract of a patient without the need for invasive procedures.

However, there have been recent advances in ingestible sensors that have expanded their potential capabilities to a wider range of applications. These applica-

tions include ingestible sensors that may be used to monitor adherence to a specific drug or dietary regimen (Hafezi et al., 2015), sensors for intraluminal gases, including oxygen, hydrogen, carbon dioxide, and methane, sensors that can detect changes in color of luminal tissues, and even those that could monitor specific electrochemical signals (Kalantar-zadeh, Ha, Ou, & Berean, 2017). Capsules have also been developed that can be activated to deliver a drug or other functional cargo at a specific location within the gastrointestinal tract (van der Schaar et al., 2013). With sensor development and testing for these different products underway, their integration into future in vivo studies will be critical to increase our understanding of specific changes in the physiological environment at different locations in the gastrointestinal tract.

In the future, these advances in imaging and sensing may help increase our understanding of changes in physicochemical properties of food during its passage through the gastrointestinal tract in humans without the need for invasive testing. Such information could also inform individual-specific responses to consumption of certain food products, knowledge that is necessary for future development of personalized nutrition regimens. Along with this information, specific consumer perception and behavior will be critical to integrate into future food development, as discussed in chapter "Consumer Psychology and Eating Behaviour".

In addition to strengthening our understanding of specific digestion processes and individual responses to consumption of certain foods in humans, it will be critical to integrate representative animal models into future research, such as those discussed in chapter "Tools and Methods to Quantify the Digestion of Protein, Lipid, Starch and Fibre from a Chemistry/Microbiology Perspective". Appropriate utilization of animal models can not only increase our understanding of food digestion processes, but may also be applicable to animal health and disease prevention. This concept is illustrated in the "one-health" approach, where advances in animals and humans are treated synergistically to tackle both animal and human health challenges (Rock, Buntain, Hatfield, & Hallgrímsson, 2009). Although this approach has not been a focus of food digestion research, future interactions with animal scientists, veterinarians, and clinicians will be critical to integrate knowledge across fields and work towards future innovations.

2 In Vitro Models

Due to their ease of use and lower cost compared to in vivo studies, in vitro digestion models will likely continue to play a key role in the advancement of knowledge on food digestion. In vitro models allow for mechanistic studies that help to understand specific factors that are important in food digestion, both from a food and a physiological standpoint, as discussed in chapters "In vivo, In vitro, and In silico Studies of the GI Tract", "Quantitative Characterization of Digestion Processes", and "Tools and Methods to Quantify the Digestion of Protein, Lipid, Starch and Fibre from a Chemistry/Microbiology Perspective" There are opportunities in the

development of in vitro model systems, both to develop systems with increasing complexity that come closer to physiological reality and to develop simple systems that can be easily implemented but still provide useful information. These models also need standardization across the research community to allow for comparison of results, as well as enhanced and standardized in vitro–in vivo correlations to increase their applicability across food products and for potential future regulatory uses.

Since the gastrointestinal tract is a complex system, as described in chapter "The Digestive Tract: A Complex System", it is nearly impossible to encompass all of the intricate elements food digestion into an in vitro model system. There is some question as to how much of the complexity is necessary to include in an in vitro model system, depending on the desired results and application. However, developments in the next-generation of in vitro model systems will likely incorporate advanced instrumentation, such as real-time quantification and control of pH, temperature, secretions, and feedback loops to modulate these parameters based on digesta properties, such as rheological properties or biochemical composition.

In addition, the configuration of the gastrointestinal organs, such as the stomach, esophagus, or intestine, may be developed using 3D printing of flexible polymers to more adequately mimic the interior tissue structure and overall shape of human or animal organs. These developments are possible due to recent advances in 3D printing technology, which was previously limited to certain structures and materials, but has now been applied to different polymers and has even been applied to develop scaffolds that can be utilized for growth of various cell lines (Chia & Wu, 2015). The use of 3D printing techniques in the advancement of in vitro models may be utilized either to create realistic or personalized gastrointestinal vessels, which could be based on specific CT scans, or even to develop similar systems that, for example, encompass the three-dimensional microstructure of the intestinal lumen and are colonized with appropriate cell lines.

Complementary to the development of sophisticated "near-real" in vitro digestion models that encompass the latest instrumentation and structural features, it will also be important to develop simple systems that can be utilized to broadly categorize the behavior of a wide variety of food products, such as the Food Breakdown Classification System (Bornhorst, Ferrua, & Singh, 2015; Drechsler & Bornhorst, 2018). Such systems, while admittedly lacking the complexity of the in vivo environment, have extensive potential to be utilized in food product development if it is not practical to utilize specialized in vitro systems or in vivo studies for many product iterations. If these type of simple systems are developed with appropriate in vitro–in vivo correlation data and a standard methodology that can be widely applicable to different types of foods, they may provide a powerful tool for the food industry, similar to the Biopharmaceutics Classification System utilized in the pharmaceutical industry (Amidon, Lennernäs, Shah, & Crison, 1995; Dahan, Miller, & Amidon, 2009).

Along with development of complex and simple digestion models, additional standardization in model parameters is critical between models and research groups. The Infogest network has proposed a consensus digestion model (Minekus et al.,

2014) that provides recommendations on sample preparation, simulated gastrointestinal fluid composition, and other experimental conditions for use in conducting in vitro digestion experiments, discussed in more detail in chapter "Quantitative Characterization of Digestion Processes". While these standard methods are useful in their ability to compare different food products with the same experimental conditions, their utility in understanding key factors controlling the digestion process may be limited due to the physiological variability of conditions with different food products. For example, the Infogest consensus model recommends mixing 1:1 (v:w) simulated saliva to food for 120 s (Minekus et al., 2014). However, when in vivo studies are consulted, values of saliva–food vary considerably based on food products. For example, mastication of a piece of cake may elicit saliva secretion of 0.35 mL saliva: 1 g food, while mastication of a dry piece of toast may elicit saliva secretion of 1.07 mL saliva: 1 g food (Gavião, Engelen, & van der Bilt, 2004). Although it is necessary to utilize a value that is applicable to a wide variety of food products in a standard method, such as that proposed by Infogest, not including variations that can be adapted for different types of foods may result in overestimation or underestimation of certain food digestion endpoint measurements. Similarly, it is necessary to increase our knowledge on the specific impact of food material properties on gastrointestinal conditions, such as amount and composition of gastrointestinal secretions, specific pH profiles at different locations after a meal, gastric emptying rate, and transit time in each phase of the digestion process. With this knowledge, more precise methods can be developed that allow for varying method conditions for different food products, but still provide standardization across the research community.

Development of standardized in vitro model systems will provide powerful tools for the research community, although these tools will only become widely applicable if they are accompanied by rigorous correlations to in vivo systems. Many of the current in vitro systems that have been developed have correlated certain results to values that were determined in vivo, such as disintegration of manufactured beads of known properties, gastric emptying rate, gastric pH, bacterial population, protein digestion, plasma glucose profile, and drug absorption profile (Dupont et al., 2018). The specific in vitro and in vivo parameters compared will clearly depend on the in vitro model and its target functionality. In many of these studies, specific output measures between the in vitro and in vivo systems are compared, but standard methods for asserting the validity of an in vitro model have not been established and consistently applied across the food digestion research community. In the future, definitive methods and comparison levels to develop in vitro–in vivo correlations need to be developed for in vitro food digestion models, such as those that have been established in the pharmaceutical industry and are described in guidance issued by the US Food and Drug Administration (Cardot, Beyssac, & Alric, 2007; Emami, 2006; Food and Drug Administration Center for Drug Evaluation and Research, 1997). Once in vitro food digestion models can be rigorously compared with in vivo systems, their applicability to food product development will increase, as well as their utility for regulatory claims on future food products.

3 In Silico Models

Recent advances in computational power have allowed for development of advanced computational models of flow and mixing in the gastrointestinal tract which are discussed in chapter "An Engineering Perspective on Human Digestion" To date, such models include flow and mixing in the human stomach, and flow and mixing in the intestines, both at a macro-scale as well as micro-mixing that may occur at the villi level in the intestine (Ferrua & Singh, 2010; Ferrua, Xue, & Singh, 2014; Kozu et al., 2010; Lentle et al., 2013; Love, Lentle, Asvarujanon, Hemar, & Stafford, 2013). The knowledge gained in these studies is important to form the foundation for future computational studies, as well as in vitro model development, but in the future, it will be critical to expand such computational models.

Such computational models can be modified to simulate conditions closer to real physiological systems through improvement of the gastrointestinal geometry. To date, gastrointestinal sizes and shapes have been typically estimated by approximate values taken from the literature. However, using computed tomography or magnetic resonance imaging scans, advanced and/or personalized geometries for the organs of the gastrointestinal tract can be developed to understand how variations in gastrointestinal geometry may impact flow, mixing, and breakdown during these physiological processes. Similar approaches have previously been used in developing models of airflow into the lungs; CT scans from individual patients can be utilized to generate patient-specific models (McClelland et al., 2006).

In addition to expansion on the complexity and specificity of gastrointestinal geometries utilized in computational models, it will be critical to integrate realistic solid and fluid properties and their evolution during the digestion process into future in silico modeling efforts. Most of the computational fluid dynamics models that have been generated to date have simulated flow of either Newtonian or shear-thinning fluids of relatively low viscosity. In reality, the material that passes through the gastrointestinal tract is a complex, multiphase system composed of particles which are continuously changing in size and shape, and a fluid phase that is diluted by gastrointestinal secretions. To integrate this complex behavior into a computational model, additional information is needed on digesta physical properties and their evolution for various meals, which can be determined using both in vitro and in vivo systems. Some such information has previously been gathered for certain meals using in vivo (pig) models (Bornhorst, Ferrua, Rutherfurd, Heldman, & Singh, 2013; Bornhorst, Ströbinger, Rutherfurd, Singh, & Moughan, 2013; Shelat et al., 2015; Wu, Dhital, Williams, Chen, & Gidley, 2016), but property information from a wider variety of meals will be critical for integration into advanced computational models.

The changes in food properties as they pass through the gastrointestinal tract is complicated because multiple processes are occurring, including particle breakdown, enzymatic hydrolysis, and dilution by gastrointestinal secretions, as discussed in Chap. 2 ("The Digestive Tract: A Complex System"). To allow for a comprehensive understanding of the impact of food material on the changes that occur during the digestion process, it is critical to utilize a quantitative approach,

such that kinetic parameters from these breakdown and hydrolysis processes can be compared between studies. Quantification of enzyme hydrolysis kinetics has been frequently conducted as part of studies on starch digestion, as is discussed in Chaps. 13, 14, and 15 ("Starchy Foods: Human Nutrition and Public Health", "Kinetics of α-Amylase Action on Starch", and "Influence of Physical and Structural Aspects of Food on Starch Digestion."). Expansion and utilization of these kinetic parameters as part of advanced computational and predictive models will allow for a holistic view of the food digestion process.

In the future, it will be critical to expand on in silico modeling efforts, and to utilize modeling tools as part of the food design process when developing new food products with targeted health benefits. Integration of computational models into the product design process has been a key part of other engineering industries, such as the aerospace industry (Curran et al., 2005; Keane & Nair, 2005). Similar approaches in the food industry can be coupled with in vitro systems to streamline the food product development process for functional food products.

4 From Beginning to End: Why Interdisciplinary?

Based on the contents of this book and the authors of the various chapters, it can be easily observed why an interdisciplinary approach is necessary to tackle the challenges in understanding food digestion processes to advance design of health-promoting foods. Many of the advances to date have been promoted through collaborative work between chemical and biological engineers with chemists, nutritionists, and food scientists. Nevertheless, to further advance our knowledge and develop transformative future solutions to the current challenges, our interdisciplinary links must reach further. Current research in food digestion will need to connect with electrical engineers who develop advanced sensors and instrumentation; mechanical engineers who have developed models for complex, multiphase flows; veterinarians who may help provide links between needs for human nutrition with similar needs in animals; and medical doctors with knowledge of advanced noninvasive diagnostic techniques and who can ultimately help communicate recent scientific advances with patients. Development of these connections across the food production–consumption supply chain will allow not only for evidence-based advances in the understanding of how food impacts health and disease but also for translation of these solutions to consumers worldwide.

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