Strategic choices for *in vitro* food digestion methodologies enabling food digestion design

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Abstract

Background: In the past decades, the great interest in food digestion research led to a wide array of *in vitro* digestion (IVD) methods. Each of these methods have the potential of providing specific valuable scientific insights in digestion mechanisms and the rational structural design of foods.

Scope and approach: This review paper outlines important transitions in recent IVD research and formulates important considerations relevant for the set-up of future *in vitro* experiments, especially in the context of rational food digestion design. Important transitions are discussed, including the importance of kinetic experiments for macronutrient digestion, the relevance of transition towards more complex (semi)-dynamic digestion conditions, the shift from single nutrients in simplified systems towards real foods and meals, and the emerging trend to adapt methods to mimic the gastrointestinal (GI) tract of specific populations.

Key findings and conclusions: Notwithstanding the recent shift towards more complex IVD methods, the possibilities and advantages of more simple digestion methods should not be overlooked for mechanistic understanding or for sample screening purposes. Since the information retrieved from a simulation experiment depends on the applied conditions, the appropriate *in vitro* protocol should be chosen depending on the research question. In this context, the harmonization of digestion methods such as the standardized INFOGEST protocols can play a notable role in food digestion research and the development of tailored foods for all different strata of the population.

Keywords

In vitro digestion models; macronutrients; complex foods; dynamics; kinetics; population groups

Abbreviations

GI	Gastrointestinal
FSD	Food structural design
GE	Gastric emptying
SCFAs	Short chain fatty acids
HSA	Human salivary amylase
IVD	*In vitro* digestion

Introduction

The widely acknowledged impact of food consumption on health has led to the need for better mechanistic insights into the behavior of foods along the digestive tract. To acquire insights into these processes, as well as the bioaccessibility and bioavailability of food components, a wide array of *in vitro* digestion (IVD) methods has been developed and applied. These methods vary greatly in the manner of simulating the complex, transient and dynamic nature of *in vivo* digestion (Lucas-González *et al.*, 2018). *In vitro* methods have the potential to provide specific, valuable scientific insights and the appropriate protocol should be chosen strategically, depending on the purpose and research question.

Rapid advances in digestion methods have contributed to the elucidation of the relation between food processing, food structure, and digestion and release patterns of nutrients. After all, the structural organization of a food and its transformation throughout the gastrointestinal (GI) tract play a governing role in the digestive behavior of foods (Singh, Ye and Ferrua, 2015). Interestingly, a detailed understanding of structural transformations occurring throughout the food supply chain (food storage and processing) as well as during digestion, could be applied to design foods with enhanced digestive properties (Aguilera, 2005). In this context, processing and product formulation can then be reversely engineered to rationally design targeted food molecular, micro-, and macrostructures (food structural design, FSD) resulting in particular digestive properties, i.e., food digestion design (Calvo-Lerma *et al.*, 2018; Lucas-González *et al.*, 2018).

An introduction into the *in vivo* human GI system and the current state-of-the-art static and semi-dynamic IVD models is given. Then, the current review discusses important strategic considerations for setting up IVD experiments. Recent reviews on IVD approaches have been focusing on one specific macronutrient, food type, specific population group, or deliver a descriptive overview of the current state of the art (Shani-Levi *et al.*, 2017; Calvo-Lerma *et al.*, 2018; Sensoy, 2021; Xavier and Mariutti, 2021). However, an

integrated overview of the recent progress and evolution in the context of food science and technology and specifically food structural design is still missing. As illustrated in *Figure 1,* the current work aims to be a practical guideline for the strategic selection of appropriate IVD approaches, depending on the research question to be answered. After all, the selected method and applied experimental conditions affect the generated data and therefore the lessons which can be learned from such a simulation. The general aim of this review was to indicate four important aspects and transitions in the field of digestion studies, explain advantages and limitations of different applied approaches, and highlight future steps to be taken. In this case, the focus is on the case of food structural design with the aim of impacting food digestion behavior. The four discussed trends are:

(i) IVD studies evolve from endpoint towards kinetic evaluations (*Section 2.1*). While endpoint evaluations may be a straightforward approach, not only the extent of macronutrient digestion is of importance. Digestive protocols applying kinetic approaches improve understanding of the time-dependent evolution of macronutrient hydrolysis as well as structural changes along the digestive tract.

(ii) *In vitro* food digestion research is more and more evolving from simplified models towards more (semi-)dynamic models. While *in vitro* food digestion studies always entail some level of simplification, these studies should be carried out under physiologically relevant conditions to provide useful insights into the mechanisms of food digestion. Standardized, static methods are simple tools, impeccable for screening purposes and (mechanistic) hypothesis building and testing. Especially in the context of food digestion design, the easy-to-handle static procedures have special merit for investigating the effect of certain structuring efforts on digestion. However, since *in vivo* digestion is not a static process, efforts were carried out to simulate digestion in an increasingly realistic, complex, and (semi-)dynamic manner (*Section 2.2*). These experiments can provide useful insights into likely structural transformations, digestive trends, and final levels of bioaccessibility

and bioavailability if applied under relevant physiological conditions. In this regard, the INFOGEST semi-dynamic protocol (Mulet-Cabero *et al.*, 2020) can be considered a 'best-of-both-worlds' approach, combining advantages of static and dynamic methods. Due to the specific relevance in the context of food digestion design, this review focuses on static to semi-dynamic IVD approaches, rather than the more complex dynamic protocols reviewed in detail elsewhere (Lucas-González *et al.*, 2018; Li *et al.*, 2020; Mackie, Mulet-Cabero and Torcello-Gomez, 2020; Sensoy, 2021).

(iii) Digestion studies are evolving from simplified food model systems to real complex foods and meals (*Section 2.3*). While the study of simplified systems delivers crucial mechanistic insights, real meals are complex and (multilevel) structured systems in which different co-ingested nutrients are heterogeneously distributed, possibly interacting upon digestion.

(iv) Research focus is shifting from an approach only considering 'average healthy adults' towards digestion methods adapted to the functioning of the GI tract of specific population groups, such as infants, elderly, and people suffering from GI disorders (*Section 2.4*). In a rapidly growing and aging population increasingly suffering from nutrition-related pathologies, the need for a better understanding of the relation between food digestion and health in these population groups is crucial. These insights could lead to better nutritional recommendations, improved pharmaceuticals, and even personalized foods with targeted digestion properties.

1. Simulating human gastrointestinal digestion from bite to metabolite

1.1. *Understanding in vivo* human gastrointestinal digestion: the logical starting point

To study human digestion using simplified IVD methods, one first needs to understand the real human digestion system. A very complete overview of the current insights on human digestion was recently given by Sensoy (2021). Therefore, this section delivers a brief overview of human digestion factors, important for developing *in vitro* models describing/mimicking the initial interactions between the human body and the food matrix.

126 The human GI tract is built up of 4 main so-called 'reactors' in series: mouth, stomach, small intestine, and
127 large intestine (Boland, 2016). In the oral cavity, the food is transformed both physically and chemically,
128 largely depending on food structure, into a cohesive bolus (Guerra *et al.*, 2012; Singh, Ye and Ferrua,
129 2015). Liquid foods are diluted with saliva and slightly altered by oral temperature, pH, salts, and mucins.
130 Additionally, solid foods are reduced in particle size (< 2 mm), leading to an increased surface area.
131 Moreover, starch hydrolysis is initiated in the oral phase by the secretion and action of human salivary
132 amylase (HSA) (Guerra *et al.*, 2012). While oral food processing and digestion are highly relevant,
133 especially for solid and semi-solid foods, this review focuses solely on (the simulation of) gastro-intestinal
134 digestion.

135 Swallowed boluses pass through the esophagus into the stomach, where protein and lipid digestion is
136 initated by the gradual secretion of gastric acid (HCl), pepsin, and gastric lipase, regulated by neural,
137 hormonal, paracrine, mechanical, and chemical stimuli (Schubert, 2010). Pepsin is an aspartic protease
138 that is active in an acidic environment (pH range of 1.5 to 5), and responsible for gastric protein hydrolysis
139 (around 10 to 15% depending on the food). Due to its broad specificity, heterogeneous mixtures of
140 peptides and polypeptides of different polymerization degrees are predominantly formed (Goodman,
141 2010). Gastric lipase is active at pH 4 to 6 and responsible for around 5 to 30% of lipolysis (Carrière *et al.*,
142 1993; Armand *et al.*, 1999). Upon ingestion of a meal, the gastric pH rises from the pH of the fasted state
143 (1 to 2) to a pH close to the pH of the food (5 to 7), as a result of the food buffering capacity (Dressman *et*
144 *al.*, 1990; Sams *et al.*, 2016). The pH decrease is buffered by the secretion and action of digestive enzymes,
145 reaching a pH between 4 and 7 at about 50 to 60% gastric emptying (GE) (Bornhorst and Singh, 2012;
146 Sams *et al.*, 2016). This dynamic pH has several consequences e.g., on enzyme activity. To give an example,
147 *in vitro,* HSA can retain its hydrolytic activity for up to 45 min as the result of a gradually decreasing pH
148 (Dressman *et al.*, 1990; Freitas *et al.*, 2018), leading to significant gastric amylolysis. Food structure,
149 compositional, and personal factors, determine the timespan in which the fasted state is reached again,

150 approximately 60 to 120 minutes after ingestion (Dressman *et al.*, 1990). The rate of GE determines the
151 residence time in the stomach and thus the level of gastric digestion.

152 The small intestine is considered the major area for nutrient absorption, as it has a large external surface
153 area of about 30 m^2 lined with villi and microvilli (Boland, 2016; Jaime-Fonseca *et al.*, 2016). Nutrient
154 absorption competes with the transit through the intestine, which takes between 2 and 5 hours (Boland,
155 2016). In the duodenum, pancreatic secretions containing digestive enzymes and carbonate neutralize
156 the pH (6 to 7.5) (Guerra *et al.*, 2012). Bile salts play a crucial role in small intestinal lipid hydrolysis by *(i)*
157 facilitating the adsorption of lipase and colipase to the lipid substrate, and *(ii)* generating mixed micelles,
158 which solubilize and transport lipid- soluble compounds allowing the continuation of interfacial lipid
159 digestion (Feher, 2012; Boland, 2016). Pancreatic lipases further hydrolyze diverse lipid species to free
160 fatty acids and 2-monoglycerides, after which lipolytic products are incorporated into mixed micelles,
161 which are further delivered to the small intestinal enterocytes (Boland, 2016). Pancreatic α-amylases
162 hydrolyze carbohydrates into maltose, maltotriose, and α-limit dextrins (Feher, 2012; Boland, 2016).
163 Soluble starch hydrolysis products require additional breakdown by disaccharidases to monosaccharides
164 which can be absorbed (e.g., glucose) at the brush border. Moreover, proteins and polypeptides are
165 converted into peptides with lower polymerization degree and amino acids by a mixture of pancreatic
166 exo- and endopeptidases (e.g., trypsin, chymotrypsin). While amino acid and di- and tripeptide absorption
167 occurs directly at the epithelium, peptides with a larger polymerization degree are further hydrolyzed by
168 aminopeptidases located at the brush border (Goodman, 2010; Feher, 2012). However, some food-
169 derived bioactive peptides can endure proteolysis, finally exerting health-promoting or adverse (e.g.,
170 allergy) effects (Ferranti *et al.*, 2014).

171 Non-absorbed material (e.g., fibers as well as non-digested and/or non-absorbed nutrients and
172 metabolites) reaches the anaerobic colon, where it is fermented by gut microbiota (Guerra *et al.*, 2012;
173 Boland, 2016). Nutrient fermentation can have significant effects on microbial communities and cause the

formation of several health-promoting and disease-related metabolites. While this is an emerging field of study (Rampelli *et al.*, 2016; Pérez-burillo *et al.*, 2021), it is outside of the scope of the current review, focusing on (simulations of) the processes occurring in the upper GI tract.

1.2. How do *in vitro* digestion data enable structural design of foods with steered digestion functionalities?

The rational structural design of foods requires insights into various factors controlling nutrient bioaccessibility. Ideally, food digestion should be studied *in vivo*, but this is often practically, ethically, and financially impossible (Li *et al.*, 2020). The collection of *in vivo* data is challenging and various invasive and non-invasive *in vivo* techniques have been applied (Schwizer, Steingoetter and Fox, 2006; Marciani *et al.*, 2007). However, these techniques are cost- and time-intensive and susceptible to important person-to-person variation (Golding and Wooster, 2010). In contrast, *in vitro* methods allow the widespread study of food digestion mechanisms as well as kinetics in a cheap(er), easy-to-use, and high-throughput manner compared to *in vivo* approaches. *In vitro* methods are based on human physiology; yet, they are simpler, economical, and reproducible.

Data obtained through different IVD methods should be validated with *in vivo* data. While a correlation between *in vitro* and *in vivo* data was confirmed in some cases, *in vitro* methods models always employ a certain level of simplification compared to the complex GI tract morphology (e.g., neglecting certain reactors and/or peristaltic movements), enzymatic interplay and hormonal regulation (Li *et al.*, 2020). Therefore, IVD models of all levels of complexity show important limits in mimicking digestive processes and predicting *in vivo* responses. Therefore, IVD outcomes and trends should be interpreted carefully and validation is necessary to make predictions regarding *in vivo* behavior. However, even though *in vitro* methods do not fully replicate the precise physiology of a living organism, they have been proven very useful tools for food technologists, for obtaining fundamental insights as well as comparing digestion trends in differently structured foods.

In particular, the importance of valid correlations between *in vitro* and *in vivo* studies, linked physiological processes and consequences should be emphasized and advances in this area have been extensively reviewed elsewhere (Bohn *et al.*, 2018). In most cases, establishing a correlation between *in vitro* and *in vivo* responses remains difficult. Often, there is no clear direct link between bioaccessibility established *in vitro* and probable *in vivo* responses, since the data are obtained at different levels during the digestion process. Nonetheless, correlation between mostly static *in vitro* and *in vivo* digestion outcomes has been proven in multiple cases, however, with varying accuracies for different nutrients depending on the complexity and adequacy of the model (Brodkorb *et al.*, 2019; Edwards *et al.*, 2019; Egger *et al.*, 2019). The attempt to predict *in vivo* outcomes has been most prominently studied in the context of starch digestion. Different *in vitro* digestibility parameters (e.g., degree of hydrolysis at a specific digestion times) showed different levels of correlation with *in vivo* glycemic index (Edwards *et al.*, 2019). Additionally, Egger et al. (2019) compared insights obtained using the *in vitro* static INFOGEST method with insights of *in vitro* dynamic methods, and validated those using *in vivo* pig and animal studies. This integrated approach demonstrated clear correlations and thus strong physiological relevance of the considered methods for the digestion of milk proteins (Egger *et al.*, 2019). However, *in vivo* validation of results obtained for a specific food and/or nutrient using a specific IVD method can not be generalized towards other IVD methods and foods.

It is important to note that *in vivo* and *in vitro* data can and should complement one another to gain mechanistic insights into *in vivo* outcomes which would be impossible to obtain using only *in vivo* set-ups. In the evolution towards more complex IVD methods, especially approaches combining *in vitro*, *in vivo, and in silico* digestion seem promising to offer superior mechanistic insights as compared to the ones provided by one approach only (Mackie, Mulet-Cabero and Torcello-Gomez, 2020). Even artificial intelligence methods, although in their infancy, in combination with the results provided by

abovementioned methods have the potential to become powerful predictive tools (Le Feunteun, Mackie and Dupont, 2020).

2. Overview of important strategic choices to be taken *in vitro* digestion simulation approaches

2.1. From endpoint to kinetic approaches

The evaluation of reaction endpoints is a simple and informative approach that has been widely applied. For example in the context of micronutrients such as minerals, vitamins, polyphenols, and carotenoids (Rodríguez-Roque *et al.*, 2013; Lemmens *et al.*, 2014; Gwala *et al.*, 2020; Rousseau *et al.*, 2020), the bioaccessibility at the end of static IVD can give valuable information about the nutrient bioaccessibility with limited efforts. For minerals, correlations between *in vitro* bioaccessibility based on endpoint assessments *and in vivo* outcomes were established (Bohn *et al.*, 2018). In contrast to macronutrients, biochemical conversion of micronutrients is not needed. Hence, micronutrient absorption is time-independent and endpoint assessment can be an appropriate measure to evaluate and compare the non-bound mineral content of differently processed samples (Gwala *et al.*, 2020; Rousseau *et al.*, 2020).

In contrast, macronutrient digestion is largely driven by time and enzyme-dependent processes. Food digestion research is therefore focusing more and more on hydrolysis kinetics, nutrient release patterns, and structural transformation rather than only on final extent of hydrolysis at a particular endpoint (Dupont *et al.*, 2018). In what follows, arguments are elaborated which support the use of a kinetic approach in the study of food digestion and food structural digestion design.

2.1.1. *Why consider a kinetic approach?*

Firstly, the physiological relevance of patterns of macronutrient breakdown and the release of constituting nutrients into the GI tract where they are presented for absorption, has been widely accepted

(Mackie, Mulet-Cabero and Torcello-Gomez, 2020). After all, a high or low rate of nutrient breakdown (and availability for absorption) can be beneficial or disadvantageous, depending on the nutrient and the nutritional health status of the individual (Dupont *et al.*, 2018). Despite the simplification entailed in *in vitro* experiments, for the example of starch digestion, it can be hypothesized that a lower starch digestion rate *in vitro* increases the probability of (s)lower increase of the blood sugar level and a remaining amount of undigested starch reaching the colon *in vivo* (Warren *et al.*, 2015). Considering proteins and lipids, digestion kinetics determine physiological regulatory mechanisms controlling appetite and energy intake (Wilde, 2009). Therefore, a kinetic approach is necessary to understand the time-dependence of biochemical conversions of macronutrients and unravel to unravel digestion patterns.

Secondly, a kinetic approach has been successfully applied to provide mechanistic insights into nutrient hydrolysis and release patterns. As mentioned in *Section 2.2,* the use of a highly simplified system can be suitable to study specific interactions between substrates and enzymes. Applying a kinetic approach, *in vitro* studies focusing on lipid digestion elucidated the effect of emulsion stability on lipid digestion (Verkempinck, Salvia-Trujillo, Moens, Charleer, *et al.*, 2018), and even postulated a plausible reaction mechanism for lipolysis (Infantes-Garcia *et al.*, 2021). In the field of macronutrient digestion in pulses, a strong relation between thermal processing time, food structure (hardness), cell wall properties, and starch and protein digestion kinetics could be established (Duijsens *et al.*, 2021; Pälchen *et al.*, 2022). These examples emphasize the relevance of following a kinetic approach to generate mechanistic understanding and cause-effect relations. Moreover, research shifted from studying single nutrients and simplified model systems towards studying more complex and realistic foods consisting of multiple macronutrients, as will be elaborated on in *Section 2.3.* Since interactions can occur in these systems, the digestive behavior cannot be seen as the simple sum of the digestive behaviors of the different single macronutrients (Le Feunteun, Verkempinck, *et al.*, 2021). In this context, a kinetic approach can especially contribute to a better understanding of interactions occurring among the digestive behaviors of different

nutrients present in a food during digestion. In this regard, it should be noted that the macro- and microstructure of the food/digesta can also change dynamically throughout gastrointestinal digestion, significantly affecting nutrient hydrolysis kinetics. To give an example, a clot can be formed during gastric digestion of milk affecting the rate of proteolysis (Ye *et al.*, 2016). Moreover, lipolysis kinetics can be affected by competing mechanisms such as droplet degradation, droplet flocculation, and phase separation (Golding and Wooster, 2010).

Thirdly, the more relevant information to be retrieved from an *in vitro* assay is the time-dependent nutrient digestibility of a food at a given enzyme activity, rather than reaction endpoints (Warren *et al.*, 2015). Especially for the case of macronutrient hydrolysis *in vivo*, inter- and intra-individual variations in variables such as transit time, enzyme secretion, and enzyme/substrate ratio highly affect reaction rates and endpoint digestibility levels. Of course, a simplified *in vitro* digestion protocol (i.e., INFOGEST) cannot capture this complexity, applying standardized enzymatic activities based on available literature (Minekus *et al.*, 2014). The applied enzymatic activity considered *in vitro* as well as product inhibition will affect the obtained reaction rate as well as the final extent of substrate hydrolysis. Therefore, IVD assays are generally not suited to predict the exact endpoint (extent) of enzymatic macronutrient hydrolysis *in vivo* (Warren *et al.*, 2015). Nevertheless, digestive behavior (rates, patterns) of different foods, established through IVD, can conveniently be compared if food digestion and nutrient hydrolysis are simulated under standardized conditions (i.e., same enzyme activity) (Brodkorb *et al.*, 2019; Mulet-Cabero *et al.*, 2020). This, however, can only be established when following a kinetic approach.

Fourthly, especially in the context of the rational structural and compositional design of foods, kinetic experiments can provide insight into the effect of certain structuring efforts (Le Feunteun, Verkempinck, *et al.*, 2021) and enable the screening and comparison and of foods with different process-induced structural properties and the potentially distinct digestive responses as a result thereof. To give an

example, this approach has been recently applied to study the impact of processing and resulting structural properties on protein and lipid digestion kinetics (Guevara-Zambrano *et al.*, 2022).

Finally, modeling of kinetic data shows great potential as a useful starting point for the study of digestion *in silico* (Le Feunteun, Verkempinck, *et al.*, 2021). To give an example, a mechanism-based *in silico* model was recently developed, describing the formation of several reaction products (fatty acids, di- and monoglycerides as well as glycerol) during triglyceride hydrolysis (Verkempinck *et al.*, 2019). Together with mechanism-based (multi-response) modeling, kinetic *in vitro* experiments help establish insight into reaction mechanisms.

2.1.2. *How to generate kinetic data?*

To study digestion kinetics, the design of endpoint approaches should be adapted. Although not yet widely applied, the use of a 'single reactor per time point' approach to studying digestion is advised to generate independent and reliable kinetic data (Brodkorb *et al.*, 2019; Le Feunteun, Verkempinck, *et al.*, 2021). Using this approach, each reactor can be seen as an independent evaluation of the digested system at a predetermined digestion time. For each reactor, enzymatic reactions are stopped upon attaining the predetermined reaction time, after which hydrolysis products (degree of hydrolysis) are determined. Since each reactor represents evaluations of the same system at different time points, they can be seen as repetitions from a statistical point of view (Verkempinck, Salvia-Trujillo, Moens, Carrillo, *et al.*, 2018). Subsequently, appropriate mathematical modeling should be applied to integrate and understand the trends established by these experiments. While outside the scope of this work, this subject was recently thoroughly reviewed (Le Feunteun, Verkempinck, *et al.*, 2021).

In contrast, when a single reactor is used to simulate digestion, as proposed by Mulet-Cabero *et al.* (2020), uniform sampling can be difficult or impossible (e.g., in an emulsion that shows creaming with increasing digestion time). Moreover, multiple sampling from a single reactor as a function of digestion time can

significantly alter the composition, substrate and metabolite concentration, and enzyme-substrate ratios. Even when using very large reactors, sampling from a single vessel yields strongly correlated data which can be highly dependent on the sampling method.

2.2. From simple static to more complex semi-dynamic *in vitro* methods: why (not)?

Even though the goal of *in vitro* methods is not to fully replicate *in vivo* conditions, the selected parameters should be chosen with care and as accurately and relevantly as possible to represent the real situation. This is especially challenging but relevant in highly dynamic phases (i.e., gastric digestion), where enzymatic activities and pH vary significantly both as a function of time and position in the gastric reactor (Carrière *et al.*, 2005; Sams *et al.*, 2016). To the present day, most IVD studies follow a static approach, mimicking digestion using fixed parameters in the different reactors (i.e., oral, gastric, and small intestinal). These methods vary greatly in the level of complexity and experimental digestion parameters, such as pH, digestion time, concentration, and sources of enzymes and bile salts, and have been extensively reviewed in literature (Li *et al.*, 2020; Wojtunik-Kulesza *et al.*, 2020).

The great diversity of conditions makes the comparison of results between research groups and the deduction of general findings impossible (Minekus *et al.*, 2014; Bohn *et al.*, 2018; Lucas-González *et al.*, 2018). Since 2014, a consortium of international scientists attempted to standardize and harmonize a static protocol to evaluate nutrient digestion and came to a consensus approach (Minekus *et al.*, 2014). Updated in 2019 (Brodkorb *et al.*, 2019), this standardized protocol has been widely used to evaluate (multi-)nutrient digestion (547 citations according to Scopus® since the update). To draft this protocol, physiologically relevant conditions (such as gastric digestion time, pH, fixed ratios and concentrations of food, and enzymatic activities) had to be selected based on vast amounts of *in vivo* data (Brodkorb *et al.*, 2019; Mulet-Cabero *et al.*, 2020). Since the transient nature of human digestion cannot be completely reproduced in an *in vitro* experiment, strategic choices had to be made for the sake of representability

and comparability of the gathered data. Firstly, the standardized INFOGEST protocols propose fixed ratios and concentrations of food and enzyme (Brodkorb *et al.*, 2019; Mulet-Cabero *et al.*, 2020), based on relevant *in vivo* data. To give an example, for the gastric phase, the enzyme to substrate ratio was fixed to correspond approximately to the half gastric emptying time (Minekus *et al.*, 2014). A static pH of 3 was selected for the gastric phase, considering the activity of digestive enzymes (significant gastric lipase activity at pH 3 while pepsin is mainly active below pH 2) as well as the *in vivo* pH profile upon food ingestion (gradually decreasing to values below 2 upon reaching the fasted state).

To mimic digestion *in vitro,* not only the choice of appropriate parameters is important. The accurate and reproducible determination of bile salt concentration and enzymatic activity is another critical step leading to major inter-laboratory variations. Standardized methods to assess enzymatic activity have therefore been reported in the INFOGEST protocols (Minekus *et al.*, 2014; Brodkorb *et al.*, 2019), and a commercial kit has been adopted to assess bile salt concentration (Brodkorb *et al.*, 2019). In this context, enzymatic activities should be determined under physiologically relevant conditions, i.e., pH, temperature, ionic strength. Logically, errors in the activity determination the used enzymes may result in over- or underdosing of the used enzymes, ultimately leading to over- or underestimated digestion trends.

Overall, the standardized static IVD method is a basic and easy-to-apply tool that has been widely used for digestion studies aiming to obtain mechanistic insights into enzyme-substrate interactions and to screen samples for differences in digestibility as a result of different processing history. While static experiments provide relevant insights, these protocols inherently omit part of the physiological complexity of the digestive process. To study food digestion in a way more closely resembling the *in vivo* situation, researchers tried to include some physiologically relevant conditions and dynamic parameters into dynamic and semi-dynamic IVD methods, as discussed in the next section.

2.2.1. *Beyond static in vitro methods: increasing the complexity*

While the simplicity of static approaches is a major advantage, these approaches fail to capture the dynamic nature of *in vivo* digestion processes. Therefore, a diverse range of efforts were initiated to study digestion under more physiologically relevant conditions. The aim of these efforts is to provide more physiologically relevant, kinetic data on nutrient breakdown patterns and structural changes in food. Moreover, these studies aim to predict macronutrient digestion, and absorption, more accurately (e.g., glycemic index in the case of starch) (Mulet-Cabero *et al.*, 2020). Often, these more dynamic models include two or more GI compartments with digestion conditions closer to the *in vivo* situation such as pH, enzymatic profiles, transit times, mixing, and/or (passive) absorption of digestion products, as has been extensively reviewed elsewhere (Lucas-González *et al.*, 2018; Li *et al.*, 2020; Mackie, Mulet-Cabero and Torcello-Gomez, 2020; Sensoy, 2021). To bring harmonization, an international standardized semi-dynamic IVD method was published by the INFOGEST consortium (Mulet-Cabero *et al.*, 2020), incorporating relevant dynamic parameters linked to the gastric phase, including *(i)* gradual acidification, *(ii)* fluid and enzyme secretion, and *(iii)* emptying. For a wide range of applications, it could be appropriate to opt for this semi-dynamic digestion simulation, combining the advantages of both static and dynamic approaches.

From the above, one should acknowledge the abilities and limits of both static and (semi-)dynamic methods. Approaches with different levels of complexity are suited to answer different research questions. In the context of food structural design, both static and more dynamic methods can play an important role. Cheap, feasible, and rapid screening tools assessing structuring strategies are of great importance. On the other hand, food structural and digestion design rely heavily on integrated knowledge of single nutrient hydrolysis patterns, relationships between macronutrients, and matrix effects involved throughout the digestion (Capuano *et al.*, 2018; Do *et al.*, 2018). In the context of food structural design, (standardized) static simulations can be suitable for extensive high-throughput comparison and screening

for differences in digestive behavior between samples with different processing history. In the later stages of food design, when the aim is to extract specific physiologically relevant predictions of actual levels of digestion, (semi-)dynamic IVD methods can be highly relevant.

It can be concluded that, while digestion models with increasing complexity are emerging, they have some important drawbacks such as complex and time-consuming protocols and data analysis. This ultimately raises the question of whether the increased complexity automatically results in more relevant and better data, i.e., a better prediction of the *in vivo* situation. Therefore, there is an important need for *in vivo* validation of more dynamic *in vitro* methods.

2.2.2. *Hybrid in vitro methods: as simple as possible, but as complex as needed*

The implementation of more dynamic factors into a digestion simulation always entails an additional level of complexity, both in experimental handling and data analysis. Therefore, the need for more physiologically relevant digestion data, combined with the need for easy-to-handle and standardized protocols, led a significant number of authors to introduce modifications to the standardized static protocol. In practice, it can be an approach to choose and adapt a certain digestion method, applying conditions suitable for the specific research need and current laboratory infrastructure. The hybrid combination of digestion models allows the performance of IVD with acceptable throughput, small sample volumes, and without the need for sophisticated lab tools such as bioreactor systems. In this context, experimental approaches should be 'as simple as possible, but as complex as needed' (Pälchen *et al.*, 2021), to answer the specific research question under investigation.

A possible strategy is the adaptation of a static digestion protocol by the stepwise introduction of certain dynamic parameters of interest (Freitas *et al.*, 2018; Fernandes *et al.*, 2020; Hiolle *et al.*, 2020; Colombo *et al.*, 2021; Pälchen *et al.*, 2021) by the incorporation of the dynamic nature of a compartment (Opazo-Navarrete *et al.*, 2018; Freitas and Le Feunteun, 2019; Pälchen *et al.*, 2021), or by mimicking a series of

406 variable factors and compartments of the GI tract (Shani-Levi *et al.*, 2017; Lucas-González *et al.*, 2018;
407 Mulet-Cabero *et al.*, 2020; Wojtunik-Kulesza *et al.*, 2020). In this manner, insights can be obtained into
408 the effects of specific dynamic parameters, while the set-up remains simple and cost-efficient (Pälchen *et*
409 *al.*, 2021). Different reasons to adapt the static model exist: *(i)* to study digestion processes under more
410 physiologically relevant conditions, *(ii)* to test specific mechanistic hypotheses, and *(iii)* to understand and
411 obtain mechanistical insight into the relevance/effect of specific dynamic parameters on structural and
412 digestive properties. To give an example, gradual gastric acidification was introduced to a static digestion
413 protocol to study its effect on starch and protein digestion patterns in chickpea cells (Pälchen *et al.*, 2021).

414 Current research interests focus on several factors proven and/or hypothesized to be important for the
415 mechanisms of food digestion. These parameters include but are not limited to adjustments at the level
416 of oral disintegration and the use of human saliva (Hiolle *et al.*, 2020), the use of specific enzymes of
417 different origin (Capolino *et al.*, 2011) and/or incorporation of brush-border enzymes (Ozorio *et al.*,
418 2020)), dynamic evolution of the gastric pH (Dekkers *et al.*, 2016; Mat *et al.*, 2016; Pälchen *et al.*, 2021),
419 gradual secretion of bile, simulated fluids, and the modification of digestion times (i.e., gastric emptying)
420 (Wickham *et al.*, 2012; Mulet-Cabero *et al.*, 2020). These types of experiments can answer very specific
421 research questions and the insights gained can help select parameters relevant to include in future IVD
422 methods. A detailed discussion of the applications of these dynamic parameters in digestion research is
423 outside the scope of this review, but was provided recently (Mackie, Mulet-Cabero and Torcello-Gomez,
424 2020; Colombo *et al.*, 2021).

425 Importantly, the evolution towards applying methodologies adapted to the specific research question
426 and/or research-unit specific infrastructures can lead to difficulties in the interpretation and comparison
427 of data between research facilities. This is in contrast to the efforts which are being made pursuing the
428 standardization of digestion methods to achieve comparable data (Brodkorb *et al.*, 2019; Mulet-Cabero
429 *et al.*, 2020). While adaptation of single parameters in digestive methods may be suitable for in-depth

mechanistic research, the implementation of the standardized semi-dynamic INFOGEST protocol could offer an affordable alternative to dynamic methods and may therefore seem a 'best-of-both worlds' solution to obtain relevant and comparable data for many applications. However, some issues remain to be resolved. To give an example, the proposed 'single reactor' approach does not allow the straightforward generation of independent data following a kinetic approach (*Section 2.1.2*).

2.2.3. *Case studies and perspectives for macronutrient digestion simulated under (semi-) dynamic conditions*

In the context of a particular research question, the model's set-up is only as good as the physiological relevance of the selected parameters in that specific context. In what follows, the relevance of incorporating some dynamic factors into the digestion model in the context of the digestion of a particular macronutrient is elaborated.

In the case of lipid digestion, the use of static IVD conditions to evaluate overall lipid hydrolysis has some limitations. Firstly, for the gastric phase, the standardized (INFOGEST) static IVD simulates lipid digestion at pH 3.0 (Brodkorb *et al.*, 2019) while the optimal pH for gastric lipase activity is around 5.4 (Golding and Wooster, 2010). This limitation could be overcome by introducing physiologically relevant (semi-)dynamic parameters, such as gradual acidification in the gastric reactor. The effect of incorporating dynamic parameters into gastric digestion studies is however little studied (Zaeim *et al.*, 2022). Secondly, for the small intestinal phase, the addition of digestive components (enzymes, bile salts) is carried out at once at the beginning of this phase during a static simulation. As a result, an excess of both enzyme and bile salts might be introduced. In turn, this overdosage could cause rapid initial lipolysis. Moreover, it is important to mention that the applied concentrations (and possible stepwise addition) of gastric and pancreatic lipase, colipase, and bile salts, as well as structural characteristics of emulsions, such as the nature of

emulsifiers and oil droplet size, could be critical parameters determining lipid digestion and/or solubilization of lipolysis products (Giang *et al.*, 2016; Verkempinck *et al.*, 2022).

Some relevant physiological parameters to consider for the case of protein digestion are both the pH dependency of pepsin activity and the presence of brush border enzymes. In contrast to the behavior of gastric lipase, pepsin activity increases with decreasing pH. Therefore, the use of a dynamic gastric pH has major implications for *(i)* pepsin activity and thus gastric proteolysis, and *(ii)* structural modifications of proteins which may increase/hinder proteolysis (e.g., coagulation of milk proteins upon decreasing gastric pH hindering proteolysis (Ye *et al.*, 2016)). After gastric digestion, proteolysis is continued in the small intestine, under the influence of a mixture of small intestinal peptidases. *In vivo,* brush border peptidases play a major role in the final processing of polypeptides by continuing the degradation of oligopeptides to bioaccessible free amino acids, di- and tripeptides. Despite the major physiological role of the brush border in the hydrolysis of protein *in vivo*, these brush border proteases have mostly been omitted from IVD studies (Claude *et al.*, 2019). A limited amount of studies consider the simulation of brush border membrane proteases. These studies have provided important insights highlighting the relevance of supplementing IVD models with intestinal brush border membrane hydrolases to realistically determine the bioaccessibility of dietary protein (Picariello, Ferranti and Addeo, 2016).

For the study of starch digestion, the incorporation of HSA and a dynamic gastric pH profile, mimicking physiological conditions more accurately, can significantly affect the rate and extent of amylolysis. *In vivo,* starch digestion is initiated in the oral phase, by the addition of HSA (Adebiyi and Aluko, 2011). Up until now, the contribution of this enzyme has been neglected in most static *in vitro* experiments, as the oral phase is very short and followed by an instant pH drop upon initiation of the gastric phase, immediately and irreversibly inactivating HSA (Bornhorst and Singh, 2012; Brodkorb *et al.*, 2019). However, the incorporation of HSA becomes of major significance considering a dynamic pH profile in the stomach. As a result of the dynamic gastric pH profile, HSA is not instantaneously inactivated upon reaching the

476 stomach but can stay active and hydrolyze starch significantly until the pH drops below 3.8 to 3.3
477 (Bornhorst and Singh, 2012; Freitas *et al.*, 2018). In this context, the buffering capacity and pH of the food
478 highly determine the pH in both the oral and gastric phase, and thus orogastric HSA activity (Freitas and
479 Le Feunteun, 2018). The importance of orogastric amylolysis is highlighted by a study by Freitas et al.
480 (2018), who showed that, when applying a dynamic gastric pH, HSA is responsible for hydrolyzing up to
481 80% of bread starch during the gastric phase (Freitas and Le Feunteun, 2018). However, co-ingestion of
482 acid lemon juice with bread led to complete interruption of orogastric amylolysis (Freitas and Le Feunteun,
483 2019). Interestingly, another study on chickpea cells showed differences in amylolysis kinetics but not final
484 extent (Pälchen *et al.*, 2021). In the static experiment (without HSA), no orogastric amylolysis could take
485 place and starch digestion occurred only during the small intestinal phase. In contrast, upon incorporating
486 HSA in the oral phase and applying a stepwise gradual pH decrease in the gastric phase, amylolysis
487 occurred in two distinct (oro-gastric and small intestinal) phases, though reaching a similar final digestion
488 extent as compared to the static experiment (Pälchen *et al.*, 2021). These examples clearly show the
489 importance of HSA in combination with a dynamic gastric pH profile for the study of starch digestion
490 kinetics.

491 To conclude, for the study of macronutrient digestion, several physiologically and mechanistically relevant
492 dynamic factors can be discerned. The incorporation of these factors into the IVD model is expected to
493 significantly alter digestion kinetics. While for some parameters, such as gastric pH profile, this has been
494 studied for different foods (Dekkers *et al.*, 2016; Freitas and Le Feunteun, 2018; Pälchen *et al.*, 2021), the
495 effect is less studied for other parameters (i.e., gastric emptying). Especially in this context, it becomes
496 important to set up kinetic experiments (*Section 2.1*), in which the evolution of macronutrient digestion
497 can be evaluated as a function of time, and hereby as a function of the inherently transient digestion
498 parameter as well. Since time is not the only determining parameter evolving throughout the experiment,
499 adapted data analysis and mathematical modeling should be applied to unravel the exact effect of the

dynamic parameter (e.g., pH profile as a function of time) on the kinetics of macronutrient digestion, as reviewed in detail elsewhere (Le Feunteun, Al-Razaz, *et al.*, 2021; Le Feunteun, Verkempinck, *et al.*, 2021).

2.3. From single nutrient evaluations to real food systems or meals

Nutrient digestibility, bioaccessibility, and bioavailability were often studied for one major nutrient in mostly simplified systems. As mentioned earlier, studies with a simplified set-up allow obtaining insights into the effect of specific process-induced food structures on nutrient release patterns, nutrient digestibility, and bioaccessibility (Singh, Ye and Ferrua, 2015; Aguilera, 2019). On the microstructural level, for example, several studies on isolated pulse cells showed that their cell wall provides a barrier diminishing the rate of starch hydrolysis (Duijsens *et al.*, 2021). To give an example, on a larger scale, the bioaccessibility of lipophilic carotenoids in oil-in-water emulsions increases with decreasing oil droplet size (Salvia-Trujillo *et al.*, 2017). Based on these examples, it can be concluded that for structured food systems, substrate accessibility generally is the rate-limiting step for enzymatic hydrolysis (Le Feunteun, Verkempinck, *et al.*, 2021).

However, most real foods are more complex and consist of an array of macro- and micronutrients as well as antinutrients, arranged heterogeneously throughout different structures from the molecular to the microscopic scale. Instead of studying simplified systems, the role of the complete food matrix received much attention recently (Aguilera, 2019; Capuano and Pellegrini, 2019; Pellegrini, Vittadini and Fogliano, 2020; Le Feunteun, Verkempinck, *et al.*, 2021). Several textural and/or rheological properties were found to play a role in the digestion of real foods. To give an example, diffusion of and thus interactions between substrates and digestive enzymes are limited in viscous digesta (Singh, Dartois and Kaur, 2010). Therefore, systems with a higher viscosity, for example, due to the presence of co-ingested fiber, were correlated to reduced postprandial glucose levels, both *in vitro and in vivo* (Singh, Dartois and Kaur, 2010; Jaime-Fonseca *et al.*, 2016). Moreover, textural properties such as the physical state (solid *versus* liquid) of the

food have been confirmed to affect digestion with higher lipolysis rates for a solid food (biscuit) compared to a liquid with an identical composition (Hiolle *et al.*, 2020).

Moreover, multiple co-ingested components are present in real foods. In this case, hydrolysis kinetics of a macronutrient may be influenced by the presence of one component (e.g., antinutrient) or the prepending or simultaneous digestion of another macronutrient. An example of interaction between different nutrients can be found for the case of emulsions. Several components surrounding the lipid phase (e.g., dietary fibers, proteins, carbohydrates, surfactants, and minerals) can interfere with emulsification and subsequently delay the digestion and absorption of these macronutrients (Golding and Wooster, 2010; Calvo-Lerma *et al.*, 2018). In another example, the digestibility of starch can be affected through entrapment within protein networks in the food matrix. This effect could be observed in bread, where the continuous gluten matrix entraps starch granules, thus hindering their digestibility (Capuano *et al.*, 2018). As a result, gradual proteolysis of the protein matrix in the GI tract could increase the accessibility of starch to amylolytic enzymes, facilitating amylolysis. A similar effect could be observed during the digestion of cooked pulse cotyledon cells. In these systems, the cell wall entraps an intracellular protein matrix which, in turn, entraps starch granules and hinders amylolysis. Upon gradual hydrolysis of the protein matrix, amylolysis is facilitated (Pälchen *et al.*, 2021). In this context, the study of digestion kinetics is indispensable for unraveling digestion patterns and mechanisms and the interplay between (macro-)nutrients in more complex structured foods. The complexity of real foods gives rise to challenges at the level of data acquisition (sampling) and mathematical modeling, e.g., for foods with simultaneous protein and starch hydrolysis, but also for foods in which phase separation occurs, and solid foods disintegrated by a relevant oral phase causing a broad range of particle sizes (Le Feunteun, Mackie and Dupont, 2020).

Current food digestion research even goes one step further. Most foods are not consumed as such but as a part of a meal, consisting of many other ingredients which may affect each other's digestive behavior.

In vivo, the complex interplay of different constituents and antinutrients ultimately affects the overall physiological response. Therefore, some efforts aimed at studying nutrient release patterns in complete meals (Calvo-Lerma *et al.*, 2018). For example, co-ingestion of an acidic beverage (lemon juice completely interrupted gastric amylolysis by HSA, while the effect of tea polyphenols was limited (Freitas and Le Feunteun, 2019). Importantly, experiments at each of the abovementioned levels of complexity (simple systems, complex foods, and real meals) can deliver crucial insights which can potentially be applied in the development of structured foods (and meals) with targeted nutrient release patterns.

2.4. From healthy adults to simulations relevant for specific populations

To generate a relevant and standardized IVD protocol, choices and priorities had to be made regarding the digestive conditions. Food scientists have developed standardized digestion methods incorporating physiological data of healthy adults to study the biochemical and physiological processes occurring during digestion and to develop healthy foods. The selection of digestion conditions based on healthy adults seems obvious, even if large inter- and intrapersonal variation exists within this group.

However, logically, the human population is not only made up of healthy adults. Research efforts should therefore be directed towards appropriately feeding the growing world population, increasingly suffering from (food-related) GI pathologies (e.g., chronic pancreatitis and cystic fibrosis) and/or altered GI conditions (e.g., elderly and infants). The functioning of the GI tract depends largely on age and health status (food-related pathologies), with the most important changing parameters being salivary secretion, mastication, enzyme secretion (i.e., amylases, proteases, and lipases), bile salt production, stomach contractions and bowel movements (Colombo *et al.*, 2021; Lee *et al.*, 2021). Variations in these parameters might lead to inefficient and/or incomplete nutrient digestion and absorption, leading to differences in the person's dietary needs. The standardized *in vitro* digestion approach, mimicking the digestive tract of healthy adults, is therefore not be suited for studying digestion in specific population

groups. However, since these people rely heavily on adapted nutrition, it is crucial to understand the effect of these altered parameters on micro- and macronutrient digestion.

Until recently, probably due to the restricted *in vivo* data available, little effort was put into studies on IVD in populations different from healthy adults. In 2015 however, the workshop held by the European Federation of Food Science and Technology (EFFoST) concluded that, due to important advances in the corresponding literature, important opportunities could arise from developing digestion models simulating the GI conditions of specific population groups (Shani-Levi *et al.*, 2017). These efforts could lead to a better understanding of the macronutrient hydrolysis and absorption phenomena in these population groups, which could lead to better nutritional guidelines. Moreover, a deeper understanding of the mechanisms occurring in the targeted populations could be a first step in the development of adapted (personalized) foods helping to preserve good health. Food structural design could enable the development of foods with *(i)* maximized macronutrient digestion to stimulate an optimal absorption (e.g., to improve protein uptake in elderly), or *(ii)* attenuated macronutrient digestion to control satiety and/or limit caloric absorption (e.g., for people suffering from obesity).

2.4.1. *How to adapt digestion models to include specific population groups*

The recent interest in food digestion in specific populations led researchers to the development and/or adaptation of *in vitro* models, as extensively reviewed elsewhere (Shani-Levi *et al.*, 2017). The first prominent challenge is collecting relevant *in vivo* data on GI conditions of the considered population group. Generally, procuring *in vivo* data is difficult and costly. However, several additional challenges arise from collecting data from specific population groups such as neonates, infants, elderly or ill people due to *(i)* practical, financial, and ethical issues, *(ii)* limited reproducibility of the results (e.g., due to large intra- and interpersonal variation), and *(iii)* the limited control over these human subjects (Ménard *et al.*, 2014). Amara et al. (2019) compiled available data on the levels of digestive lipase, gastric and intestinal pH, and

bile salt production at different ages and health conditions. Once available, the relevant *in vivo* data should appropriately be translated into experimental *in vitro* conditions.

In recent years, the standardized static INFOGEST protocol has been slightly adapted to mimic the GI tract of several population groups, as shown in *Table 1*. In the following paragraphs, an overview is given of the specific adaptations necessary for the *in vitro* study of food digestion in infants, elderly, and people suffering from GI disorders.

2.4.2 Infants

Understanding the GI conditions of infants might lead to the development of formulas (breastmilk substitutes) with health benefits for neonates. To this end, monogastric animal models were often used, with disadvantages such as the price of the set-up, work intensity, and variability and transferability of results. To overcome these limitations, researchers tried to develop IVD models simulating the digestive tract of infants (Passannanti *et al.*, 2017; Ménard *et al.*, 2018).

The digestive function of infants highly differs from that of adults, with many mechanisms still immature, as extensively reviewed (Bourlieu *et al.*, 2014) with the prospect of developing *in vitro* models. In short, infants have a lower gastric acid secretion than adults with a fasting pH of around 3.1 to 3.4 (Armand *et al.*, 1996). Upon meal ingestion, the pH increases to around 6, to gradually decrease again (Mitchell, McClure and Tubman, 2001). Enzymatic secretions occur very differently in infants. Gastric lipase activity is about 4 times higher in infants than in healthy adults, while pepsin activity is about 8-fold lower in infants (Henderson *et al.*, 2001). In the small intestine, the secretion of pancreatic amylase is very low. Moreover, the contribution of pancreatic lipase to lipid digestion is limited, in contrast to its major significance in adults (Hamosh, 1996).

Infant digestion was mimicked using highly varying parameters, rendering comparison of results among studies impossible. To standardize simulation efforts, Ménard et al. (2018) proposed a static *in vitro* model

simulating digestion in infants born at term and aged 28 days. Similar to the standardized *in vitro* protocol developed for healthy adults (Minekus *et al.*, 2014), the gastric phase of the model is based on digestive parameters found at the gastric emptying half-time (Ménard *et al.*, 2018). The model was applied using infant formula and the results were compared to *in vivo* data confirming the physiological relevance of the model. A comparison with adult data highlighted the importance of considering the specific (immature) characteristics of the infant's digestive system. It was concluded that the model could be extrapolated to older infants if the progressing maturity of the digestive system (e.g., enzyme secretion) is considered.

2.4.2. *Elderly*

The nutritional intake of older adults decreases by about 25% due to a combination of factors, such as changes in eating habits, changes in smell and taste affecting enjoyment, physiological responses, health-related, social and/or economic factors as well as limitations in preparing, ingesting, and tolerating certain foods (Rémond *et al.*, 2001; Milan and Cameron-Smith, 2015). While the energy requirement gradually decreases from a certain age, this is not the case for protein and (micro-)nutrient requirements (Nowson and O'Connell, 2015), shifting the dietary needs towards more nutrient-dense foods. The reduced food intake of older adults can thus cause a risk of insufficient nutrient and especially protein intake, possibly leading to undernutrition, loss of muscle mass, and development of 'anorexia of aging' (Milan and Cameron-Smith, 2015; Nowson and O'Connell, 2015; Rémond *et al.*, 2015).

All GI functions are affected by aging (Dumic *et al.*, 2019), including changes in chewing capacity, a decrease in gastric emptying times, gastric, pancreatic, and gall bladder secretions and suppressed gastric and small intestinal motility, possibly affecting macronutrient digestion and absorption (Milan and Cameron-Smith, 2015). The functional decline of the GI tract mainly includes altered pH and reduced pepsin level in the stomach, and altered secretions of bile and pancreatic enzymes in the small intestine (Levi and Lesmes, 2014). These changes may significantly affect the hydrolysis of food nutrients, primarily

lipids, and proteins as demonstrated for (meat) proteins and emulsions using dynamic *in vitro* GI methods (Denis *et al.*, 2016; Shani Levi *et al.*, 2017).

While there are clear effects of aging, alterations of physiological digestion capacities largely depend on health status and medication intake. To give a common example, proton pump inhibitors (PPIs) are used to treat acid-related diseases (e.g., gastroesophageal reflux) by decreasing gastric acid secretion. However, some nutrients such as proteins (and protein-bound vitamins such as B-12) require the acidic conditions of the stomach for hydrolysis (and release of B-12 from food proteins) (Maes, Fixen and Linnebur, 2017). The effect of aging is therefore often difficult to separate from the effect of pre-existing illness (Dumic *et al.*, 2019). Moreover, some GI diseases (e.g., diabetes type II) are more prevalent in older adults, but their occurrence might also be correlated with other factors such as eating habits, lifestyle, BMI, and physical fitness (Milan and Cameron-Smith, 2015). Interestingly, a high disease burden, as often present in elderly individuals, increases the protein requirement even more (Nowson and O'Connell, 2015). In this regard, to answer the specific nutritional needs of elderly, appropriate nutritional recommendations are necessary. Considering that elderly (a very diverse group, often considered as people from an age of about 60 to 65 years old (Rémond *et al.*, 2001)) are becoming an increasingly large part of the population, the changes occurring in the GI tract upon aging and their effect on the mechanisms of digestion should be studied. In this context, *in vitro* models relevantly recreating the physiological conditions of the elderly GI tract are essential and could contribute to the development of nutritious, appealing, and enjoyable foods with tailored digestive properties.

Hernández-Olivas et al. (2020) evaluated the effect of the GI conditions of elderly during digestion of different foods *in vitro* and concluded that proteolysis and micronutrient bioaccessibility were significantly reduced compared to healthy adult conditions. The model developed in this study could be applied as a screening tool to evaluate commercially available products and to determine whether their digestive properties are in line with the needs of elderly (e.g., high amounts of readily digestible protein).

2.4.3. *People with gastrointestinal disorders*

Every year, around 1 million people die from GI disorders (e.g., chronic pancreatitis, cystic fibrosis) in Europe (United European Gastroenterology, 2016), highlighting the importance of understanding the processes occurring during digestion in these patients to prevent and combat the consequences. *In vivo,* these populations show a functional decline of the GI tract, mainly including altered duodenal pH and altered secretions of carbonate, bile salts, and pancreatic enzymes in the small intestine (Carrière *et al.*, 2005; Calvo-Lerma *et al.*, 2019). These changes can significantly affect the hydrolysis of food nutrients such as lipids and proteins.

IVD models adapted to match digestion conditions of people with GI disorders can play a crucial role in understanding macronutrient digestion patterns, and the subsequent development of pharmaceuticals (e.g., enzymatic supplements) improving macronutrient digestion and absorption. However, only limited data are currently available on *in vitro* simulation of the GI conditions of people with GI pathologies, and possible correlation of *in vitro* with *in vivo* data. Calvo-Lerma *et al.* (2018) applied an IVD method using the conditions recorded in patients suffering from cystic fibrosis to study the effect of the lipid organization and interactions with different components on *in vitro* lipid digestion. Moreover, the theoretically optimal dose of an enzymatic supplement, required to reach optimal lipolysis of a selection of foods in people with cystic fibrosis, was determined (Calvo-Lerma *et al.*, 2019). These interesting results showed great potential for application in *in vivo* clinical trials for Pancreatic Enzymatic Replacement Therapy (Calvo-Lerma *et al.*, 2019).

Overall, the development of *in vitro* methods simulating digestion conditions of specific population groups is a step forward towards nutrition targeted at specific populations (i.e. population-oriented nutrition) and even personalized foods. Advances in food science and relevant *in vivo* data give researchers the tools necessary to build appropriate and flexible IVD models able to mimic physiological parameters such as pH, gastric and intestinal secretions, digestion time, absorption of digested products, and peristaltic

movements in the different GI compartments. Further testing of these models and development of models for other population groups (such as diabetics, obese people, and people with gastric bypass) is necessary to study the digestive function of people in other population groups which are becoming more and more prevalent in our society. In turn, this can help towards the development of appropriate nutritional recommendations, improved and innovative pharmaceuticals, and intelligently designed foods with directed digestive properties.

Concluding remarks and future directions

Rational food digestion design requires a detailed understanding of underlying principles guiding food digestive fate. The INFOGEST IVD method is most widely used, and enabled researchers to obtain detailed mechanistic insights. IVD methods are increasingly being adapted to better represent physiological conditions and/or answer specific research questions. These methods of distinct complexity can be considered complementary as they deliver distinct scientific insights. The present paper discusses important strategic considerations for the appropriate set-up of digestion experiments. Overall, an evolution from simplified IVD systems for obtaining mechanistic understanding towards more complex, realistic systems was observed.

The continuous evolution of *in vitro* simulation methods requires further improvement of analytical platforms, statistical data analysis, and mathematical modeling, to appropriately study and interpret digestion patterns. Importantly, integration of static to dynamic *in vitro*, *in vivo,* and potentially *in silico* and *in situ* microscopic digestion data (Do *et al.*, 2020) could provide unique mechanistic insights which could not be provided by a single approach.

Future *in vitro* digestion research should gather new insights by employing more complex digestion approaches (e.g., dynamic digestion factors, complex foods), and take these into account in the design of new experimental set-ups which should be ‘as simple as possible, but as complex as needed’. While the

inclusion of dynamic digestion factors may be crucial for studying digestion processes under more physiologically realistic conditions, the importance of simple screening methods should not be minimalized, especially in the context of food structural design. In addition, to answer the challenges the world is currently facing, future digestion methods should include multiple population groups such as obese but also malnourished people. Moreover, since nutrient labels are still based on concentrations, widely implementable and easy-to-use methods allowing for the straightforward comparison of the digestion functionality of foods, should receive sufficient attention. These combined efforts could lead to appropriate nutritional advice and, building on vast knowledge of process-structure-function relations, well-designed (personalized or population-oriented) foods with tailored digestive properties.

Declaration of interests

None

CRediT authorship contribution statement

D. Duijsens: Conceptualization; Formal analysis; Investigation; Visualization; Roles/Writing - original draft. **K. Pälchen:** Conceptualization; Formal analysis; Investigation; Visualization; Roles/Writing - original draft. **J.M. Guevara-Zambrano:** Conceptualization; Formal analysis; Investigation; Visualization; Roles/Writing - original draft. **S.H.E. Verkempinck:** Writing - review & editing. **M.R. Infantes-Garcia:** Writing - review & editing. **M.E. Hendrickx:** Conceptualization; Funding acquisition; Supervision. **A.M. Van Loey:** Conceptualization; Funding acquisition; Supervision. **T. Grauwet:** Conceptualization; Funding acquisition; Supervision, Writing – review & editing.

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