INFOGEST static in vitro simulation of gastrointestinal food digestion

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INFOGEST static in vitro simulation of gastrointestinal food digestion

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Developing a mechanistic understanding of the impact of food structure and composition on human health has increasingly involved simulating digestion in the upper gastrointestinal tract. These simulations have used a wide range of different conditions that often have very little physiological relevance, and this impedes the meaningful comparison of results. The standardized protocol presented here is based on an international consensus developed by the COST INFOGEST network. The method is designed to be used with standard laboratory equipment and requires limited experience to encourage a wide range of researchers to adopt it. It is a static digestion method that uses constant ratios of meal to digestive fluids and a constant pH for each step of digestion. This makes the method simple to use but not suitable for simulating digestion kinetics. Using this method, food samples are subjected to sequential oral, gastric and intestinal digestion while parameters such as electrolytes, enzymes, bile, dilution, pH and time of digestion are based on available physiological data. This amended and improved digestion method (INFOGEST 2.0) avoids challenges associated with the original method, such as the inclusion of the oral phase and the use of gastric lipase. The method can be used to assess the endpoints resulting from digestion of foods by analyzing the digestion products (e.g., peptides/amino acids, fatty acids, simple sugars) and evaluating the release of micronutrients from the food matrix. The whole protocol can be completed in ~7 d, including ~5 d required for the determination of enzyme activities.

Introduction

The worldwide prevalence of diet-related diseases has been on the increase for the past few decades[1]. Large-scale human intervention trials have been used to correlate diet with the health of different demographic groups. However, to understand the physiological response to specific foods, it is necessary to follow the complex digestive processes within the human digestive tract in more detail. This can be achieved with invasive procedures, such as aspiration from the stomach[2] or small intestine[3] or with less invasive imaging technologies (e.g., magnetic resonance imaging[4]) and wireless

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telemetric systems[2,5]. Animal models are also widely used, although their use generally involves animal death or surgical approaches in which cannulas are placed into digestive organs to access the contents of the gastrointestinal tract. The relevance of animal models for understanding food digestion in humans is also regularly questioned. In summary, in vivo (human or animal) intervention trials can be difficult to undertake, unsuitable and expensive or are not justifiable on ethical grounds. For these reasons, in vitro models have been used for many decades to simulate the digestion of food.

Development of the protocol

There are several types of in vitro digestion methods that are commonly used for food; these can be divided into static and dynamic methods. These models aim to simulate the physiological conditions of the upper gastrointestinal tract, namely the oral, gastric and small intestinal phases. Most dynamic models[6–10] have been shown to be suitable for simulating the digestion of foods and pharmaceutical products in different population groups and for different purposes[11]. However, these models are relatively complex, expensive to set up and maintain, and therefore may not be available to the majority of food researchers.

Owing to its simplicity, static models, which use a constant ratio of food to enzymes and electrolytes, and a constant pH for each digestive phase, have been widely used for many decades for food, animal feed and pharmaceutical purposes[12–14]. Static in vitro digestion models have been shown to be very useful in predicting outcomes of in vivo digestion[15,16]. There are standardized static models[17] that vary in complexity[18,19]; these are used for simulating the gastrointestinal behavior of pharmaceutical products (United States Pharmacopeia methods)[17]. Other static methods were developed for assessing the in vitro bioaccessibility of soil contaminants[20], heavy metals in particular, or mycotoxins in food[21]. These methods, developed and standardized[22] by the Bioaccessibility Research Group of Europe (BARGE) were based on available physiological data reported by landmark papers such as Dressman et al.[23] or the Geigy tables[24]. The static methods of the BARGE group and United States Pharmacopeia procedures were important milestones in the evolution of standardized in vitro digestion methods. However, their experimental conditions, purpose and endpoints were found to be unsuitable for digesting food because of the complexity and variability of food structures, as well as very different research questions in food science. This resulted in the use of a great number of digestion methods, reviewed by Hur et al.[25], with slight but important variations in parameters such as pH, duration, enzyme concentration and activity, and composition of simulated digestive fluids.

Hence, the need for a harmonization of digestion conditions was identified, and the international INFOGEST[26] network (http://www.cost-infogest.eu) of multidisciplinary experts (in, e.g., food science, nutrition, gastroenterology, engineering and enzymology) from more than 35 countries was established. One of the primary outcomes of this network was an international consensus on a set of digestion parameters for a static in vitro simulation of adult digestion suitable for food. The method, generally referred to as the INFOGEST method, was published[27], and experimental parameters were justified and discussed in great detail in relation to available in vivo physiological data. Some of the previous digestion methods outlined above were used as a starting point. Since its publication in 2014, this in vitro digestion method has received a Highly Cited Paper status for agricultural sciences, with more than 650 citations in Web of Science and has been extensively used all over the world for numerous purposes, with a variety of foods and different endpoints. The current article builds on that publication and clarifies a number of aspects of the original protocol, leading to an improved INFOGEST 2.0 protocol, which is described here.

Overview of the procedure

The digestion procedure is summarized in Fig. 1. It can be divided into three phases: preparation, digestion procedure and sample treatment with subsequent analysis. For preparation of the in vitro digestion, the activities of all digestive enzymes and the concentrations of bile salts should be determined experimentally, using the recommended standardized assays for amylase, pepsin, lipase (both gastric and pancreatic), trypsin and chymotrypsin, outlined in Box 1 and described in detail in the Supplementary Information, based on Minekus et al.[27]. This first preparation step is of the utmost importance, and failure to correctly assay enzyme activity will lead to incorrect rates of digestion of components (e.g., proteins)[28], potentially changing the overall digestion of the food.

The digestion involves the exposure of the food to three successive digestive phases: oral, gastric and intestinal. For static in vitro digestion methods, the experimental conditions are constant during

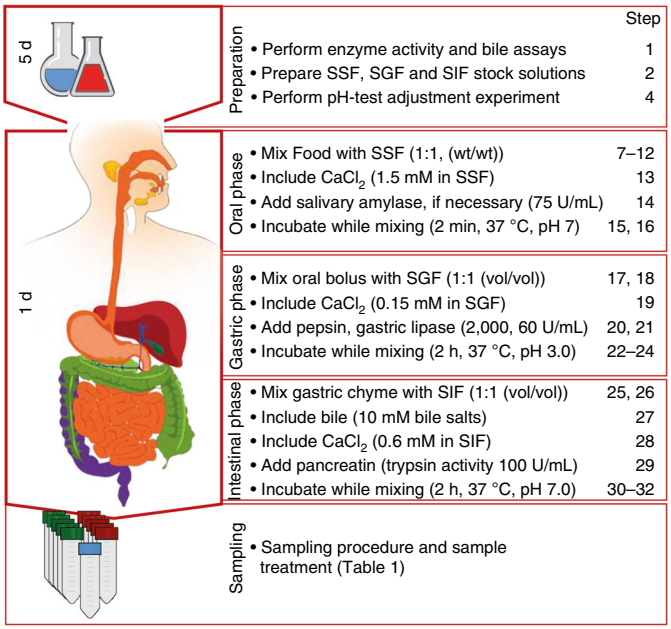

Fig. 1 | Flow diagram of the INFOGEST 2.0 digestion method. Timing and flow diagram of the INFOGEST 2.0 in vitro digestion method for food. Expected time frame (left) and stages and corresponding step numbers in the Procedure (right) are given. SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SSF, simulated salivary fluid.

each phase. The oral phase involves dilution of the food 1:1 (wt/wt) with simulated salivary fluid (SSF), with or without salivary amylase, and for solids or semisolids, simulated mastication of the food. If used, exposure of the food to salivary amylase is limited to 2 min at pH 7. The oral phase must be included in all simulated digestion procedures, regardless of the state of the food (liquid or solid) in order to provide consistency of dilution. Further clarification regarding the preparation of the food and the oral phase can be found in the 'Experimental design' section.

The oral bolus is then diluted 1:1 (vol/vol) with simulated gastric fluid (SGF) and gastric enzymes (pepsin and gastric lipase) and incubated under agitation at pH 3.0 for 2 h. The gastric chyme is then diluted 1:1 (vol/vol) with simulated intestinal fluid (SIF), bile salts and pancreatic enzymes (pancreatin based on the activity of trypsin or as individual enzymes) and incubated at pH 7 for a further 2 h.

The experimental conditions for the digestion procedure, such as pH, time of digestion and enzyme activity, were based on available physiological data of the fed state for a typical meal and were described and justified in detail in Minekus et al.[27]. For this improved INFOGEST 2.0 method, the use of gastric lipase is recommended; hence, a detailed justification of the type and activity of the gastric lipase is provided in the 'Experimental design' section.

The last step of the digestion procedure involves sampling, sample treatment, storage and subsequent analysis of samples. This step should be carefully considered before digestion, as it may differ from case to case because of different endpoints, purposes of the digestion experiment and type of analysis. A description of sample treatment can be found in the 'Experimental design' section and Table 1.

Advantages and limitations

Static in vitro digestions are the simplest methods for simulation of in vivo food digestion. Although there are clear weaknesses in these simple models, they have obvious advantages over more complex methods. The main strengths of static in vitro models are the good intra- and inter-laboratory reproducibility, robustness, simplicity, relatively low cost and easy assessment of each digestion phase. This latter point makes them quite suitable for mechanistic studies, hypothesis building and screening. It was one of the aims of the INFOGEST network to not only standardize in vitro methods but to agree on experimental conditions based on available physiological data that are as close as possible to the in vivo equivalent, while keeping the method sufficiently simple to reproduce all over the world. The clear definition of standardized experimental conditions and procedures is one of the major advantages of the INFOGEST method. Egger et al.[28] showed very good lab-to-lab

Box 1 | Enzyme activity assays

Detailed assays are given in the Supplementary Methods for each enzyme and are based on Minekus et al.[27].

Pepsin activity assay
Principle: hemoglobin plus H_2O produces TCA-soluble tyrosine peptides when pepsin is applied.
Unit definition: one unit produces a ΔA280 of 0.001 per min at pH 2.0 and 37 °C, measured as TCA-soluble products.
Substrate: 2% (wt/vol) hemoglobin in water at pH 2.
Enzyme solution: pepsin in 10 mM Tris buffer, 150 mM NaCl, pH 6.5. Before the assay, dilute it in 10 mM HCl at concentrations ranging from 5 to 30 μg/mL.
Mix 500 μL of hemoglobin with 100 μL of each pepsin solution (5–30 μg/mL) and incubate for 10 min at 37 °C. To stop the reaction, add 1 mL of 5% (wt/vol) TCA. Centrifuge at 6,000*g* for 30 min at room temperature and read the absorbance at 280 nm in quartz cuvettes.

Lipase activity assay
Principle: tributyrin plus H_2O produces butyric acid and *sn*-2 monobutyrin when lipase is applied.
Unit definition: one unit releases 1 μmol of butyric acid per min at 37 °C at the pH of the assay.
Substrate: tributyrin purity ≥99%.
Enzyme solution: Lipase 1 mg/mL in H_2O.
Assay solution for gastric lipase: 2 mM sodium taurodeoxycholate, 150 mM NaCl and 1 μM BSA.
Assay solution for pancreatic lipase: 4 mM sodium taurodeoxycholate, 150 mM NaCl and 1.4 mM $CaCl_2(H_2O)_2$.
In a pH-stat at 37 °C, mix 14.5 mL of assay solution with 0.5 mL of tributyrin, and stir until it forms a fine oil-in-water emulsion. Add 50 or 100 μL of enzyme solution (1 mg/mL) and monitor the rate of titrant (0.1 N NaOH) to maintain pH 6.0 (human gastric lipase), pH 5.5 (rabbit gastric lipase) or pH 8 (pancreatic lipase) for 5 min.

Trypsin activity assay
Principle: TAME plus H_2O produces p-toluene-sulfonyl-L-arginine plus methanol when trypsin is applied.
Unit definition: One unit hydrolyzes 1 μmol of p-toluene-sulfonyl-L-arginine methyl ester (TAME) per min at pH 8.1 and 25 °C.
Substrate: 10 mM TAME in H_2O.
Enzyme solution: Trypsin in 1 mM HCl at concentrations ranging from 10 to 20 μg/mL.
Mix 2.6 mL of 46 mM Tris–HCl buffer (pH 8.1) with 300 μL of the substrate at 25 °C. Add 100 μL of each trypsin assay solution. Read the absorbance increase at 247 nm during 10 min.

Amylase activity assay
Principle: Starch plus H_2O produces reducing groups (e.g., maltose) when α-amylase is applied.
Unit definition: One unit releases 1.0 mg of maltose equivalent from starch in 3 min at pH 6.9 and 20 °C.
Substrate: 1.0 % (wt/vol) soluble potato starch in 20 mM sodium phosphate buffer with 6.7 mM NaCl, adjusted to pH 6.9.
Enzyme solution: 1 mg/mL amylase in H_2O.
Incubate 1 mL of substrate at 20 °C, add the enzyme solution (0.5–1 mL, with an estimated activity of 1 unit/mL) and incubate at 20 °C for 3 min. Stop the reaction with a color reagent (96 mM 3,5-dinitrosalicyclic acid, 5.3 M sodium potassium tartrate). Bring the volume to 1 mL with H_2O, cap the tube and boil for 15 min. Add 9 mL of H_2O and read the absorbance at 540 nm. Calculate the activity against a maltose standard curve.

reproducibility of results with regard to peptide patterns from the in vitro digestion of skim milk from powder. Some weaknesses were identified and subsequently addressed. The recommendation of standardized enzyme assays (including units) substantially added to the precision and reproducibility of the digestion procedure, as previously a number of common but slightly different enzyme assays were being used, resulting in the application of a wide range of enzyme activities during digestion experiments. The endpoint of this INFOGEST method was recently compared to digests obtained in human jejunum after casein and whey protein ingestion[16] and showed excellent correlation of protein degradation and peptide patterns, as explained below in the 'Applications' section.

However, static digestion methods have known limitations and cannot mimic the complex dynamics of the digestion process or the physiological interactions with the host. For example, for the gastric phase, the pH is kept constant, and there is a lack of the gradual addition of gastric fluid (acid, minerals and pepsin) and an absence of gradual gastric emptying. In addition, the enzyme activity in each digestive phase is kept constant, regardless of the type of food and whether the food contains high or low amounts of substrate, e.g., proteins, lipids and carbohydrates. The intestinal phase is treated as one phase rather than as the sequential duodenal, jejunal and ileal phases, which exhibit different dilutions, mineral content, pH, enzyme activities and microbial content. These shortcomings render the method unsuitable for detailed kinetic analysis of the different stages of the digestion process. However, in vivo comparison shows good correlation with the INFOGEST method at the

Table 1 | Examples of how to preserve and treat samples after in vitro digestion

Application	Objectives	Method/description	Sample preparation	Ref.
Food structure	Microscopy Rheology Particle size	Keep on ice and perform microscopy observations immediately after sampling	Fresh samples for standard microscopy sample preparation (e.g., resin embedding, chemical fixation and drying)	75,102
Breakdown of nutrients: proteins	Protein hydrolysis or resistant protein analysis	**To stop gastric digestion:**		
		Raise the pH to 7 for partial inactivation of pepsin; use pH 8 for complete inactivation	Addition of 1 M $NaHCO_3$ or 1 N NaOH	28
		Addition of pepstatin A for pepsin inhibition	Add pepstatin A at a 0.5–1.0 μM final concentration	103
		To stop intestinal digestion:		
		Addition of Pefabloc SC for serine protease (trypsin and chymotrypsin) inhibition	Add 50 μL of Pefabloc (0.1 M) in water per milliliter of intestinal digesta (5 mM final concentration)	28
		Addition of BBI from soybean with ability to inhibit both trypsin and chymotrypsin	Add 100 μL of a BBI solution (0.05 g/L) in water per milliliter of intestinal digesta	104
		Heat-shock treatment	Sample treatment: 100 °C, 5 min, but this is detrimental to food structure, especially protein and carbohydrate structures	41
Breakdown of nutrients: lipids	Lipid hydrolysis	**To stop lipase activity in the gastric phase:**		
		Addition of Orlistat	Add 10 μL/mL of a 100 mM Orlistat solution in ethanol (1 mM final concentration)	105
		Raise the pH to 8	—	59
		To stop lipase activity in the intestinal phase:		
		Addition of lipase inhibitor (4-bromophenylboronic acid)	Add 5 μL/mL of a 1 M solution of Orlistat in methanol to 1 mL of digesta (5 mM final concentration)	69
		Addition of methanol/chloroform	Addition of methanol/chloroform mixture used for Folch extraction	77
Breakdown of nutrients: carbohydrates	Starch hydrolysis	**To stop amylase activity:**		
		Addition of Na_2CO_3	Dilute digesta in equal volume of 0.3 M Na_2CO_3	106
		Heat-shock treatment	100 °C for 5 min, see above	
		TCA precipitation	Add 700 μL of 100% (wt/vol) TCA to 5 mL of digesta	107
		Ethanol	Add sample to four volumes of ethanol	108
Breakdown of oxygen-sensitive phytochemicals	Degradation of polyphenols and carotenoids	**To prevent contact with oxygen:**		
		Flushing with Ar or N_2, pyrogallol addition (carotenoids) before small intestinal digestion	Flush the sample for 1 min with Ar or N_2	88
Bioaccessibility	Bioaccessibility of digested nutrients	**To stop pancreatic activities (see 'To stop intestinal digestion' above):**		
		Use of inhibitors, e.g., Pefabloc Test whether the use of enzyme inhibitors affects the results of the experiment	See 'To stop intestinal digestion' above	28
		Use of dialysis membranes/centrifugation tubes having a cutoff of 3–10 kDa	—	109
		To dilute the digested samples to maintain the epithelium integrity of cell monolayers and avoid cytotoxicity	Dilution (several folds) of digested samples to reach osmolarity values at physiological levels (285–300 mOsm/kg H_2O)	110,111
		Extraction of compounds by using either solvents or acidic solutions	Different procedures for a wide range of compounds are employed	112
	Bioaccessibility of digested phytochemicals	Removal of unavailable constituents such as those bound to macromolecules or complexed form	Ultracentrifugation and filtration with certain cutoff filters (e.g., 0.2 μm)	111
		Cleavage of glucosides and esters	Addition of brush-border vesicles	91
Probiotic survival	To determine the survival rates of probiotic bacteria in digestion conditions	Immediate use of samples after digestion	To serially dilute the digested samples and plate for bacterial growth	113
Colonic fermentation and modulation of intestinal microbiota	Biotransformation of compounds and their effects on bacterial growth	**To stop enzymatic activities:**		
		Heat shock	Heat treatment: 100 °C for 5 min, but this is detrimental to food structure, especially protein and carbohydrate structures	114
		Immediate storage in ice before batch culture fermentation		115

endpoints of each digestion phase[16,29]. For this reason, the static model should be used only to assess digestion endpoints and not kinetics.

In some cases, a slight alteration of the procedure can be considered to more accurately reflect physiological conditions. For example, during the gastric in vivo digestion of food containing probiotic bacteria, the bacteria are exposed to a range of pH values, which can be as low as 1 at the end of the gastric emptying. Hence, a static method with a constant pH of 3.0 for the gastric phase may fail to accurately predict probiotic survival, and a lower pH or a dynamic gastric model should be chosen. In the study of the bioaccessibility of phytochemicals such as polyphenols and carotenoids, the model allows the realistic release from a food into the aqueous phase. However, specific hydrolytic processes occurring at the brush border are currently not simulated, and additional steps, such as centrifugation of the digesta, are needed to separate the bioaccessible phases. An extension including colonic fermentation, an important step in the bioactivation of several phytochemicals, would further enhance the physiological appropriateness. Finally, for the assessment of the bioaccessibility of small amounts of contaminants in food, such as heavy metals, environmental pollutants or mycotoxins, alternative methods reflecting extensive digestion and worst-case scenarios[20] can be applied.

Applications

The method described has been used to assess the release of carotenoids and phenolic compounds from different matrices, such as carotenoids in fruits[30,31], carotenoids in tomatoes compared to tomatoes subjected to pulsed electric fields[32], β-carotene protected by microencapsulation[33] and resveratrol encapsulated in protein nanoparticles[34]. However, most studies have been dedicated to the evaluation of protein, lipid and starch digestion in foods or modified carriers. Protein digestion has been widely assessed in different dairy products[35,36] and in isolated milk proteins, such as lactoferrin, with different iron contents and after mild heat treatment[37]. The stability of proteins to gastrointestinal digestion has been proposed as an additional piece of information for the allergenicity assessment of novel proteins[38]. With this focus, the INFOGEST method was also applied to the study of the immunogenic potential of peptides from pasta[39], hazelnut[40] and peanut[41], which are resistant to gastrointestinal digestion. Using a pH-stat to monitor enzymatic hydrolysis, it was shown that solid emulsions led to a lesser extent of lipolysis but a greater degree of proteolysis as compared to liquid emulsions because of the higher sensitivity of denatured whey proteins to gastrointestinal enzymes[42]. The tendency of dairy rennet gels to form compact protein aggregates during gastric digestion has also been assessed[43]. Other applications of this protocol include the evaluation of novel biopolymers designed for a controlled nutrient release[44,45] and the digestive stability of transgenic microRNAs in genetically modified plants[46].

An inter-laboratory trial applying different in vitro digestion protocols clearly demonstrated a good reproducibility obtained by using the standardized INFOGEST protocol. It also highlighted the importance of correctly applying standardized pepsin activity assays, which is a key factor for proper gastric protein hydrolysis[28]. A special effort was made to validate and compare the results from this in vitro digestion protocol with in vivo data. For instance, β-cryptoxanthin bioavailability of pasteurized orange juice was found to be higher than that of fresh oranges in a randomized crossover human study, and from the in vitro digestion an increased bioaccessibility could also be inferred[47]. Several studies have focused on protein digestion and the comparison with in vivo digestion in human or animal models. The results from the in vitro gastrointestinal digestion of skim milk powder were compared with in vivo porcine samples collected from the stomach and several sites in the intestine[29]. Protein degradation and peptides generated at the end of the gastric phase correlated well with in vivo gastric peptides, and the in vitro intestinal phase correlated well with the in vivo samples taken in the median jejunum. Human jejunal digests after the oral ingestion of casein and whey protein were compared with the intestinal digests obtained using the standardized INFOGEST method[16]. In vivo and in vitro intestinal digests showed common protein regions that are resistant to digestion and a high number of identical peptide sequences, and the researchers concluded that the INFOGEST in vitro method is a good approximation of the endpoints of gastrointestinal digestion of milk proteins in vivo.

Alternative methods

A wide variety of static in vitro digestion models can be found in the literature[25], but they all exhibit different conditions (e.g., pH, duration of each step, ratio of enzymes to substrate), making the comparison between studies impossible. The static methods published by Versantvoort et al.[21],

Garrett et al.[48] and Oomen et al.[20] are among the most highly cited approaches. However, most of the static in vitro digestion methods found in the literature simulate the fasted state, which is quite far from the physiological conditions when food is digested in the gastrointestinal tract. The advantages and limitations of static in vitro digestion models were recently reviewed by a group of experts within the INFOGEST network[15]. Although static methods can be useful for understanding trends or performing a screening of samples, they fall short in their ability to assess some of the important dynamic processes occurring during gastrointestinal digestion, namely the pH gradients and the gradual addition of enzymes and gastric fluid, as well as continuous gastric emptying. More physiologically relevant dynamic digestion methods[6–10] take these and other factors into account. However, these models are highly complex, require substantial hardware and software and are still expensive to set up and maintain; hence, they are often not available to food researchers. It has recently been shown that, when human data are available to set up the system, these models can be physiologically relevant[11]. In an effort to improve in vitro digestion methods, a low-cost semi-dynamic method was recently developed[49] and described in detail[50], in which parameters were based on the equivalent in vivo data from the digestion of dairy products. Here, the SGF and pepsin are slowly added to the food in a suitable reaction vessel with manual, stepwise gastric emptying. A harmonization of experimental conditions is currently ongoing, and a standardized semi-dynamic method will be published in 2019 by INFOGEST members, coordinated by A.R. Mackie.

Even though they are expensive and must be ethically justifiable, in vivo models have been widely used for studying the digestive process. The pig model can closely simulate the upper part of the human digestive tract (stomach and small intestine)[51]. Conventional pigs or mini-pigs can be used for this purpose and can be equipped with cannulas, in order to allow sampling of the effluents throughout digestion, and a catheter to collect blood, whereas piglets can be used for all the questions related to neonatal nutrition[29,52,53].

Finally, human volunteers can be equipped with naso-gastric or naso-intestinal probes to access and sample digestive effluents[3]. Ileostomy patients have been used to study digestion[54–56] but can hardly be considered as models of a healthy human because they are affected by digestive pathologies.

Experimental design

Enzyme assays

The determination of the standard units of activity of the enzyme used in the protocol is a critical step and one of the main sources of variation in results between different laboratories[37]. Enzyme activity determination is recommended for each new batch of enzyme or after prolonged storage.

Enzyme and bile assays were previously described in protocol format in the supplementary materials of Minekus et al.[27], namely α-amylase (EC 3.2.1.1), pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), pancreatic lipase (EC 3.1.1.3) and bile salts (according to supplier's protocol). To improve the reproducibility of the pepsin activity assay for this revised INFOGEST 2.0 protocol, it is now recommended to dissolve pepsin in 10 mM Tris buffer (tris-hydroxymethyl-aminomethane), 150 mM NaCl (pH 6.5), instead of in sodium chloride solution adjusted with sodium hydroxide. The buffering capacity of Tris buffer reduces the variability in the measurement of the pepsin activity, as shown previously[37]. The detailed protocols for the complete set of enzyme and bile assays, including that of the gastric lipase assay (EC 3.1.1.3), can be found in the Supplementary Methods and are summarized in Box 1.

Spreadsheets for the enzyme assays and the volumes for the digestion procedure are provided in Supplementary Data 1 and 2. The enzyme assay spreadsheets (Supplementary Data 1) can be used to calculate the enzyme activities of all digestive enzymes. The digestion spreadsheets (Supplementary Data 2) provide help in calculating all volumes of simulated digestive fluids, enzyme and bile solutions on the basis of the initial amount of digested food; one example is shown in Table 2. The corresponding online spreadsheets can also be used, and are available at http://www.proteomics.ch/IVD and on the INFOGEST website https://www.cost-infogest.eu/. In addition, videos of the digestion procedures (Supplementary Videos 1 and 2) and all enzyme activity assays (Supplementary Videos 3 to 7) are available in the Supplementary Information. The videos are also available online on the YouTube channel 'In vitro food digestion - COST action INFOGEST' (https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg) and on the INFOGEST website (https://www.cost-infogest.eu/).

Table 2 | Example of an in vitro digestion experiment with 5 g of food

Input	5 g of liquid or solid food				
Digestion phase	**Oral (SSF)**	**Gastric (SGF)**		**Intestinal (SIF)**	
Food or digesta	5 g of food	10 mL from oral phase		20 mL from gastric phase	
1.25× electrolyte stock solutions (mL)	4[a]	8		8[b]	
$CaCl_2(H_2O)_2$ (0.3 M) (mL)	0.025	0.005		0.04	
Enzymes	Salivary amylase[c]	Pepsin	Gastric lipase[d]	Trypsin in pancreatin	Bile salts
Enzyme activity (U/mL) or bile concentration (mM) in total digesta (final volume in milliliters at each digestion phase, see row below)	75 U/mL	2,000 U/mL	60 U/mL	100 U/mL	10 mM
Specific activity[e] (U/mg), concentration (bile) mmol/g	100 U/mg	3,000 U/mg	25 U/mg	6 U/mg	0.667 mmol/g
Concentration of enzyme/bile solution (mg/mL)	10	20	100	133.3	200
Volume of enzyme/bile to be added (mL)	0.75	0.667	0.48	5[b]	3[b]
H_2O (mL)	0.225	0.448		3.16	
HCl (5 M) for pH adj. (mL)	–	0.4		–	
NaOH (5 M) for pH adj. (mL)	–	–		0.8	
Final volume (mL)	10	20		40	

[a]1:1 (wt/wt) dilution with simulated salivary fluid (SSF) should result in a paste-like consistency; add more water if necessary. Some foods may not be digested as expected due to a high substrate-to-enzyme ratio in the static digestion method and may need to be further diluted with water before the oral phase; see Table 4. [b]Total volume of simulated intestinal fluid (SIF 1.25×): 16 mL, including pancreatin (5 mL) and bile (3 mL), both of which are dissolved in SIF. [c]Use salivary amylase only for food containing starch. [d]Rabbit gastric extract (RGE) contains gastric lipase and pepsin, i.e., the pepsin content needs to be accounted for in the total pepsin activity. [e]Specific enzyme activity or bile concentration: measured for each batch of enzymes or bile extract according to standard assays (see Box 1 and Supplementary Information).

Food preparation and oral phase

It is important to plan the preparation of the food and the oral phase before in vitro gastrointestinal digestion to determine the food to digestive enzyme ratio throughout the in vitro digestion process. First, consideration should be given as to whether the food to be digested in vitro is consumed as a meal, a meal portion or even a food ingredient. Some foods, such as milk, are often consumed on their own or as part of a meal. Other foods or food ingredients are nearly always consumed as part of a meal rather than on their own (e.g., coconut milk, spices, pure proteins and oils). Hence, these foods should be prepared in a way that reflects real food or a meal, e.g., dilution, emulsification, integration into other foods. High-solid foods such as powders must be reconstituted in liquids to make them a consumable food.

An optional oral phase with a standardized 1:1 (wt/wt) ratio of food to simulated oral fluid for all foods (solid and liquid foods) was recommended by the INFOGEST method[27] in 2014. Although in vivo data vary greatly (Supplementary Fig. 1), this dilution ratio enables the formation of a swallowable bolus with almost all types of foods. For this revised INFOGEST 2.0 protocol, a standardized, easy-to-follow approach for the oral phase is necessary. Hence, it is now recommended to dilute all food 1:1 (wt/wt) with simulated oral fluid to achieve a swallowable bolus with a paste-like consistency that is no thicker than that of tomato paste or mustard at the end of the oral phase. If the consistency of the bolus is thicker than such a paste, add water to achieve the proper consistency (see also Table 2 and the 'Troubleshooting' section).

Use of lipase in the gastric phase

Lipid digestion starts in the stomach with the action of preduodenal lipase (gastric lipase in humans; lingual lipase in rodents) on triacylglycerides (TAGs) and some other esters[57]. Gastric lipolysis not only contributes to the overall digestion of TAGs (10% with a solid–liquid test meal to 25% with an emulsified liquid test meal) but it also triggers the subsequent action of pancreatic lipase on lipid substrates that may be poorly digested by pancreatic lipase alone; examples include milk fat droplets and lecithin-stabilized TAG emulsions[58]. It is therefore recommended to add gastric lipase during the gastric phase of in vitro digestion. The mean gastric lipase concentration in human gastric juice is 100 μg/mL, which is equivalent to 120 U/mL, using tributyrin as the reference substrate for gastric lipase[59,60]. In some static digestion models, a concentration of ~16 μg of gastric lipase/mL (20 U/mL)

has been used to reproduce gastric conditions at the halfway point of gastric emptying[61,62], which corresponds to a gastric juice to meal ratio of 1:5 (vol/vol). In the INFOGEST method, the gastric phase of digestion includes a 1:1 dilution of the oral bolus with SGF, which would correspond to a dilution of gastric juice by half and thus a gastric lipase concentration of 60 U/mL. To date, access to commercially available gastric lipase or an appropriate equivalent has been limited; hence, gastric lipase has been omitted or lipases from alternative sources have been widely used. However, caution should be applied regarding the specific biochemical properties of these alternative lipases. Human gastric lipase (HGL), encoded by the *LIPF* gene, is stable and active between pH 2 and 7, with an optimal activity between pH 4 and 5.4. HGL displays an S_N3 stereospecificity for TAG hydrolysis (where S_N defines stereospecific numbering in positions 3 of the triglycerol moiety), leading to the preferential release of short/medium-chain fatty acids from milk TAGs[61]. It is resistant to pepsin hydrolysis and is not inhibited by bile salts. HGL can, however, be replaced by other preduodenal lipases from the acid lipase gene family of various mammalian species such as dog[63] and rabbit[64]. Rabbit gastric lipase is now commercially available (Lipolytech, http://www.lipolytech.com). Preduodenal lipases originating from the oropharyngeal tissues of young ruminants (pharyngeal lipase of calf, kid goat and lamb) may also be used and are commercially available for applications in the dairy industry (DSM for Capalase K and Capalase KL lipases; CHR Hansen for Lipase Kid-Goat ST20, Lipase Calf 57 LFU, Spice IT AC and Spice IT AG; DuPont Danisco, Clerici-Sacco). These preduodenal lipases are, however, less resistant to acid denaturation (threshold at around pH 3.5 (ref. [65])) than gastric lipase, and pH conditions may have to be adapted. Their contents and activity should be estimated before use in in vitro digestion experiments, using the recommended standard gastric lipase assay[27]; see the Supplementary Information. So far, no commercially available lipase of microbial origin combines all the above properties of gastric lipase[61,66], and their use is not recommended at this time. For this revised INFOGEST 2.0 protocol, the authors recommend using rabbit gastric lipase, commercially available as rabbit gastric extracts (RGEs), at 60 U/mL in the final gastric digestion mixture. However, because these extracts also contain pepsin[67], the pepsin concentration/activity in the gastric phase must be accordingly adjusted to the recommended value.

Sampling, controls and test tube

Before performing the protocol (time-lagged before the digestion experiment or 1 d before the digestion experiment), it is recommended to run one preliminary experiment, the pH-test adjustment experiment, with the relevant amount of food, enzymes and bile for the entire digestion process. The aim of this pH-test adjustment experiment is to measure and record the amounts of HCl and NaOH used to reach the target pH in order to perform more efficient pH adjustments when running the digestion protocol. These volumes are indicative of the necessary volume of acids and bases needed for the gastric and intestinal phases. Note that, for solid food, the pH changes are generally slower in response to addition of HCl or NaOH. It is important to remain patient and wait long enough for the pH to become stable; this may take >5 min, depending on food particle size and buffering capacity.

If one intends to take samples at different time points during digestion, it is recommended to prepare one tube per time point, e.g., prepare six digestion tubes for six time points. As most foods are heterogeneous mixtures during digestion, sampling is more reproducible by starting digestion with individual tubes per time point. If the food sample has special requirements in terms of nutrient stability (e.g., light sensitivity, oxidation), the characteristics of the tubes should be adapted to these particular situations (e.g., opaque tubes, maintenance of the food samples on ice). The end volume of the digest should be calculated in advance in order to use the most suitable reaction vessel (e.g., 50-mL tubes) to allow proper mixing during all digestion phases.

Optionally, a replicate test tube (stability test tube) can be prepared to evaluate food stability during exposure to simulated digestive fluids without enzymes or bile, for example, after the oral, gastric and intestinal phases. It can also be advisable to prepare an enzyme-blank tube, i.e., a digestion tube with all enzymes and bile but without food. This may be helpful in identifying enzyme, bile salts or degradation products thereof during analysis of the digests. It is important to highlight that owing to proteolytic enzyme autolysis, especially that of pepsin, enzyme-derived peptides can be detected in digesta, which can be easily monitored with this blank-enzyme tube.

Intestinal phase, stop reaction and readout

The intestinal phase of the protocol starts with the mixing of the gastric chyme with the same volume of the pre-warmed SIF. The pH is adjusted with the amount of NaOH previously calculated in the

pH-test adjustment experiment. In this phase, two different options are given, (i) the use of pancreatin and (ii) the use of individual enzymes: porcine trypsin (100 U/mL), bovine chymotrypsin (25 U/mL), porcine pancreatic α-amylase (200 U/mL), porcine pancreatic lipase (2,000 U/mL) and porcine pancreatic colipase in molar excess to lipase. The amount of pancreatin to be used in the intestinal phase of digestion is based on trypsin activity to achieve 100 U/mL in the final mixture. This calculation may result in low lipase activity for high-fat-containing foods or if fat digestion is the aim of the study. In this case, it is recommended to include additional lipase to obtain 2,000 U/mL of lipase activity in the final mixture and colipase in a molar ratio of 2:1 (colipase to lipase), which corresponds approximately to a mass ratio of 1:2 (colipase to lipase). Because this will require the measurement of the lipase activity in the pancreatic extract and in the lipase preparation, the use of individual enzymes could be the preferred option. Similarly, because the activity of amylase in pancreatin can vary between batches and the activity can be too low to digest starch-rich foods, the use of individual enzymes could also be a good option when following carbohydrate digestion. Bile salts are added to the intestinal mixture to reach 10 mM in the final mixture, after determination of the bile salt concentration in the commercial product (Enzyme assays). There are several commercial options for bile salts, but bovine bile is preferred because its composition is similar to that of human bile[64]. Bile solubilization requires exhaustive mixing, which can be achieved, for instance, in a rotating wheel mixer at 37 °C for 30 min.

In vitro digestion is carried out for a wide range of purposes and with different endpoints. In all cases, sampling, sample preservation and the post treatment of samples after food digestion are critical, and some adaptations could be needed depending on the particular requirements of each experiment (Table 1). For example, to stop pepsin activity, the pH of gastric samples must be raised to 7.0, either by the addition of 1 M sodium bicarbonate or 1 N NaOH solution. The pH shift after the gastric phase is very effective in stopping pepsin activity and similar to in vivo conditions found in the duodenum[56]. If the pH increase is not desired, the use of pepstatin A, a highly selective inhibitor of aspartyl proteases such as pepsin (inhibition constant $K_i = 0.1$ nM), has also been suggested[68]. When gastric digestion is considered as an endpoint, sample snap freezing in liquid nitrogen followed by freeze-drying is recommended. Raising the pH to 7.0 strongly reduces the activity of gastric lipase on long-chain triglycerides[58–60]. Alternatively, the use of Orlistat (tetrahydrolipstatin) is also recommended (gastric lipase half-inhibition time of <1 min) to block gastric lipolysis[61]. Add Orlistat at a final concentration of 0.6 mg/mL (1 mM) to obtain an inhibitor to lipase molar ratio of 1,000, taking into account that the gastric lipase activity of 60 U/mL corresponds to 50 μg/mL or 1 μM lipase.

After gastrointestinal digestion and in order to inhibit the different enzymatic activities of the digested samples, immediate snap freezing after sampling is necessary. However, when thawing the sample for subsequent analysis, residual enzymatic activities could substantially affect the stability of the samples. Therefore, addition of sufficient amounts of enzyme inhibitors against target digestive enzymes is strongly recommended. In the case of proteases, the addition of 5 mM Pefabloc SC (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride), with the ability to irreversibly inhibit trypsin and chymotrypsin, is recommended due to its lower toxicity in comparison with phenylmethylsulfonyl fluoride[40]. Alternatively, the use of Bowman–Birk inhibitor (BBI) from soybean, a potent inhibitor of both trypsin and chymotrypsin that has K_i values at the nanomolar level, has also been recommended[62]. To inhibit lipolysis by pancreatic lipase, the use of 5 mM 4-bromophenylboronic acid has been reported[69]. Inhibition of pancreatic lipase by Orlistat is too slow (half-inhibition time >5 min) to be used here[61]. For amylase inhibition heat-shock treatment, inactivation by ethanol or inhibition with 12% (wt/vol) trichloroacetic acid (TCA) has been used[64], depending on the downstream sample analysis. Once the target inhibition occurs, the digests should be immediately snap frozen in liquid nitrogen and freeze-dried.

When biological activity of digested samples has been evaluated, heat-shock treatment (in boiling water for 5 min) to irreversibly inactivate proteases can also be considered[28]. However, it should be noted that heat treatment is detrimental to the food structure, to proteins in particular, as heat treatment generally causes irreversible denaturation and aggregation. For cell culture assays, consider whether the use of Pefabloc or other enzyme inhibitors can affect the readout of the experiment, and whether the osmolarity must be corrected by dilution to physiological values (285–300 mOsm/kg H_2O, pH 7–7.5) in order to avoid cell osmotic shock. Other combined procedures for removal or enrichment of certain food components, such as defatting, centrifugation, dialysis, filtration and size-exclusion chromatography, are also commonly used.

Materials

Reagents

- Ultrapure type I water, generated by a Milli-Q system or similar (referred to in the text as 'water')
- Human salivary α-amylase (Sigma-Aldrich, cat. no. 1031)
- Porcine pepsin (Sigma-Aldrich, cat. no. P7012 or P6887)
- Rabbit gastric extract for gastric lipase (see 'Use of lipase in the gastric phase' section above; Lipolytech, cat. no. RGE 25-100MG) ▲ **CRITICAL** Rabbit gastric extract contains both gastric lipase and pepsin.
- Bovine bile (Sigma-Aldrich, cat. no. B3883, preferred option because composition is closest to that in humans); alternatively, porcine bile (Sigma-Aldrich, cat. no. B8631) can be used
- Porcine pancreatin (Sigma-Aldrich, cat. no. P7545) or individual intestinal porcine enzymes (trypsin, chymotrypsin, amylase, lipase and colipase; see 'Reagents for optional protocol with individual enzymes' below)
- $CaCl_2(H_2O)_2$ (Merck, cat. no. 2382)
- NaOH (Merck, cat. no. 9141) **! CAUTION** NaOH is corrosive and causes severe skin burns and eye damage. Use proper personal protective equipment.
- HCl (J. T. Baker, cat. no. 6081) **! CAUTION** HCl is corrosive, causes burns and is irritating to the respiratory system. Use proper personal protective equipment and work in a fume hood while handling it.
- KCl (Merck, cat. no. 4936)
- KH_2PO_4 (J. T. Baker, cat. no. 0240)
- $NaHCO_3$ (Merck, cat. no. 6329)
- NaCl (Merck, cat. no. 6404)
- $MgCl_2(H_2O)_6$ (Merck, cat. no. 5833)
- $(NH_4)_2CO_3$ (Sigma-Aldrich, cat. no. 207861)
- $CaCl_2$ $(H_2O)_2$ (Sigma-Aldrich, cat. no. C3881)
- Amyloglucosidase (Sigma-Adrich, cat. no. A1602)
- Tris–HCl buffer (Sigma-Aldrich, cat. no. T3251)
- 3,5-Dinitrosalicyclic acid (Sigma-Adrich, cat. no. D0550)
- Sodium potassium tartrate (Sigma-Aldrich, cat. no. 1551140)

Enzyme inhibitors options (see Experimental design and Table 1)

- Pefabloc SC (4-(2-aminoethyl)benenesulfonyl fluoride; Sigma-Aldrich, cat. no. 76307) **! CAUTION** Pefabloc SC is corrosive. Use proper personal protective equipment.
- Pepstatin A (Sigma-Aldrich, cat. no. P5318)
- Bowman–Birk inhibitor (BBI; Sigma-Aldrich, cat. no. T9777)
- 4-Bromophenylboronic acid (Sigma-Aldrich, cat. no. B75956) **! CAUTION** 4-Bromophenylboronic acid is hazardous and corrosive, causes eye damage and is harmful to the respiratory system. Use proper personal protective equipment and work in a fume hood while handling it.

Chemicals for pepsin activity assay

- Hemoglobin from bovine blood (Sigma-Aldrich, cat. no. H6525-25G)
- Trichloroacetic acid (TCA; Sigma-Aldrich, cat. no. T6399-5G) **! CAUTION** Trichloroacetic acid is corrosive and causes severe burns to skin and eyes. It is soluble in water with release of heat. Use proper personal protective equipment.

Chemicals for gastric lipase activity assay

- Taurodeoxycholate (Sigma-Aldrich, cat. no. T0875-1G)
- Tributyrin (≥99% (vol/vol); Sigma-Aldrich, cat. no. T8626)
- Bovine serum albumin (≥98% (wt/wt); Sigma-Aldrich, cat. no. A7030)

Chemical for trypsin activity assay

- p-Toluene-sulfonyl-L-arginine methyl ester (TAME; Sigma-Aldrich, cat. no. T4626-5G)

Chemicals for amylase activity assay

- Maltose standard (Sigma-Aldrich, cat. no. M5885-100G)
- Soluble potato starch (Sigma-Aldrich, cat. no. S5651-500G)

• 3,5-Dinitrosalicylic acid (DNS; Sigma-Aldrich, cat. no. D0550-10G) **! CAUTION** DNS is harmful if swallowed and causes acute oral toxicity.

Chemical for chymotrypsin activity assay
• *N*-benzoyl-L-tyrosine ethyl ester (BTEE; Sigma-Aldrich, cat. no. B6125-5G)

Chemicals for pancreatic lipase activity assay
• Sodium taurodeoxycholate (Sigma-Aldrich, cat. no. T0875-1G)
• Tributyrin (Sigma-Aldrich, cat. no. W222305-1KG)

Chemical for bile acid determination
• Bile Acid Assay Kit (Sigma-Aldrich, cat. no. MAK 309) or ECOLINE Acides Biliaires (DiaSys, cat. no. 122129990313) or equivalent assay

Reagents for optional protocol with individual enzymes
• Porcine trypsin (Sigma-Aldrich, cat. no. T0303)
• Bovine chymotrypsin (Sigma-Aldrich, cat. no. C7762)
• Porcine pancreatic α-amylase (Sigma-Aldrich, cat. no. A3176)
• Porcine pancreatic lipase (Sigma-Aldrich, cat. no. L3126)
• Porcine pancreatic colipase (Sigma-Aldrich, cat. no. C3028)

Food (for further examples, see 'Anticipated results' section)
• Skim milk powder (SMP; low-heat, organic, protein= 42.34%, fat = 0.89% and lactose = 49.8% (wt/wt); Fonterra)[28]

Equipment
• Standard laboratory centrifuge (suitable for 50-mL tubes, 5,000*g*; Heraeus Megafuge 40R; Thermo Fisher, model no. 75004519)
• Standard laboratory vortex (Genius 3, IKA; HuberLab, model no. 17.1377.01)
• Standard laboratory pH meter (827 pH lab; Metrohm, model no. 2.827.0214)
• pH electrode designed for food systems (SenTix HWD, WTW, model no. X-103731)
• Overhead shaker/rotator (small volume, up to 50 mL; Rotator SB Stuart; Huberlab, model no. 17.0014.02)
• Incubator large enough to hold the above rotator, adjustable to 37 °C (Termaks; Labtec, model. no. B9000)
• Electric or manual mincer (Eddingtons Mincer Pro; Amazon, model no. 86001)
• Eppendorf tubes (2 mL; VWR, model no. 211-2120)
• Plastic centrifuge tubes (15 and 50 mL; VWR, cat nos. 391-3450 and 525-0399)
• Micropipettes and tips (Gilson P10-P1000; VWR)
• Volumetric flasks for solutions
• Glass beakers

Reagent setup
Simulated digestion fluids
Minimum volumes of stock solutions needed for the preparation of 400 mL of simulated digestion fluid at a 1.25× concentration:
• 0.5 mL of $CaCl_2(H_2O)_2$ (0.3 M)
• 30 mL of KCl (0.5 M)
• 6 mL of KH_2PO_4 (0.5 M)
• 65 mL of $NaHCO_3$ (1 M)
• 25 mL of NaCl (2 M)
• 2 mL of $MgCl_2(H_2O)_6$ (0.15 M)
• 2 mL of $(NH_4)_2CO_3$ (0.5 M)
• 1 M NaOH and 1 M HCl (a minimum of ~5 mL): for pH adjustment of stock solutions of simulated digestion fluids

Stock solutions can be prepared and stored in aliquots at −20 °C for 1 year.

Simulated digestion fluids for the oral (SSF), gastric (SGF) and intestinal (SIF) digestion phases are mixed at a 1.25× concentration using the electrolyte stock solutions and water according to Table 3

Table 3 | Volumes of electrolyte stock solutions of digestion fluids for a volume of 400 mL diluted with water (1.25× concentrations)

			SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
Salt solution added	Stock concentrations		Milliliters of stock added to prepare 0.4 L (1.25×)	Final salt concentration in SSF	Milliliters of stock added to prepare 0.4 L (1.25×)	Final salt concentration in SGF	Milliliters of stock added to prepare 0.4 L (1.25×)	Final salt concentration in SIF
	(g/L)	(M)	(mL)	(mM)	(mL)	(mM)	(mL)	(mM)
KCl	37.3	0.5	15.1	15.1	6.9	6.9	6.8	6.8
KH_2PO_4	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
$NaHCO_3$[a]	84	1	6.8	13.6	12.5	25	42.5	85
NaCl	117	2	-	-	11.8	47.2	9.6	38.4
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
$(NH_4)_2CO_3$*	48	0.5	0.06	0.06	0.5	0.5	-	-
HCl		6	0.09	1.1	1.3	15.6	0.7	8.4
$CaCl_2(H_2O)_2$[b]	44.1	0.3	0.025	1.5	0.005	0.15	0.04	0.6

[a]The use of carbonate salts in the electrolyte solutions requires the use of sealed containers with limited headspace, see also CRITICAL STEP in Step 24. [b]$CaCl_2(H_2O)_2$ should be added immediately before use. Volumes in Table 2 are indicated for a typical experiment of 5 mL of SSF.

and can be stored at −20 °C for 1 year. ▲**CRITICAL** $CaCl_2(H_2O)_2$ should be added immediately before the digestion experiment to avoid precipitation during storage. ▲**CRITICAL** All the volumes (Table 3) are calculated for 400 mL of a 1.25× concentrated storage solution; just before use, they are mixed with the necessary quantities of enzyme and finally diluted to a 1× concentration working solution (i.e., 4 parts of electrolyte solution + 1 part consisting of enzymes and water results in a 1× concentration of the digestion fluids). ▲**CRITICAL** Simulated digestion fluids (1.25× concentrates) can be stored at −20 °C for 1 year in small aliquots of appropriate size; e.g., for the experiment shown in Box 1, using 5 g of food, at least 48 mL of SSF, 88 mL of SGF and 96 mL of SIF are needed. ▲**CRITICAL** Dilute enzymes in cold solutions and keep them on ice until used. This will keep enzyme activity to a minimum. ▲**CRITICAL** Pre-warm electrolyte solutions (SSF, SGF and SIF) to 37 °C before using them in the digestion procedures.

Procedure

Preparation of reagents and digestion tubes ● Timing 4–5 d for all assays

1 Perform all enzyme and bile assays (Box 1) according to the protocols in the Supplementary Information for each new batch of enzymes or after prolonged storage.
▲**CRITICAL STEP** For the pepsin assay, dissolve pepsin in 10 mM Tris, 150 mM NaCl, pH 6.5, which improves the reproducibility of the assay (Supplementary Method).
▲**CRITICAL STEP** Spreadsheets for the enzyme assays and the volumes for the digestion procedure are provided in Supplementary Data 1 and 2. In addition, online spreadsheets are available at http://www.proteomics.ch/IVD and on the INFOGEST website at https://www.cost-infogest.eu/.
▲**CRITICAL STEP** Prepare one tube per time point and food; e.g., for one food and six time points, prepare six tubes.
? TROUBLESHOOTING

2 Pre-warm the electrolyte stock solutions at 37 °C, initially only SSF, SGF and SIF.

3 Prepare all enzyme and bile solutions immediately before the digestion experiment.
▲**CRITICAL STEP** Keep all enzyme solutions on ice.

4 To perform more efficient pH adjustments during the digestive phases, prepare one replicate tube (pH-test adjustment experiment) with the relevant amount of food, enzymes and bile for the entire digestion process (time-lagged before the digestion experiment or 1 d before the digestion experiment) and measure and record the volumes of HCl and NaOH used to reach the target pH. These volumes are indicative of the necessary volume of acids and bases needed for the gastric and intestinal phases.

5 (Optional) Prepare one replicate test as a food stability control to assess the behavior of the food during exposure to simulated digestive fluids without enzymes or bile, for example, after the oral, gastric and intestinal phases.

6 Prepare one replicate test tube as a blank for digestion without food (replaced by water) but with all required enzymes and bile. See videos of enzyme assays (Supplementary Videos 3–7), as well as the digestion procedures (Supplementary Videos 1 and 2). Videos are also available online on the YouTube channel 'In vitro food digestion - COST action INFOGEST', https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg, and on the INFOGEST website, https://www.cost-infogest.eu/.

Digestion procedure ● Timing 5–8 h

7 *Oral phase (30 min)*. Dilute food with SSF at a ratio of 1:1 (wt/wt) to achieve a swallowable bolus with a paste-like consistency similar to that of tomato paste or mustard at the end of the oral phase. If the consistency of the bolus is thicker than such a paste, add water to achieve the proper consistency. Salivary amylase is needed only to digest starch-containing food. It can be omitted if the food does not contain starch. Do not use lower-purity salivary amylase or pancreatic amylase.

8 Mix food with SSF to achieve a final ratio of 1:1 (wt/wt), e.g., 5 g of food to 5 g of SSF.

9 Measure the volume of the final digestion mixture of the food + SSF mixture. Record this volume, as it will be used in Step 17.

10 If necessary, simulate mastication by mincing the food in an electric or manual mincer.

11 Depending on the food (e.g., bread), mincing can be done together with the SSF electrolyte (without enzymes).

12 Add SSF electrolyte stock solution to the food, if not done in the previous step.

13 Add $CaCl_2(H_2O)_2$ in order to achieve a total concentration of 1.5 mM in SSF.

14 Add the salivary amylase, if necessary, prepared in water to achieve an activity of 75 U/mL in the final mixture.

15 Add the remaining water in order to achieve a 1× concentration of the SSF.

16 Incubate while mixing for 2 min at 37 °C.

▲ **CRITICAL STEP** Electrolyte concentrations are given for the simulated digestive fluids (SSF, SGF and SIF), and accumulation in consecutive digestion phases is not considered; enzyme activities are expressed as units per milliliter in the final digestion mixture.

17 *Gastric phase (2–3 h)*. Pre-warm the SGF electrolyte stock solution at 37 °C. Add SGF electrolyte stock solution to the oral bolus to achieve a final ratio of 1:1 (vol/vol).

18 Adjust the pH to 3.0 by adding a defined volume of HCl previously determined during a pH-test adjustment experiment, see 'Experimental design'.

▲ **CRITICAL STEP** For solid food, the pH changes are generally slower in response to the addition of HCl; it is important to remain patient and wait until the pH is stable; this usually takes >5 min, depending on food particle size and buffering capacity.

19 Add $CaCl_2(H_2O)_2$ solution in order to achieve a final concentration of 0.15 mM in SGF.

20 Add the porcine pepsin solution prepared in water to achieve an activity of 2,000 U/mL in the final digestion mixture.

21 Add the gastric lipase solution prepared in water to achieve an activity of 60 U/mL in the final digestion mixture.

22 Verify the pH and adjust to 3.0 if necessary.

23 Add water in order to achieve a 1× concentration of SGF.

24 Incubate the samples at 37 °C, mixing the digestive mixture sufficiently (e.g., rotating wheel, shaking incubator) for 2 h from the point at which pepsin was added. If there are large precipitates or clogs form, see the 'Troubleshooting' section.

▲ **CRITICAL STEP** Rabbit gastric extracts contain both gastric lipase and pepsin[67]. The pepsin activity in RGE must be determined and taken into account together with the porcine pepsin to reach a combined pepsin activity of 2,000 U/mL in the final digestion mixture.

▲ **CRITICAL STEP** The use of carbonate salts in the electrolyte solutions requires that sealed containers with limited headspace be used. In open vessels, CO_2 will be released and the pH will progressively increase with time. If open vessels are used, such as when using the 'pH-stat' approach or for sampling purposes, we suggest replacing the sodium bicarbonate ($NaHCO_3$), the main source of carbonates, with NaCl at the same molar ratio in order to maintain the ionic strength of the electrolyte solutions (oral, gastric and intestinal). Such adjustments have already proven effective in avoiding unwanted pH drift in open vessels in both the gastric[70] and intestinal[42] phases of digestion (Table 3).

? TROUBLESHOOTING

25 *Intestinal phase (2–3 h).* Pre-warm the SIF electrolyte stock solution in a 37 °C water bath. Add SIF electrolyte to the gastric chyme to achieve a final ratio of 1:1 (vol/vol).

26 Adjust the pH to 7.0 by adding a defined volume of NaOH previously determined during a pH-test adjustment experiment, see 'Experimental design'.
▲ **CRITICAL STEP** For solid food, the pH changes are slower in response to the addition of NaOH; see remarks in Step 18; this may take several minutes.

27 Add the bile solution to the SIF/gastric chime solution in order to reach a final concentration of 10 mM. Place the solution in a rotating wheel mixer at 37 °C for at least 30 min to achieve complete bile solubilization.

28 Add $CaCl_2(H_2O)_2$ solution in order to reach a concentration of 0.6 mM in SIF.

29 Perform the intestinal phase with pancreatin (option A) or with individual enzymes (option B).
(A) **Pancreatin**
(i) Add the pancreatin suspension in SIF solution to achieve a trypsin activity of 100 U/mL in the final mixture. Additional pancreatic lipase may be needed for the digestion of fat-containing food to reach the required lipase activity to achieve a lipase activity of 2,000 U/mL in the final mixture.
▲ **CRITICAL STEP** Measure trypsin activity in pancreatic lipase powder and subtract it from the needed trypsin activity.
(B) **Individual enzymes**
(i) Add trypsin, chymotrypsin, pancreatic α-amylase, pancreatic lipase and the colipase solutions in SIF in order to reach 100, 25, 200 and 2,000 U/mL and colipase at a molar excess (ratio of 2:1 colipase:lipase), respectively, in the final digestion mixture.

30 Verify the pH and adjust to 7.0 if necessary.

31 Add water in order to achieve a 1× concentration of the SIF.

32 Incubate the samples at 37 °C, using a rotating wheel or shaking incubator to mix the digestive mixture sufficiently for 2 h, starting at the point when pancreatic enzymes were added. For difficulties with sampling, see the 'Troubleshooting' section.
▲ **CRITICAL STEP** If open vessels are used ('pH-stat' approach), $NaHCO_3$ should be replaced with NaCl in the electrolyte solutions to avoid unwanted pH drift (Step 24, CRITICAL STEP note).
? TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 4.

Table 4 | Troubleshooting table

Step	Problem	Possible reason	Solution
1 (enzyme activity)	Pepsin activity results in a lower amount of activity units than specified	Enzyme activity measurement performed incorrectly	Follow the standardized procedure, using hemoglobin as substrate. Dissolve pepsin in 10 mM Tris, 150 mM NaCl, pH 6.5
	Amylase activity very low	DNS (3,5-dinitrosalicylic acid) does not react with the product	DNS solution must be freshly prepared
24 (gastric phase)	Food is not digested as expected. It forms a big clog and it is not digested at the end of the gastric phase	Excessive amount of substrate	Revise the amount of food introduced into the system. Realistic food consumption should be targeted. Dilute or suspend food in an appropriate amount of water, if necessary. For example, to mimic the porcine in vivo digestion of cheese[29] at the end of the gastric phase, the cheese must be diluted with water at a 1:2 (wt/wt) ratio before the oral phase
	It is difficult to adjust the pH during gastric digestion	Quick pH drift during the gastric phase	Run a pH-test adjustment experiment with the same food to determine volumes and times for HCl addition
24, 32 (gastric/intestinal phases)	Difficulties taking a homogeneous sample during digestion	Presence of different phases (lipids, water and solids)	Use individual sample tubes for each time point rather than withdrawal of samples from the digestion vessel

Table continued

Table 4 (continued)

Step	Problem	Possible reason	Solution
	Poor mixing during digestion	Tube shape, volume or shaking is insufficient	Check the volume of the sample and the tube or vials to allow sufficient mixing of the sample
32 (intestinal phase)	Intestinal samples affect cell viability in cell culture studies	Presence of bile salts, enzyme inhibitors	Avoid the use of enzyme inhibitors to stop the digestion reaction. Reduce the bile salt concentration during the intestinal phase. Sufficiently dilute the digestion mixture
	Presence of insoluble material at the end of the intestinal phase	Non-digestible material	Use an individual sample tube for each time point
	Poor lipid digestion at the end of digestion	Food contains a high amount of lipids	Add porcine pancreatic lipase and colipase to achieve 2,000 U/mL lipase activity in the final mixture. Consider additional trypsin activity present in the pancreatic lipase
	Starch digestion is too low	Incorrect method was used for quantification of starch digestion products	Add amyloglucosidase to samples before measuring glucose or use a reducing sugar assay to measure starch digestion products. Check the activity of the amylase
	Starch digestion product concentration does not change over time	Starch digestion is completed before the samples are collected	Take more samples at earlier time points. Consider using less amylase to slow the reaction. Check the feasibility of results by expressing findings as percentages of starch digested

Timing

Step 1, enzyme activity and bile assays: ~4 d for all assays
Steps 2 and 3, preparation of solutions: 2 h
Step 4, pH-adjustment experiment: 5 h (time-lagged before the digestion experiment)
Steps 5 and 6, preparation of replicate tests as control: 20 min
Steps 7–32, whole digestion experiment: 5–8 h, depending on number of food samples and time points; e.g., one food sample and five time points takes ~5 h; two food samples and five time points (two gastric and three intestinal points) takes ~8 h
Steps 7–16, oral phase: 30 min
Steps 17–24, gastric phase: 2–3 h
Steps 25–32, intestinal phase: 2–3 h

Anticipated results

Protein digestion

Without standardized digestion methods, the main difficulties in the field of food digestion were the absence of comparable results from different laboratories and the lack of physiological relevance of experimental data. The INFOGEST method was tested with respect to these two aspects, focusing on protein digestion.

The robustness of the protocol and comparability of experimental data were assessed in several inter-laboratory trials in which the participants were asked to digest a standardized SMP by applying their existing in-house protocols first, and then by using the harmonized protocol[28]. The first critical step in protein hydrolysis is assessment of the pepsin activity in the gastric phase. The heterogeneous pattern observed with the in-house digestion protocols (Fig. 2a, gastric phase) was improved substantially by the correct implementation of the harmonized protocol (Fig. 2b, gastric phase), except for in laboratories 6 and 7, which showed incomplete casein hydrolysis. Adjustments in the pepsin assay (addition of Tris buffer, see Step 1 CRITICAL STEP note and Box 1) improved the reproducibility and reduced lab-to-lab variability[28]. This improved pepsin assay is now recommended for the INFOGEST 2.0 method. Figure 2b shows improved homogeneity between samples, as related to the gastric phase, when the harmonized protocol was applied. Increased protein degradation in the intestinal phase was observed in laboratories 4 and 7. Subsequent recommendations for correct sample preparation, in particular the correct inhibition of enzymes at the end of the digestion experiment (Table 1), improved lab-to-lab variability[28].

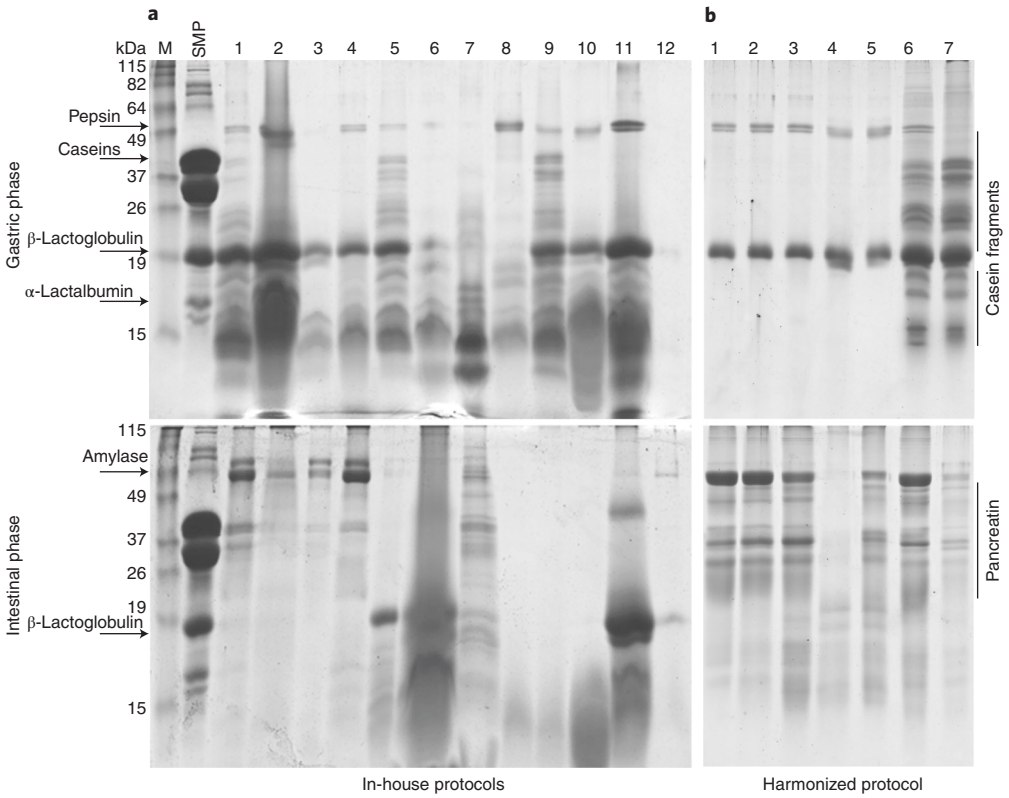

Fig. 2 | Protein separation by gel electrophoresis of in vitro–digested skim milk powder. a,**b**, Comparison of results from in-house protocols performed in individual laboratories 1–12 (**a**), with the harmonized protocol, performed in seven different laboratories (**b**), after the gastric and the intestinal phases of in vitro digestion. Undigested SMP is shown as a control; specific protein bands are highlighted with arrows: Casein fragments, partly hydrolyzed casein; pancreatin, bands originating from pancreatin. M, molecular weight marker; SMP, skim milk powder. Adapted from Egger et al.[28] under a Creative Commons Attribution 4.0 license (https://creativecommons.org/licenses/by/4.0/legalcode).

Physiological relevance of the protocol was evaluated by comparing in vitro SMP digestion with that of an in vivo pig trial[29]. Pigs were fed reconstituted SMP from the same batch as that applied in the in vitro tests, and samples were collected from the stomachs and in several sections of the small intestine (jejunum, I1–I3 to ileum, I4) after sacrifice. Milk peptides were identified with mass spectrometry, and overall peptide patterns were visualized by summing the number of times each individual amino acid was identified within a milk peptide. Overlay of the average peptide patterns for α_{s2}-casein from the harmonized in vitro digestion ($n = 7$) and in vivo pig digestion ($n = 8$) showed that at the end of the gastric phase, the peptide pattern corresponded well to that of the pig sample collected from the stomach; the peptide pattern in the in vitro intestinal phase sample was most similar to that of the pig sample collected in the median jejunum (I3). This comparison showed that protein hydrolysis at the endpoints of the harmonized INFOGEST digestion method was in agreement with that of the in vivo digestion (Fig. 3).

In conclusion, both inter-laboratory comparability and physiological relevance were improved by the correct application of the harmonized in vitro digestion protocol.

Lipid digestion

To date, most published digestion experiments using this INFOGEST method did not include a gastric lipase because of the lack of commercially available, acceptable substitutes for HGL. This situation changed with the availability of RGEs containing gastric lipase; see 'Use of lipase in the gastric phase' in the 'Experimental design' section. Both HGL and rabbit gastric lipases exhibit, at the recommended gastric pH of 3.0, ~50% of their maximum activity measured at pH 4–5.4[71,72]. Moreover, the in vitro gastric lipolysis of infant formula by rabbit gastric lipase was consistent with in vivo data, with the degree of lipolysis reaching 10% after 60 min of gastric digestion[73]. These data therefore suggest that gastric lipolysis could be studied using this INFOGEST 2.0 method with RGE as a source of gastric lipase[64] or HGL, if available[61].

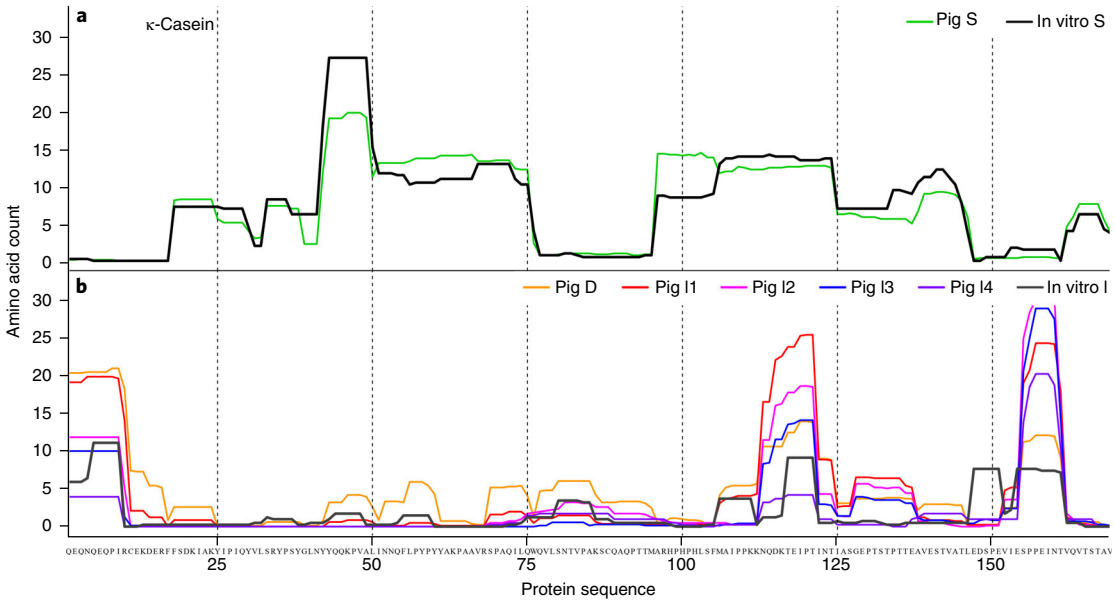

Fig. 3 | Comparison of in vitro–digested skim milk powder peptide patterns of κ-casein with in vivo (pig) digestion. a, Gastric in vitro digestion samples (in vitro S) were compared to gastric pig samples (pig S, $n = 8$, as previously published by Egger et al.[29], approval no. 2015_04_FR;26115). **b**, Intestinal in vitro digestion samples were compared to pig sampling sections collected along the digestive tube from duodenum (D), proximal jejunum intestinal section 1 (I1), median jejunum (I2), distal jejunum (I3) and ileum (I4)[29]. The *x* axis shows the amino acid sequence (one-letter code) of κ-casein, and the *y* axis shows the number of times each amino acid was identified within a κ-casein peptide of ≥5 amino acids. Adapted with permission from Egger et al.[28], Elsevier.

The INFOGEST method has also been used to study intestinal lipid digestion, for example, in oil-in-water emulsions stabilized by milk or soya lecithin[74]. However, human gastric analog and phospholipase A2 (PLA_2) were added in this procedure. The degree of hydrolysis (percentage of TAG disappearance) ranged between 73% and 87% (±5%) at the end of the intestinal phase (120 min). In addition, in vitro digestion was also performed with more complex systems, such as whole-fat dairy products or protein/polysaccharide emulsions. Depending on the structure of the food matrix and the state of dispersion of the lipids, the reported degrees of hydrolysis at the end of the intestinal phase ranged from moderate (66% of remaining lipids in poorly digestible raw oat flakes due to limiting matrix structure)[75] to an almost complete disappearance of triglycerides[76].

Intestinal lipid digestion can be assessed by chemical analyses of collected samples. The protocol recommends analyzing the entire volume of digestive tubes to prevent sampling errors (see Step 1, CRITICAL STEP note, one tube per time point and food). This precaution is particularly useful in the presence of lipids[75], as they often tend to destabilize and phase-separate (cream) during the gastric and/or intestinal phases of digestion. If aliquots are taken as sample points, great care should be taken to represent the whole digested solution. The best way to analyze the extent of lipolysis is to conduct Folch extractions[77] on the samples in the presence of internal standards before the analysis of classes of the lipids (residual triglycerides, free fatty acids, diglycerides and monoglycerides) by thin-layer chromatography combined with densitometry or gas chromatography with a flame ionization detector (GC-FID)[78] or HPLC coupled to a light-scattering detector[79]. Free fatty acids can also be quantified after solid-phase extraction with GC-FID, using fatty acids as internal standards[73,80]. The pH-stat method, one of the most commonly used methods for monitoring pancreatic lipolysis, can also be used, but three sources of errors should be taken into consideration: (i) the pH-stat measurements can be impaired by the high concentrations of carbonate salts recommended for the simulated digestion fluids (Step 24, CRITICAL STEP note); it is therefore advised to replace $NaHCO_3$ salts with NaCl at the same molarity in all electrolyte solutions (oral, gastric and intestinal) when planning to use pH-stat experiments during the intestinal phase of digestion[42]; (ii) protein hydrolysis also contributes to the pH-stat signal in the intestinal conditions (pH = 7), meaning that this approach is suitable for studying pancreatic lipolysis only when the contribution of proteins is either neglected or subtracted[42]; and (iii) some fatty acids, especially long-chain fatty acids, are not ionized at pH 7. A back titration at pH 9.0 should be performed to measure all the free fatty acids released[81].

Digestion of starch

The structure of starch in a ready-to-eat plant-based food is a function of a multitude of factors. These include its botanical origin, growing conditions, processing, food preparation (mainly cooking) and storage. These all have a major impact on salivary and pancreatic amylase-catalyzed starch digestion. The rate of the loss of starch and the appearance of the digestion products (maltose and maltooligosaccharides) are the most common measures of in vitro starch digestibility. To help in the understanding of the physiological effects of starch digestion, such as on glycemic response in humans, measurements should also include (i) the accurate dose and nature of the starch in the food as eaten; (ii) the characterization of the food matrix (microstructure, macro- and micronutrient composition) and (iii) a measure of the degree of starch gelatinization and/or retrogradation.

It is recommended that starch amylolysis be quantified only in the intestinal phase by measuring the appearance of the starch digestion products over time, e.g., the concentration of reducing sugars in the liquid phase. Salivary amylase will have a minor impact on starch digestion in the static model, where the gastric pH is instantaneously adjusted to 3. After terminating amylase activity by mixing the sample with four volumes of ethanol (final concentration = 80% (wt/vol)), for example (see different options in Table 1), undigested starch is often separated from digested starch by centrifugation. Analysis of reducing sugar concentration in the supernatant is often done with common colorimetric assays (e.g., using DNS or PAHBAH (4-hydroxybenzhydrazide) reagents). Another more common method is to treat an aliquot of the amylase digestion products from the 80% (wt/vol) ethanol supernatant with buffered amyloglucosidase to convert all amylase digestion products to glucose. Glucose can then be determined through a whole host of methods, including colorimetric and enzymatic assays (e.g., D-glucose assay kit) or by direct chromatography analysis, to name just a few. The data collected can then be used as input variables to a wide variety of simple to complex kinetic-based mathematical models that seek to quantify starch digestion and give predictions on the physiological effects of the food.

Bioaccessibility of phytochemicals

The main challenges for investigating common dietary phytochemicals such as hydrophilic polyphenols and hydrophobic carotenoids are (i) the physiological appropriateness of the digestion conditions, such as reproducible matrix release and the sufficient presence of enzymes required for cleavage and cellular uptake and (ii) separation of the bioaccessible phase from unavailable phytochemicals (e.g., precipitated or in complexed form), which can be achieved by centrifugation and/or filtration/dialysis.

Good correlations between bioaccessibility and in vivo bioavailability have been obtained for certain phytochemicals, such as carotenoids[82,83]. However, slight alterations of the digestion parameters suggested by the original INFOGEST method[27] can markedly influence bioaccessibility. For instance, increasing the amount of pancreatin and/or bile[84] or increasing the speed of shaking/stirring can considerably enhance the bioaccessibility of carotenoids by improving mixing, disrupting oil droplets and increasing micellization. Thus, careful consideration and the possible further standardization of these parameters are vital. Additional important factors to consider are light and oxygen, as they can result in the oxidative degradation of carotenoids[85] and polyphenols[86], as well as polymerization, of the latter[87]. It is recommended to flush samples with Ar or N_2 for a few minutes before small intestinal digestion to remove oxygen[83,88] or to use pyrogallol. However, the latter is unsuitable for polyphenolic samples, as it is a potential metabolite. Another often-neglected factor is the potential effect of brush-border membrane (BBM) enzymes (e.g., lactase–phlorizin–hydrolase) on phytochemical bioaccessibility, especially for polyphenols[89,90]. The inclusion of BBM vesicles in in vitro gastrointestinal digestion may increase the physiological relevance of the model, especially for polyphenols[91]. However, BBMs are not commercially available nor is there any standard method available to date.

For polyphenols, dialysis is often performed to remove macromolecular-bound compounds[92], but for carotenoids, a combination of centrifugation (e.g., 4,000*g* for at least 30 min at room temperature) and a filtration step (0.2 μm) has become the most widely used method[31] to separate the bioaccessible aqueous phase from larger lipid droplets or crystals that would not be taken up by the enterocytes.

When combining in vitro digestion with cellular assays (e.g., cellular uptake/transport), the toxicity of the bile salts must be accounted for, by including a cleanup step, e.g., solid-phase extraction[93–95], or at least the sufficient dilution of samples (e.g., 4× dilution).

Finally, it should be considered that the colon may play an important role for the bioavailable fraction. Although it is well known that polyphenols can undergo many changes in the colon[89], and may be absorbable in the colon, little is known about carotenoids, although a significant fraction would be bioaccessible in the colon[96].

Ongoing developments and future perspectives for in vitro food digestion

The establishment of the INFOGEST digestion protocol is a good starting point in the standardization and harmonization of food digestion methods. Henceforth, results from different research groups can be compared in a meaningful manner. However, users must be aware of the shortcomings of this method, and considerable efforts are being made around the world to improve or add to the existing method.

The INFOGEST method is for adult digestion only. However, there is a strong need to apply this method to specific human population groups, the most important being infants and the elderly, but also adolescents and patients with cystic fibrosis or gastric bypass surgery, to name but a few. A recent review[97] summarizes the existing literature and provides some recommendations on experimental digestion parameters, with the INFOGEST method being the starting point for all other methods.

Although static methods can be useful, they can be inadequate for simulation of the dynamic processes during digestion (e.g., pH gradients, gradual addition of enzymes and gastric fluid, continuous gastric emptying). As mentioned earlier, various dynamic digestion methods[6–10] account for some of these factors. A low-cost semi-dynamic method based on equivalent in vivo data from the digestion of dairy products was recently developed[49] and described in detail[50]. International INFOGEST members are currently working on a consensus method.

Enzymes from the small intestinal BBMs are recognized as playing a major role in the activation of trypsinogen (enterokinase) and the further degradation of proteins/peptides and carbohydrates, as well as improving the bioaccessibility of phytochemicals. The use of brush-border enzymes falls into the gray area between bioaccessibility (potentially absorbable) and bioavailability (available at the site of action), and, to date, it is not clear how they should be applied. BBMs of animal origin have recently been included in static digestion methods[39,98,99] and can provide physiologically consistent information[100]. However, to date, BBM enzymes are not commercially available and are extracted from fresh animal intestines[101] or used as intestinal extracts. There is still a lack of reliable information on the correct enzymatic activities, enzyme/substrate ratio and diversity of enzymes, which further limits the use of BBM in standardized digestion methods at the moment. However, given the importance of BBMs in the digestive process, further progress in terms of defining digestive parameters is anticipated.

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Author contributions

A.B., L.E. and I.R. wrote the manuscript. M.A., S.B., T.B., F.C., A.C., D.D., C.D., C.E., S.L.F., U.L., A.M., A.R.M., O.M., M.M., R.P., C.N.S. and I.S. contributed to the writing of the manuscript. A.B., L.E., M.A., P.A., S.B., T.B., C.B.-L, R.B., F.C., A.C., M.C., D.D., C.D., C.E., M.G., S.K., B.K., S.L.F., U.L., A.M., A.R.M., S.M., O.M., M.M., R.P., C.N.S., I.S., G.E.V., M.S.J.W., W.W. and I.R. contributed to the definition of digestion parameters. R.P. wrote the online tools. R.A. and C.M. prepared the videos. M.G., D.J.M. and R.P.S. contributed to the manuscript by critical revision of the digestion parameters.

Competing interests

Rabbit lipase from rabbit gastric extract is available commercially from Lipolytech, a start-up company founded by a researcher who had previously worked in F. Carrière's (coauthor of this paper) group. The F. Carrière laboratory, a joint unit of the Centre National de la Recherche Scientifique (CNRS) and Aix Marseille University (AMU), has a research collaboration contract with Lipolytech (CNRS reference no. 163451; signed on 30 June 2017). However, the coauthor F. Carrière does not financially benefit from this contract and, as an employee of CNRS and civil servant of the French state, is not allowed to have private consulting activity for a company contracting with his own laboratory.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41596-018-0119-1.

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