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INFOGEST static *in vitro* simulation of gastrointestinal food digestion

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Abstract

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 have often very little physiological relevance and this impedes the meaningful comparison of results. The standardised protocol presented here is based on an international consensus developed by the COST INFOGEST network. The method is designed to be used with the standard laboratory equipment and 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) addresses a number of ambiguities in the original scheme such as the inclusion of the oral phase and the use of gastric lipase. The method can be used to assess the end points resulting from digestion of foods, to analyse the digestion products (e.g. peptides/amino acids, fatty acids, simple sugars, etc.) and evaluate the release of micronutrients from the food matrix. The whole protocol can be completed in ~7 days including ~5 days required for determination of enzyme activities.



40 Introduction

41 The worldwide prevalence of diet-related diseases has been on the increase for the last few
42 decades.¹ Large scale human intervention trials have been used to correlate diet with the
43 health of different demographic groups. However, to understand the physiological response
44 to specific foods, it is necessary to follow the complex digestive processes within the human
45 digestive tract in more detail. This can be achieved with invasive procedures such as
46 aspiration from the stomach² or small intestine³ or with less invasive imaging technologies
47 (e.g. magnetic resonance imaging⁴) and wireless, telemetric systems^{2,5}. Animal models are
48 also widely used, though it generally involves animal death or surgical approaches placing
49 cannulas into digestive organs to access the contents of the gastrointestinal tract. The
50 relevance of animal models for understanding food digestion in humans is also regularly
51 questioned. In summary, *in vivo* (human or animal) intervention trials can be difficult to
52 undertake, unsuitable, expensive or not justifiable on ethical grounds. For these reasons, *in*
53 *vitro* models have been used for many decades to simulate the digestion of food.

54 Development of the Protocol:

55 There are several types of *in vitro* digestion methods that are commonly used for food, which
56 can be divided into static and dynamic methods. These models aim to simulate the
57 physiological conditions of the upper gastrointestinal tract, namely the oral, gastric and small
58 intestinal phases. Most dynamic models⁶⁻¹⁰ have been shown to be suitable for simulating the
59 digestion of foods and pharmaceutical products in different population groups and for
60 different purposes¹¹. However, these models are relatively complex, expensive to set up and
61 maintain, and therefore may not be available to the majority of food researchers.

62 Owing to its simplicity, static models, which use a constant ratio of food to enzymes and
63 electrolytes, and a constant pH for each digestive phase, have been widely used for many
64 decades for food, animal feed and pharmaceutical purposes¹²⁻¹⁴. Static *in vitro* digestion
65 models have been shown to be very useful in predicting outcomes of *in vivo* digestion^{15,16}.
66 There are standardised static models¹⁷ that vary in complexity^{18,19}, which are used for
67 simulating the gastrointestinal behaviour of pharmaceutical products (Pharmacopeia
68 methods)¹⁷. Other static methods were developed for assessing the *in vitro* bioaccessibility of
69 soil contaminants²⁰, heavy metals in particular, or mycotoxins in food²¹. These methods,
70 developed and standardised²² by the Bioaccessibility Research Group of Europe (BARGE)
71 were based on available physiological data reported by landmark papers such as Dressman
72 et al.²³ or the Geigy tables²⁴. The static methods of the BARGE group and Pharmacopeia

73 procedures were important milestones in the evolution of standardised *in vitro* digestion
74 methods. However, their experimental conditions, purpose and endpoint were found to be
75 unsuitable for digesting food due to the complexity and variability of food structures as well
76 as very different research questions in food science. This resulted in the use of a great
77 number of digestion methods, reviewed by Hur et al.²⁵, with slight but significant variations in
78 parameters such as pH, duration, enzyme concentration and activity, composition of
79 simulated digestive fluids, etc.

80 Hence, the need for a harmonisation of digestion conditions was identified and the
81 international INFOGEST²⁶ network (www.cost-infogest.eu) of multidisciplinary experts (food
82 science, nutrition, gastroenterology, engineering, enzymology, etc.) from more than 35
83 countries was established. One of the primary outcomes of this network was an international
84 consensus on a set of digestion parameters for a static *in vitro* simulation of adult digestion
85 suitable for food. The method, generally referred to as the INFOGEST method, was
86 published²⁷ and experimental parameters were justified and discussed in great detail in
87 relation to available *in vivo* physiological data. Some of the previous digestion methods
88 outlined above were used as a starting point. Since its publication in 2014, this *in vitro*
89 digestion method has received a *Highly Cited Paper* status for Agricultural Sciences with
90 more than 550 citations in Web of Science and has been extensively used all over the world
91 for numerous purposes, with a variety of foods and different endpoints. The current article
92 builds on that publication and clarifies a number of aspects of the original protocol, leading to
93 an improved INFOGEST 2.0 protocol described here.

94 **Overview of the Procedure**

95 The digestion procedure is summarised in **Figure 1**. It can be divided into three phases:
96 preparation, digestion procedure and sample treatment with subsequent analysis. For
97 preparation of the *in vitro* digestion, the activity of all digestive enzymes and the
98 concentration of bile salts should be determined experimentally, using the recommended
99 standardised assays for amylase, pepsin, lipase (both gastric and pancreatic), trypsin and
100 chymotrypsin, outlined in **Box 1**, described in detail in the Supplementary Information. This
101 first preparation step is of the utmost importance and failure to correctly assay enzyme
102 activity will lead to incorrect rates of digestion of components (e.g. proteins)²⁸, potentially
103 changing the overall digestion of the food.

104 The digestion involves the exposure of the food to three successive digestive phases: oral,
105 gastric and intestinal. For static *in vitro* digestion methods, the experimental conditions are
106 constant, during each phase. The oral phase involves dilution of the food 1:1 (w/w) with

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107 simulated salivary fluid (SSF), with or without salivary amylase, and for solids or semi-solids
108 simulated mastication of the food. If used, exposure of the food to salivary amylase is limited
109 to two minutes at pH 7. The oral phase needs to be included in all simulated digestion
110 procedures, regardless of the state of the food (liquid or solid) in order to provide consistency
111 of dilution. Further clarification regarding the preparation of the food and the oral phase can
112 be found in the Experimental Design.

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

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

124 The last step of the digestion procedure involves sampling, sample treatment, storage and
125 subsequent analysis of samples. This step should be carefully considered prior to digestion
126 as it may differ from case to case due to different endpoints, purposes of the digestion
127 experiment and type of analysis. A description of sample treatment can be found in the
128 Experimental Design and Table 1.

129

130 **Advantages and limitations**

131 Static *in vitro* digestions are the simplest methods to simulate *in vivo* food digestion. While
132 there are clear weaknesses in these simple models, they have obvious advantages over
133 more complex methods. The main strengths of static *in vitro* models is the good intra- and
134 inter-laboratory reproducibility, robustness, simplicity, relatively low cost and easy
135 assessment of each digestion phase. This latter point makes them very suitable for
136 mechanistic studies, hypothesis building and screening. It was one of the aims of the
137 INFOGEST network not just to standardise *in vitro* methods but to agree on experimental
138 conditions that are based on available physiological data to be as close as possible to the *in*
139 *vivo* equivalent, while keeping the method sufficiently simple to reproduce all over the world.
140 The clear definition of standardised experimental conditions and procedures is one of the
141 major advantages of the INFOGEST method. Egger et al. ²⁸ showed very good lab to lab
142 reproducibility of results from the *in vitro* digestion of skim milk from powder, in regards to
143 peptide patterns. Some weaknesses were identified and have been addressed subsequently.
144 The recommendation of standardised enzyme assays (including units) significantly added to
145 the precision and reproducibility of the digestion procedure as previously, a number of
146 common but slightly different enzyme assays were being used, resulting in the application of
147 a wide range of enzyme activities during digestion experiments. The end point of this
148 INFOGEST method was recently compared to digests obtained in human jejunum after
149 casein and whey protein ingestion¹⁶ showing excellent correlation in protein degradation and
150 peptide patterns, as explained below in Applications.

151 However, static digestion methods have known limitations and cannot mimic the complex
152 dynamics of the digestion process or the physiological interaction with the host. For example
153 for the gastric phase, the pH is kept constant, there is a lack of the gradual addition of gastric
154 fluid (acid, minerals, pepsin) and an absence of gradual gastric emptying. In addition, the
155 enzyme activity in each digestive phase is kept constant, regardless of the type of food and
156 whether the food contains high or low amount of substrate e.g. proteins, lipids and
157 carbohydrates. The intestinal phase is treated as one phase rather than those of the
158 sequential duodenal, jejunal and ileal phases, which exhibit different dilutions, mineral
159 content, pH, enzyme activities, microbial content, etc. These shortcomings render the
160 method unsuitable for detailed kinetic analysis of the different stages of the digestion
161 process. However, *in vivo* comparison shows good correlation with the INFOGEST method
162 at the end points of each digestion phase.^{16,29} For this reason, the static model should only
163 be used to assess digestion endpoints and not kinetics.

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

179 **Applications**

180 The method described has been used to assess the release of carotenoids and phenolic
181 compounds from different matrices, such as, carotenoids in fruits^{30,31}, carotenoids in
182 tomatoes compared to tomatoes subjected to pulsed electric fields³², β -carotene protected by
183 microencapsulation³³ and resveratrol encapsulated in protein nanoparticules³⁴. However,
184 most studies have been dedicated to the evaluation of protein, lipid and starch digestion in
185 foods or modified carriers. Protein digestion has been widely assessed in different dairy
186 products^{35,36}, or in isolated milk proteins, such as lactoferrin with different iron contents and
187 after mild heat treatment³⁷. The stability of proteins to gastrointestinal digestion has been
188 proposed as an additional piece of information for the allergenicity assessment of novel
189 proteins³⁸. With this focus, the INFOGEST method was also applied to the study of the
190 immunogenic potential of peptides from pasta³⁹, hazelnut⁴⁰, and peanut⁴¹, which are resistant
191 to gastrointestinal digestion. Using a pH-stat to monitor enzymatic hydrolysis, it was shown
192 that solid emulsions led to a lesser extent of lipolysis but a greater degree of proteolysis
193 compared to liquid emulsions due to the higher sensitivity of denatured whey proteins to
194 gastrointestinal enzymes⁴². The tendency of dairy rennet gels to form compact protein
195 aggregates during gastric digestion has also been assessed⁴³. Other applications of this
196 protocol include the evaluation of novel biopolymers designed for a controlled nutrient
197 release^{44,45}, or the digestive stability of transgenic microRNAs in genetically modified plants⁴⁶.



198 An inter-laboratory trial applying different *in vitro* digestion protocols clearly demonstrated a
199 good reproducibility obtained by using the standardised INFOGEST protocol. It also
200 highlighted the importance of correctly applying standardised pepsin activity assays, which is
201 a key factor for proper gastric protein hydrolysis²⁸. A special effort was made to validate and
202 compare the results from this *in vitro* digestion protocol with *in vivo* data. For instance, β -
203 cryptoxanthin bioavailability from pasteurised orange juice was found to be higher than from
204 fresh oranges in a randomised crossover human study, and from the *in vitro* digestion an
205 increased bioaccessibility could also be inferred⁴⁷. Several studies have focused on protein
206 digestion and the comparison with *in vivo* digestion in human or animal models. The results
207 from the *in vitro* gastrointestinal digestion of skim milk powder were compared with *in vivo*
208 porcine samples collected from the stomach and several sites in the intestine²⁹. Protein
209 degradation and peptides generated at the end of the gastric phase correlated well with *in*
210 *vivo* gastric peptides while the *in vitro* intestinal phase correlated well with the *in vivo*
211 samples taken in the median jejunum. Human jejunal digests after the oral ingestion of
212 casein and whey protein were compared with the intestinal digests obtained using the
213 standardised INFOGEST method¹⁶. *In vivo* and *in vitro* intestinal digests showed common
214 protein regions that are resistant to digestion and a high number of identical peptide
215 sequences, concluding that the INFOGEST *in vitro* method is a good approximation to the
216 end points of gastrointestinal digestion of milk proteins *in vivo*.

217 **Alternative methods**

218 A wide variety of static *in vitro* digestion models can be found in the literature²⁵ but they all
219 exhibit different conditions (pH, duration of each step, ratio enzymes/substrate...) making the
220 comparison between studies impossible. The static methods published by Versantvoort et
221 al.²¹, Garrett et al.⁴⁸ and Oomen et al.²⁰ are amongst the most used, based on their citations.
222 However, most of the of static *in vitro* digestion methods found in the literature simulate the
223 fasted state, which is quite far from the physiological conditions when food is digested in the
224 gastrointestinal tract. Advantages and limitations of static *in vitro* digestion models have been
225 recently reviewed by a group of experts within the INFOGEST network¹⁵. While static
226 methods can be useful for understanding trends or performing a screening of samples, it falls
227 short in terms of some of the important dynamic processes occurring during gastrointestinal
228 digestion, namely the pH gradients and the gradual addition of enzymes and gastric fluid as
229 well as continuous gastric emptying. More physiologically relevant dynamic digestion
230 methods⁶⁻¹⁰ take these and other factors into account. However these models are highly
231 complex, require substantial hard- and software and are still expensive to set up and
232 maintain, hence are often not available to food researchers. It has recently been shown that,

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233 when human data are available to set up the system, these models can be physiologically-
234 relevant¹¹. In an effort to improve *in vitro* digestion methods, a low-cost semi-dynamic
235 method was recently developed⁴⁹ and described in detail⁵⁰, where parameters were based on
236 the equivalent *in vivo* data from the digestion of dairy products. Here, the simulated gastric
237 fluid (SGF) and pepsin are slowly added to the food in a suitable reaction vessel with
238 manual, stepwise gastric emptying. A harmonisation of experimental conditions is currently
239 on-going and a standardised semi-dynamic method will be published shortly by INFOGEST
240 members, coordinated by A.R Mackie.

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

247 Finally, human volunteers can be equipped with naso-gastric or naso-intestinal probes to
248 access and sample the digestive effluents³. Ileostomy patients have been used to study
249 digestion⁵⁴⁻⁵⁶ but can hardly be considered as a model of a healthy human since they are
250 affected by digestive pathologies.

251

252 **Experimental Design**

253 *Enzyme assays*

254 The determination of the standard units of activity of the enzyme used in the protocol is a
255 crucial step and one of the main sources of variation in results with the digestion periods or
256 between different laboratories.³⁷ Enzyme activity determination is recommended for each
257 new batch of enzyme or after prolonged storage.

258 Enzyme and bile assays were previously described in protocol format in the Supplementary
259 Materials of Minekus et al.²⁷, namely: α -amylase (EC 3.2.1.1), pepsin (EC 3.4.23.1), trypsin
260 (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), pancreatic lipase (EC 3.1.1.3) and bile salts
261 (according to supplier's protocol). In order to improve the reproducibility of the pepsin activity
262 assay for this revised INFOGEST 2.0 protocol, it is now recommended to dissolve pepsin in
263 10 mM Tris buffer (tris-hydroxymethyl-aminomethane), 150 mM NaCl, (pH 6.5), instead of in
264 sodium chloride solution adjusted with sodium hydroxide. The buffering capacity of Tris
265 buffer reduces the variability in the measurement of the pepsin activity, as shown
266 previously³⁷. The detailed protocols for the complete set of enzyme and bile assays, including



267 that of the gastric lipase assay (EC 3.1.1.3), can be found in the Supplementary Information
268 and is summarise in **Box 1**.

269 Spreadsheets for the enzyme assays and the volumes for the digestion procedure are
270 provided in the Supplementary Information of this manuscript. The enzyme assay
271 spreadsheets (Supplementary spreadsheets 1) can be used to calculate the enzyme
272 activities of all digestive enzymes. The digestion spreadsheets (Supplementary spreadsheets
273 2) provides help in calculating all volumes of simulated digestive fluids, enzyme and bile
274 solutions based on the initial amount of digested food; one example is shown in **Table 3**. The
275 corresponding online spreadsheets can also be used, and are available here:
276 www.proteomics.ch/IVD and on the INFOGEST website <https://www.cost-infoGEST.eu/>. In
277 addition, videos of the digestion procedures (Supplementary Video 1 and 2) and all enzyme
278 activity assays (Supplementary Video 3 to 7) are available in the Supplementary Information.
279 In addition, the videos are also available online on the YouTube channel “In vitro food
280 digestion - COST action INFOGEST” [https://www.youtube.com/channel/UCdc-](https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg)
281 [NPx9kTDGyH_kZCgpQWg](https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg) and on the INFOGEST website <https://www.cost-infoGEST.eu/>.

282 *Food preparation and oral phase*

283 It is important to plan the preparation of the food and the oral phase prior to *in vitro*
284 gastrointestinal digestion to determine the food to digestive enzyme ratio throughout the *in*
285 *vitro* digestion process. Firstly, consideration should be given as to whether the food to be
286 digested *in vitro* is consumed as a meal, a meal portion or even a food ingredient. Some
287 foods such as milk are often consumed on their own or as part of a meal. Other foods or food
288 ingredients are nearly always consumed as part of a meal rather than on its own (e.g.
289 coconut milk, spices, pure proteins, oils). Hence these foods should be prepared in a way
290 that reflects real food or a meal, i.e. dilution, emulsification, integration into other foods, etc.
291 High solid foods such as powders need to be reconstituted in liquids to make them a
292 consumable food.

293 An optional oral phase with a standardised 1:1 (w/w) ratio of food to simulated oral fluid for all
294 foods (solid and liquid foods) was recommended by the INFOGEST method²⁷ in 2014. While
295 *in vivo* data varies greatly (Supplementary **Figure 1**), this dilution ratio enables the formation
296 a swallowable bolus with almost all types of foods. For this revised INFOGEST 2.0 protocol a
297 standardised, easy-to-follow approach for the oral phase is necessary. Hence, it is now
298 recommended to dilute all food 1:1 (w/w) with simulated oral fluid to achieve a swallowable
299 bolus that is no thicker than a paste-like consistency similar to that of tomato paste or



300 mustard at the end of the oral phase. If the consistency of the bolus is thicker than paste-like,
301 add water to achieve it (see also **Table 3** and **Table 4** Troubleshooting).

302 *Use of lipase in the gastric phase*

303 Lipid digestion starts in the stomach with the action of preduodenal lipase (gastric lipase in
304 humans, lingual lipase in rodents) on triacylglycerides (TAG) and some other esters⁵⁷.
305 Gastric lipolysis not only contributes to the overall digestion of TAG (10% with a solid-liquid
306 test meal to 25% with an emulsified liquid test meal) but it also triggers the subsequent action
307 of pancreatic lipase on lipid substrates that may be poorly digested by pancreatic lipase
308 alone; examples include milk fat droplets and lecithin-stabilised TAG emulsions⁵⁸. It is
309 therefore recommended to add gastric lipase during the gastric phase of *in vitro* digestion.
310 The mean gastric lipase concentration in human gastric juice is 100 µg/mL, which is
311 equivalent to 120 U/mL using tributyrin as the reference substrate for gastric lipase^{59,60}. In
312 some static digestion models, a concentration of approx. 16 µg gastric lipase/mL (20 U/mL)
313 has been used to reproduce gastric conditions at half time of gastric emptying^{61,62}, which
314 corresponds to a gastric juice to meal ratio of 1:5 v/v. In the INFOGEST method, the gastric
315 phase of digestion includes a 1:1 dilution of the oral bolus by simulated gastric fluid, which
316 would correspond to a dilution of gastric juice by half and thus a gastric lipase concentration
317 of 60 U/mL. To date, access to commercially available gastric lipase, or an appropriate
318 equivalent has been limited, hence gastric lipase has been omitted or lipases from alternative
319 sources have been widely used. However, caution should be applied regarding the specific
320 biochemical properties of these alternative lipases. Human gastric lipase (HGL), encoded by
321 the LIPF gene, is stable and active between pH 2 and 7 with an optimum activity between pH
322 4 to 5.4. HGL displays a S_N3 stereospecificity for TAG hydrolysis leading to the preferential
323 release of short/medium chain fatty acids from milk TAG⁶¹. It is resistant to pepsin hydrolysis
324 and is not inhibited by bile salts. HGL can however be replaced by other preduodenal lipases
325 from the acid lipase gene family of various mammalian species like dog⁶³ and rabbit⁶⁴. Rabbit
326 gastric lipase is now commercially available (Lipolytech, www.lipolytech.com). Pre-duodenal
327 lipases originating from the oro-pharyngeal tissues of young ruminants (pharyngeal lipase of
328 calf, kid goat, lamb) may also be used and are commercially available for applications in the
329 dairy industry (DSM for Capalase[®] K and Capalase[®] KL lipases; CHR Hansen for Lipase Kid-
330 Goat ST20, Lipase Calf 57 LFU, Spice IT[™] AC and Spice IT[™] AG; DuPont Danisco, Clerici-
331 Sacco). These preduodenal lipases are however less resistant to acid denaturation
332 (threshold at around pH 3.5⁶⁵) than gastric lipase and pH conditions may have to be
333 adapted. Their contents and activity should be estimated before use in *in vitro* digestion
334 experiments, using the recommended standard gastric lipase assay²⁷, see Supplementary

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335 Information Section. So far, no commercially available lipase of microbial origin combines all
336 the above properties of gastric lipase^{61,66}, and their use is not recommended at this time. For
337 this revised INFOGEST 2.0 protocol, the authors recommend using rabbit gastric lipase,
338 commercially available as rabbit gastric extracts (RGE) at 60 U/mL in the final gastric
339 digestion mixture. However, since these extracts also contain pepsin⁶⁷, the pepsin
340 concentration/activity in the gastric phase has to be accordingly adjusted to the
341 recommended value.

342 *Sampling, controls and test tube*

343 Before performing the protocol (time-lagged before the digestion experiment or one day prior
344 to the digestion experiment), it is recommended to run one preliminary experiment, the **pH-**
345 **test adjustment experiment**, with the relevant amount of food, enzymes and bile for the
346 entire digestion process. The aim of this pH-test adjustment experiment is to measure and
347 record the amounts of HCl and NaOH used to reach the target pH in order to perform more
348 efficient pH adjustments when running the digestion protocol. These volumes are indicative
349 of the necessary volume of acids and bases needed for the gastric and intestinal phase. It
350 has to be noted that for solid food, the pH changes are generally slower in response to
351 addition of HCl or NaOH – it is important to remain patient and wait long enough for the pH to
352 become stable - >5 min depending on food particle size and buffering capacity.

353 If it is intended to take samples at different time points during digestion, it is recommended to
354 prepare one tube per time point, e.g. prepare six digestion tubes for six time points. Because
355 most foods are heterogeneous mixtures during digestion, sampling is more reproducible by
356 starting digestion with individual tubes per time point. If the food sample has special
357 requirements in terms of nutrient stability (e.g. light sensitivity, oxidation) the characteristics
358 of the tubes should be adapted to these particular situations (opaque tubes, maintenance of
359 the food samples on ice, etc). The end volume of the digest should be calculated to use the
360 most suitable reaction vessel, e.g. 50 mL tubes, which allow properly mixing during all
361 digestion phases.

362 Optionally, a replicate test tube (**stability test tube**) can be prepared to evaluate food
363 stability during exposure to simulated digestive fluids without enzymes or bile, for example
364 after oral, gastric and intestinal phase. It can also be advisable to prepare an **enzyme-blank**
365 **tube**, i.e., a digestion tube with all enzymes and bile but without food. This may be helpful to
366 identify enzyme, bile salts or degradation products thereof during analysis of the digests. It is
367 important to highlight that due to proteolytic enzyme autolysis, especially pepsin, enzyme-



368 derived peptides can be detected in digesta which can be easily monitored with this blank-
369 enzyme tube.

370 *Intestinal phase, stop reaction and read out*

371 The intestinal phase of the protocol starts with the mixing of the gastric chyme with the same
372 volume of the pre-warmed SIF. The pH is adjusted with the amount of NaOH previously
373 calculated in the *pH-test adjustment experiment*. In this phase, two different options are
374 given, (i) the use of pancreatin or (ii) the use of individual enzymes: porcine trypsin (100
375 U/mL), bovine chymotrypsin (25 U/mL), porcine pancreatic α -amylase (200 U/mL), porcine
376 pancreatic lipase (2,000 U/mL) and porcine pancreatic colipase in molar excess to lipase.
377 The amount of pancreatin to be used in the intestinal phase of digestion is based on trypsin
378 activity to achieve 100 U/mL in the final mixture. This calculation may result in low lipase
379 activity for high fat containing foods or if fat digestion is the aim of the study. In this case, it is
380 recommended to include additional lipase to get 2000 U/mL of lipase activity in the final
381 mixture and colipase in a molar ratio 2:1 colipase to lipase, which corresponds approximately
382 to a mass ratio 1:2 colipase to lipase. Since this will require the measurement of the lipase
383 activity in the pancreatic extract and in the lipase preparation, the use of individual enzymes
384 could be a preferred option. Similarly, because the activity of amylase in pancreatin can vary
385 between batches and the activity can be too low to digest starch rich foods, the use of
386 individual enzymes could also be a good option when following carbohydrate digestion. Bile
387 salts are added to the intestinal mixture to reach 10 mM in the final mixture, after
388 determination of the bile salt concentration in the commercial product (see Enzymatic
389 Assays). There are several commercial options for bile salts but bovine bile is preferred
390 because its composition is similar to that in humans⁶⁴. Bile solubilisation requires exhaustive
391 mixing which can be achieved, for instance, in a rotating wheel mixer at 37°C for 30 min.

392 *In vitro* digestion is carried out for a wide range of purposes and with different endpoints. In
393 all cases, sampling, sample preservation and the post-treatment of samples after food
394 digestion are critical and some adaptations could be needed depending on the particular
395 requirements of each experiment (**Table 1**). For example, to stop pepsin activity, the pH of
396 gastric samples must be raised to 7.0, either by the addition of 1 M sodium bicarbonate or 1
397 N NaOH solution. The pH shift after the gastric phase is very effective in stopping pepsin
398 activity and similar to *in vivo* conditions found in the duodenum⁵⁶. If the pH increase is not
399 desired, the use of pepstatin A, a highly selective inhibitor of aspartyl proteases like pepsin
400 ($K_i = 0.1$ nM) has also been suggested⁶⁸. When gastric digestion is considered as an end
401 point, sample snap freezing in liquid nitrogen followed by freeze-drying are recommended.



402 Raising the pH to 7.0 strongly reduces the activity of gastric lipase on long chain
403 triglycerides⁵⁸⁻⁶⁰. Alternatively, the use of Orlistat[®] (tetrahydrolipstatin) is also recommended
404 (gastric lipase half-inhibition time of < 1 min) to block gastric lipolysis⁶¹. Add Orlistat at a final
405 concentration of 0.6 mg/mL (1 mM) to obtain an inhibitor to lipase molar ratio of 1,000, taking
406 into account that the gastric lipase activity of 60 U/mL corresponds to 50 µg/mL or 1 µM
407 lipase.

408 After gastrointestinal digestion and in order to inhibit the different enzymatic activities of the
409 digested samples, immediate snap freezing after sampling is necessary. However, when
410 thawing the sample for subsequent analysis, residual enzymatic activities could significantly
411 affect the stability of the samples. Therefore, addition of sufficient amounts of enzyme
412 inhibitors against target digestive enzymes is strongly recommended. In the case of
413 proteases, the addition of 5 mM of Pefabloc[®] SC (4-(2-Aminoethyl) benzenesulfonyl fluoride
414 hydrochloride, AEBSF) with ability to irreversibly inhibit trypsin and chymotrypsin is
415 recommended due to its lower toxicity in comparison with phenylmethylsulfonyl fluoride
416 (PMSF)⁴⁰. Alternatively, the use of Bowman-Birk inhibitor from soybean, a potent inhibitor
417 against both trypsin and chymotrypsin having K_i values at nanomolar level, has been also
418 recommended⁶². In order to inhibit lipolysis by pancreatic lipase, the use of 5 mM of 4-
419 bromophenylboronic acid has been reported⁶³. Inhibition of pancreatic lipase by Orlistat is too
420 slow (half-inhibition time > 5 min) to be used here⁶¹. For amylase inhibition heat-shock
421 treatment, inactivation by ethanol or inhibition with 12% TCA have been used⁶⁴, depending
422 on the downstream sample analysis. Once the target inhibition occurs, the digests should be
423 immediately snap frozen in liquid nitrogen and freeze-dried.

424 When biological activity of digested samples has been evaluated, heat-shock treatment (in
425 boiling water for 5 min) to irreversibly inactivate proteases may also be considered²⁸.
426 However, it should be noted that heat treatment is detrimental to the food structure, proteins
427 in particular as heat treatment generally causes irreversible denaturation and aggregation.
428 For cell culture assays, consider whether the use of Pefabloc or other enzyme inhibitors can
429 affect the read out of the experiment, and whether the osmolarity needs to be corrected by
430 dilution to physiological values (285-300 mOsm/kg H₂O, pH 7-7.5) in order to avoid cell
431 osmotic shock. Other combined procedures for removal or enrichment of certain food
432 components such as defatting, centrifugation, dialysis, filtration and size exclusion
433 chromatography are also commonly used.

434

435

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437
438

Materials

439 Reagents:

- 440 - Ultrapure type I water, generated by a Milli-Q® system or similar (referred in text as
441 water)
- 442 - Human salivary α -amylase (Sigma-Aldrich, 1031)
- 443 - Porcine pepsin (Sigma -Aldrich, P7012 or P6887)
- 444 - Rabbit gastric extract (RGE) for gastric lipase (see section on gastric lipase above,
445 currently supplied by e.g. Lipolytech RGE 25-100MG) **Critical:** RGE contains both
446 gastric lipase and pepsin.
- 447 - Bovine bile (Sigma-Aldrich, B3883, preferred option as composition in closest to that
448 in humans), alternatively Porcine Bile (Sigma-Aldrich, B8631),
- 449 - Porcine pancreatin (Sigma-Aldrich, P7545) or individual intestinal porcine enzymes
450 (trypsin, chymotrypsin, amylase, lipase and co-lipase), see below optional reagents
- 451 - $\text{CaCl}_2(\text{H}_2\text{O})_2$ (Merck 2382)
- 452 - NaOH (Merck 9141) **! Caution: corrosive, causes severe skin burns and eye damage**
- 453 - HCl (J. T. Baker 6081) **! Caution: corrosive, causes burns, irritating to respiratory**
454 **system**
- 455 - KCl (Merck 4936)
- 456 - KH_2PO_4 (J. T. Baker 0240)
- 457 - NaHCO_3 (Merck 6329)
- 458 - NaCl (Merck 6404)
- 459 - $\text{MgCl}_2(\text{H}_2\text{O})_6$ (Merck 5833)
- 460 - $(\text{NH}_4)_2\text{CO}_3$ (Sigma-Aldrich, 207861)
- 461 - Enzyme inhibitors options (see Experimental Design and **Table 1**) :
- 462 o Pefabloc® SC (4-(2-Aminoethyl)benzenesulfonyl fluoride, Sigma-Aldrich,
463 76307) **! Caution: corrosive;**
- 464 o Pepstatin A (Sigma-Aldrich, P5318)
- 465 o Bowman-Birk inhibitor (Sigma Aldrich, T9777)
- 466 o 4-bromophenylboronic acid (Sigma Aldrich, B75956) **! Caution: hazardous,**
467 **corrosive, causes eye damage, harmful for respiratory system**

468 Chemicals for enzyme and bile tests:

- 469 - Pepsin test
- 470 o Haemoglobin from bovine blood (Sigma-Aldrich, H6525-25G),
- 471 o Trichloroacetic acid (Sigma-Aldrich, T6399-5G) **! Caution: Corrosive, causes**
- 472 **severe burns to skin and eyes. Soluble in water with release of heat.**
- 473 - Gastric lipase test:
- 474 o Taurodeoxycholate (Sigma-Aldrich, T0875-1G)
- 475 o Tributyrin (Sigma-Aldrich, T8626; ≥99%)
- 476 o Bovine serum albumin (Sigma-Aldrich, A7030; ≥98%)
- 477 - Trypsin test:
- 478 o TAME (p-Toluene-Sulfonyl-L-arginine methyl ester, Sigma-Aldrich, T4626-5G)
- 479 - Amylase test:
- 480 o Maltose Std. (Sigma-Aldrich, M5885-100G)
- 481 o Soluble Potato Starch (Sigma-Aldrich, S5651-500G)
- 482 o DNS (3,5-Dinitrosalicylic acid, Sigma-Aldrich, D0550-10G), **! Caution:**
- 483 **Harmful if swallowed, Acute oral toxicity**
- 484 - Chymotrypsin test:
- 485 o BTEE (N-Benzoyl-L-Tyrosine Ethyl Ester, Sigma-Aldrich, B6125-5G)
- 486 - Pancreatic lipase test:
- 487 o Sodium taurodeoxycholate (Sigma-Aldrich, T0875-1G)
- 488 o Tributyrin (Sigma-Aldrich, W222305-1KG)
- 489 - Bile acid determination
- 490 o Bile acid kit (Sigma-Aldrich, MAK 309) or ECOLINE Acides Biliaires, Diasys,
- 491 122129990313) or equivalent assay
- 492 Reagents for optional protocol with individual enzymes:
- 493 - Porcine trypsin (Sigma-Aldrich, T0303)
- 494 - Bovine chymotrypsin (Sigma-Aldrich, C7762)
- 495 - Porcine pancreatic α-amylase (Sigma-Aldrich, A3176)
- 496 - Porcine pancreatic lipase (Sigma-Aldrich, L3126)
- 497 - Porcine pancreatic co-lipase (Sigma-Aldrich, C3028)
- 498 Food (for further examples see Anticipated Results Section)
- 499 - Skim milk powder (SMP, Fonterra, NZ, low-heat organic, protein 42.34%, fat 0.89%,
- 500 lactose 49.8% (w/w)²⁸
- 501



502

503 **Equipment:**

- 504 - Standard laboratory centrifuge suitable for 50 mL tubes, 5,000 × *g* (e.g. Heraeus
505 Megafuge 40R, 75004519, Thermo Fisher, Switzerland)
- 506 - Standard laboratory vortex (e.g. Genius 3, IKA, 17.1377.01, HuberLab, Switzerland)
- 507 - Standard laboratory pH Meter (e.g. 827 pH lab, 2.827.0214, Metrohm, Switzerland),
508 electrode, designed for food systems (e.g. Sentek, P17/S7, pH electrode for food and
509 dairy, 11981656, Fisher Scientific)
- 510 - Overhead shaker/rotator; small volume up to 50mL (Rotator SB Stuart, 17.0014.02,
511 Huberlab, Switzerland)
- 512 - Incubator large enough to hold the above rotator (e.g. Termaks, B9000, Labtec,
513 Switzerland), adjustable at 37°C
- 514 - Electric or manual mincer (Eddingtons Mincer Pro, 86001, Amazon, or similar)
- 515 - Eppendorf tubes (2 mL, 211-2120, VWR, Deutschland)
- 516 - Centrifuge Plastic tubes (15 mL, 391-3450, 50 mL, 525-0399, VWR, Deutschland)
- 517 - Micropipettes (e.g. Gilson P10 - P1000, VWR) and tips
- 518 - Volumetric flasks for solutions
- 519 - Glass beakers

520 **Reagent setup:**

521 Minimum volumes of stock solutions needed for the preparation of 400 mL of simulated
522 digestion fluids 1.25× concentration:

- 523 - 0.5 mL of $\text{CaCl}_2(\text{H}_2\text{O})_2$ (0.3M)
- 524 - 30 mL of KCl (0.5M)
- 525 - 6 mL of KH_2PO_4 (0.5M)
- 526 - 65 mL of NaHCO_3 (1M)
- 527 - 25 mL of NaCl (2M)
- 528 - 2 mL of $\text{MgCl}_2(\text{H}_2\text{O})_6$ (0.15M)
- 529 - 2 mL of $(\text{NH}_4)_2\text{CO}_3$ (0.5M)
- 530 1 M NaOH and 1 M HCl: for pH adjustment of stock solutions of simulated digestion
531 fluids

532 Stock solutions can be prepared and stored in aliquots at -20°C for one year.

533 Preparation of simulated digestion fluids at a 1.25× concentration

534
535 Simulated digestion fluids for oral (SSF), gastric (SGF), and intestinal (SIF) digestion phase
536 are mixed at a 1.25× concentration using the electrolyte stock solutions and water according
537 to **Table 2** and can be stored at -20°C for one year. **Critical:** CaCl₂ should be added
538 immediately prior to the digestion experiment to avoid precipitation upon storage. **Critical:** All
539 the volumes (**Table 2**) are calculated for 400 mL of a 1.25× concentrated storage solution
540 and just before use they are mixed with the necessary quantities of enzyme and finally
541 diluted to a 1× concentrated working solution (i.e. 4 parts of electrolyte solution + 1 part
542 consisting of enzymes and water result in a 1× concentration of the digestion fluids).
543 Simulated digestion fluids (1.25× concentrates) can be stored at -20°C for one year in small
544 aliquots of appropriate size; e.g. for the experiment shown in **Box 1**, using 5 g of food, at
545 least 48 mL of SSF, 88 mL of SGF, and 96 mL of SIF are needed. **Critical:** Dilute enzymes
546 in cold solutions and keep them on ice until used. This will keep enzyme activity to a
547 minimum. **Critical:** Pre-warm electrolyte solutions (SSF, SGF, SIF) to 37°C prior to using
548 them in the digestion procedures.

549



550 Procedure

551 Preparation reagents and digestion tubes (5 days):

- 552 1. Perform all enzyme and bile assays (**Box 1**) according to the protocols in the
553 Supplementary Information for each new batch of enzymes or after prolonged storage;
554 **TIMING** 4-5 days for all assays
555 **Critical Step:** For the pepsin assay, dissolve pepsin in 10 mM Tris, 150 mM NaCl, pH
556 6.5, which improves the reproducibility of the assay (see Supplementary Information).
557 **Critical Step:** Spreadsheets for the enzyme assays and the volumes for the digestion
558 procedure are provided in the Supplementary Information of this manuscript
559 (Supplementary spreadsheets 1 and 2). In addition, the corresponding online
560 spreadsheets are available here: www.proteomics.ch/IVD and on the INFOGEST website
561 <https://www.cost-infogest.eu/>.
562 **Critical Step:** Prepare one tube per time point and food; e.g. for one food and six time
563 points, prepare six tubes
- 564 2. Pre-warm the electrolyte stock solutions at 37 °C, initially only SSF and SGF, SIF
565 3. Prepare all enzyme and bile solutions immediately before the digestion experiment
566 **Critical Step:** Keep all enzyme solutions on ice
- 567 4. In order to perform more efficient pH adjustments during the digestive phases, prepare
568 one replicate tube (pH-test adjustment experiment) with the relevant amount of food,
569 enzymes and bile for the entire digestion process (time-lagged before the digestion
570 experiment or one day prior to the digestion experiment) and measure and record the
571 volumes of HCl and NaOH used to reach the target pH. These volumes are indicative of
572 the necessary volume of acids and bases needed for the gastric and intestinal phase
573 **TIMING** 5h
- 574 5. Optional: Prepare one replicate test as a food stability control to assess the behaviour of
575 the food during exposure to simulated digestive fluids without enzymes or bile, for
576 example after oral, gastric and intestinal phase
- 577 6. Prepare one replicate test tube as a blank, digestion without food (replaced by water) but
578 with all required enzymes and bile. See videos of enzyme assays (supplementary videos
579 3 to 7) as well as the digestion procedures (supplementary videos 3 and 4). Videos are
580 also available online on the YouTube channel “In vitro food digestion - COST action
581 INFOGEST” https://www.youtube.com/channel/UCdc-NPx9kTDGyH_kZCgpQWg
582 and on the INFOGEST website <https://www.cost-infogest.eu/>

583



584 **Digestion procedure**

585 **TIMING** depending on number of food samples and time points, for example: 1 food sample
 586 and 5 time points - approximately 5h; 2 food samples and 5 time points (2 gastric and 3
 587 intestinal points) - approximately 8h

588 **Oral phase (30 min)**

- 589 7. Dilute food with SSF at a ratio of 1:1 (w/w) to achieve a swallowable bolus with a paste-
 590 like consistency similar to that of tomato paste or mustard at the end of the oral phase. If
 591 the consistency of the bolus is thicker than paste-like, add water to achieve it. Salivary
 592 amylase is only needed to digest starch containing food. It can be omitted if the food
 593 does not contain starch. Do not use lower purity salivary amylase or pancreatic amylase.
- 594 8. Mix food with SSF at a 1:1 ratio (w/w), e.g. 5 g of food to 5 g of SSF
- 595 9. Measure the volume of the final digestion mixture of the food + SSF mixture. Record this
 596 volume as it will be used in step 17.
- 597 10. If necessary, simulate mastication by mincing the food in an electric or manual mincer.
- 598 11. Depending on the food (e.g. bread), mincing can be done together with the SSF
 599 electrolyte (without enzymes)
- 600 12. Add SSF electrolyte stock solution to the food, if not done in the previous step
- 601 13. Add CaCl₂ in order to achieve a total concentration of 1.5 mM in SSF
- 602 14. Add the salivary amylase, if necessary, prepared in water to achieve an activity of 75
 603 U/mL in the final mixture.
- 604 15. Add the remaining water in order to achieve 1× concentration of the SSF.
- 605 16. Incubate while mixing for 2 minutes at 37°C.

606 **Critical step:** Electrolyte concentrations are given for the simulated digestive fluids
 607 (SSF, SGF and SIF) and accumulation in consecutive digestion phases is not
 608 considered whereas enzyme activities are expressed U/mL in the final digestion mixture.

609
610 **Gastric phase (3h)**

- 611 17. Pre-warm the SGF electrolyte stock solution at 37°C. Add SGF electrolyte stock solution
 612 to the oral bolus to a final ratio of 1:1 (v/v)
- 613 18. Adjust the pH to 3.0 by adding a defined volume of HCl previously determined during a
 614 pH-test adjustment experiment, see Experimental Design
- 615 **Critical step:** For solid food, the pH changes are generally slower in response to the
 616 addition of HCl – it is important to remain patient and wait until the pH is stable, usually,
 617 this takes >5 min depending on food particle size and buffering capacity.

- 618 19. Add CaCl_2 solution in order to achieve a final concentration of 0.15 mM in SGF.
- 619 20. Add the porcine pepsin solution prepared in water to achieve an activity of 2,000 U/mL in
620 the final digestion mixture.
- 621 21. Add the gastric lipase solution prepared in water to achieve an activity of 60 U/mL in the
622 final digestion mixture.
- 623 22. Verify the pH and adjust to 3.0 if necessary
- 624 23. Add water in order to achieve 1× concentration of the SGF
- 625 24. Incubate the samples at 37 °C, mixing the digestive mixture sufficiently (e.g. rotating
626 wheel, shaking incubator) for 2 h from the point when pepsin was added. In case of large
627 precipitates and formation of clogs, see Troubleshooting.
- 628 **Critical step:** Rabbit gastric extracts (RGE) contains both gastric lipase and pepsin⁶⁷.
629 The pepsin activity in RGE needs to be determined and taken into account together with
630 the porcine pepsin to reach a combined pepsin activity of 2,000 U/mL in the final
631 digestion mixture.
- 632 **Critical step:** The use of carbonate salts in the electrolyte solutions requires that sealed
633 containers with limited headspace are used. In open vessels, CO_2 will be release and
634 the pH will progressively increase with time. If open vessels are to be used, such as
635 when using the “pH-stat” approach or for sampling purposes, it is suggested to replace
636 sodium bicarbonate (NaHCO_3), the main source of carbonates, by NaCl at the same
637 molar ratio in order to maintain the ionic strength of the electrolyte solutions (oral, gastric
638 and intestinal). Such adjustment has already proven effective in avoiding unwanted pH
639 drift in open vessels in both gastric⁶⁹ and intestinal⁴² phases of digestion (see **Table 2**).

640

Intestinal phase (3h):

- 641 **Intestinal phase (3h):**
- 642 25. Pre-warm the SIF electrolyte stock solution in a 37°C water bath. Add SIF electrolyte to
643 the gastric chyme and achieve a final ratio of 1:1 (v/v).
- 644 26. Adjust to pH 7.0 by adding a defined volume of NaOH previously determined during a
645 pH-test adjustment experiment, see Experimental Design.
- 646 **Critical step:** For solid food, the pH changes are slower in response to the addition of
647 NaOH, see remarks in step 18; this may take several minutes.
- 648 27. Add the bile solution to the SIF: gastric chime solution in order to reach a final
649 concentration of 10 mM. Place the solution in a rotating wheel mixer at 37 °C for at least
650 30 min to achieve complete bile solubilisation.
- 651 28. Add CaCl_2 solution in order to reach concentration of 0.6 mM in SIF.
- 652 29. Perform intestinal phase with option (A) pancreatin or option (B) with individual enzymes

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- 653 A.
- 654 i. Add the pancreatin suspension in SIF solution to achieve a trypsin activity
- 655 of 100 U/mL in the final mixture. Additional pancreatic lipase may be
- 656 needed for the digestion of fat containing food to reach the required lipase
- 657 activity to achieve a lipase activity of 2,000 U/mL in the final mixture.
- 658 **Critical step:** Measure trypsin activity in pancreatic lipase powder and subtract it
- 659 from the needed trypsin activity
- 660 B.
- 661 i. Add trypsin, chymotrypsin, pancreatic α -amylase, pancreatic lipase and
- 662 the co-lipase solutions in SIF, in order to reach 100, 25, 200 and 2,000
- 663 U/mL, respectively, in the final digestion mixture
- 664 30. Verify the pH and adjust to 7.0 if necessary
- 665 31. Add water in order to achieve 1 \times concentration of the SIF
- 666 32. Incubate the samples at 37 °C, mixing the digestive mixture sufficiently using a rotating
- 667 wheel or shaking incubator for 2h starting at the point when pancreatic enzymes were
- 668 added. For difficulties with sampling, see **Table 4** Troubleshooting.
- 669 **Critical step:** If open vessels are used (“pH-stat” approach), NaHCO₃ should be
- 670 replaced by NaCl in the electrolyte solutions to avoid unwanted pH drift (see the step 24
- 671 critical step).
- 672



673 Anticipated Results

674 Protein digestion

675 Without the use of standardised digestion methods, the main difficulties were (i) the absence
676 of comparable results from different laboratories and (ii) the physiological relevance of
677 experimental data in the field of food digestion. The INFOGEST method was tested with
678 respect to these two aspects focusing on protein digestion.

679 (i) *Robustness of the protocol* and comparability of experimental data were assessed in
680 several inter-laboratory trials where the participants were asked to digest a standardised
681 skim milk powder (SMP) by applying their existing in-house protocols first, then by using the
682 harmonised protocol²⁸. The first critical step in protein hydrolysis is the pepsin activity in the
683 gastric phase. The heterogeneous pattern observed with the in-house digestion protocols
684 (**Figure 2a**, gastric phase) was improved significantly by the correct implementation of the
685 harmonised protocol (**Figure 2b**, gastric phase), except for laboratories 6 and 7, which
686 showed incomplete casein hydrolysis. Adjustments in the pepsin assay (addition of Tris
687 buffer, see Step 1 Critical Step and **Box 1**) improved the reproducibility and reduced lab-to-
688 lab variability²⁸. This improved pepsin assay is now recommended for the INFOGEST 2.0
689 method. **Figure 2b** shows improved homogeneity between samples, compared to the gastric
690 phase when the harmonised protocol was applied. Increased protein degradation in the
691 intestinal phase was observed in laboratories 4 and 7. Subsequent recommendation on the
692 correct sample preparation, in particular the correct inhibition of enzymes at the end of the
693 digestion experiment (see **Table 1**), improved lab-to-lab variability²⁸.

694 (ii) *Physiological relevance* was evaluated by comparing *in vitro* SMP digestion with that of
695 an *in vivo* pig trial²⁹. Pigs were fed reconstituted SMP from the same batch as applied in the
696 *in vitro* tests and samples were collected from the stomach and in several sections of the
697 small intestine (jejunum, I1- I3 to ileum, I4) after sacrifice. Milk peptides were identified with
698 mass spectrometry and overall peptide patterns were visualised by summing up the number
699 of times each individual amino acid was identified within a milk peptide. Overlay of the
700 average peptide patterns for α_{s2} -casein from the harmonised *in vitro* digestion (n=7) and *in*
701 *vivo* pig digestion (n=8) showed that at the end of the gastric phase, the peptide pattern
702 corresponded well to that of the pig sample collected from the stomach; the peptide pattern
703 in the *in vitro* intestinal phase sample was most similar to that of the pig sample collected in
704 the median jejunum (I3). This comparison showed that protein hydrolysis at the endpoints of

705 the harmonised INFOGEST digestion method were in agreement with that of the *in vivo*
706 digestion (**Figure 3**).

707 In conclusion, both critical points, inter-laboratory comparability and physiological relevance
708 were improved by the correct application of the harmonised *in vitro* digestion protocol.

709

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710

711 Lipid Digestion

712 To date, most published digestion experiments using this INFOGEST method did not include
713 a gastric lipase because of the lack of commercially available, acceptable substitutes for
714 human gastric lipase (HGL). This situation has changed with the availability of rabbit gastric
715 extracts containing gastric lipase, see Experimental Design in the Introduction: *Use of lipase*
716 *in the gastric phase*. Both HGL and rabbit gastric lipases exhibit, at the recommended gastric
717 pH of 3.0, approximately 50% of their maximum activity measured at pH 4 to 5.4^{70,71}.
718 Moreover, the *in vitro* gastric lipolysis of infant formula by rabbit gastric lipase were
719 consistent with *in vivo* data, with a degree of lipolysis reaching 10% after 60 min of gastric
720 digestion⁷². These data therefore suggest that gastric lipolysis could be studied using this
721 INFOGEST 2.0 method with rabbit gastric extract as a source of gastric lipase⁶⁴ or human
722 gastric lipase if available⁶¹.

723 The INFOGEST method has also been used to study intestinal lipid digestion, for example in
724 oil-in-water emulsions stabilised by milk or soya lecithin⁷³. However, human gastric analogue
725 and phospholipases A2 (PLA₂) were added in this procedure. The degree of hydrolysis (%
726 TAG disappearance) ranged between 73 and 87 % (± 5 %) at the end of the intestinal phase
727 (120 min). In addition, *in vitro* digestion was also performed with more complex systems such
728 as whole fat dairy products or protein/polysaccharide emulsions. Depending on the structure
729 of the food matrix and the state of dispersion of the lipids, the reported degrees of hydrolysis
730 at the end of the intestinal phase ranged from moderate (66% of remaining lipids in poorly
731 digestible raw oat flakes due to limiting matrix structure)⁷⁴ to an almost complete
732 disappearance of triglycerides⁷⁵.

733 Intestinal lipid digestion can be assessed by chemical analyses of collected samples. The
734 protocol recommends analysing the entire volume of digestive tubes to prevent sampling
735 errors (see Procedure Step 1 Critical Step, one tube per time point and food). This
736 precaution is particularly useful in the presence of lipids⁷⁴ as they often tend to destabilise
737 and phase-separate (cream) during the gastric and/or intestinal phases of digestion. If
738 aliquots are taken as sample points, great care should be taken to represent the whole
739 digested solution. The best way to analyse the extent of lipolysis is to conduct the Folch
740 extractions⁷⁶ on the samples in the presence of internal standards before the analysis of
741 classes of the lipids (residual triglycerides, free fatty acids, diglycerides and monoglycerides)
742 by thin layer chromatography⁷⁵ combined with densitometry or gas chromatography with a
743 flame ionization detector (GC-FID)⁷⁷ or HPLC coupled to a light scattering detector⁷⁸. Free
744 fatty acids can also be quantified after solid phase extraction with GC-FID, using fatty acids

745 (typically C11:0, C15:0, C17:0 or C23:0) as internal standards^{72,79}. The pH-stat method, one
746 of the most commonly used methods for monitoring pancreatic lipolysis, can also be used,
747 but three sources of errors should be taken into consideration: (i) the pH-stat measurements
748 can be impaired by the high concentrations of carbonate salts, recommended for the
749 simulated digestion fluids (see the step 24 critical step It is therefore advised to replace
750 NaHCO₃ salts with NaCl at the same molarity in all electrolyte solutions (oral, gastric and
751 intestinal) when planning to use pH-stat experiments during the intestinal phase of
752 digestion⁴²; (ii) protein hydrolysis also contributes to the pH-stat signal in the intestinal
753 conditions (pH = 7), meaning that this approach is only suitable for studying pancreatic
754 lipolysis when the contribution of proteins is either neglected or subtracted⁴²; (iii) some fatty
755 acids, especially long chain fatty acids, are not ionised at pH7. A back titration at pH 9.0
756 should be performed to measure all the free fatty acids released⁸⁰.

757

758 **Digestion of starch**

759 The structure of starch in a ready-to-eat plant-based food is a function of a multitude of
760 factors. These include its botanical origin, growing conditions, processing, food preparation
761 (mainly cooking), and not least storage. These all have a major impact on salivary and
762 pancreatic amylase catalysed starch digestion. The rate of the loss of starch and the
763 appearance of the digestion product (maltose and maltooligosaccharides) are the most
764 common measures of *in vitro* starch digestibility. To help in the understanding of the
765 physiological effects of starch digestion such as on glycaemic response in humans,
766 measurements should also include (i) the accurate dose and nature of the starch in the food
767 as eaten, (ii) the characterisation of the food matrix (microstructure, macro and micronutrient
768 composition) and (iii) a measure of the degree of starch gelatinisation and/or retrogradation.

769 It is recommended that starch amylolysis is quantified *only* in the intestinal phase by
770 measuring the appearance of the starch digestion products over time, e.g. the concentration
771 of reducing sugars in the liquid phase. Salivary amylase will have a minor impact on starch
772 digestion in the static model were the gastric pH is instantaneously adjusted to 3. After
773 terminating amylase activity by mixing the sample with 4 volumes of ethanol (final conc. 80%
774 w/v) to the sample, for example (see different options in **Table 1**), undigested starch is often
775 separated from digested starch by centrifugation. Analysis of reducing sugar concentration in
776 the supernatant is often done with common colorimetric assays (e.g. using DNS or PAHBAH
777 (4-Hydroxybenzhydrazide) reagents). Another more common method is to treat an aliquot of
778 the amylase digestion products from the 80% w/v ethanol supernatant with buffered
779 amyloglucosidase to convert all amylase digestion products to glucose. Glucose can then be

780 determined through a whole host of methods including colorimetric and enzymatic assays (e.
781 g. GOPOD) or by direct chromatography analysis to name just a few. The data collected can
782 then be used as input variables to a wide variety of simple to complex kinetic-based
783 mathematical models that seek to quantify starch digestion and give predictions on the
784 physiological effects of the food under.

785

786 **Bioaccessibility of phytochemicals**

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

793 (i) *Physiological appropriateness and pitfalls*: Good correlations between bioaccessibility and
794 *in vivo* bioavailability have been obtained for certain phytochemicals, such as
795 carotenoids^{81,82}. However, slight alterations of the digestion parameters suggested by the
796 original INFOGEST method²⁷ can drastically influence bioaccessibility. For instance,
797 increasing the amount of pancreatin and/or bile⁸³ or increasing the speed of shaking/stirring
798 can considerably enhance the bioaccessibility of carotenoids by improving mixing, disrupting
799 oil droplets and increasing micellisation. Thus, careful consideration and the possible further
800 standardisation of these parameters are vital. Additional important factors to consider are
801 light and oxygen, as they can result in the oxidative degradation of carotenoids⁸⁴ and
802 polyphenols⁸⁵ and polymerisation of the latter⁸⁶. It is recommended to flush samples with Ar
803 or N₂ for a few minutes prior to small intestinal digestion to remove oxygen^{82,87} or to use
804 pyrogallol. However, the latter is unsuitable for polyphenolic samples as this is a potential
805 metabolite. Another often neglected factor is the potential effect of brush border membrane
806 enzymes (e.g. lactase-phlorizin-hydrolase) on phytochemical bioaccessibility, especially for
807 polyphenols^{88,89}. The inclusion of brush border membranes (BBM) vesicles in *in vitro*
808 gastrointestinal digestion may increase the physiological relevance of the model, especially
809 for polyphenols⁹⁰. However, BBM are not commercially available nor is there any standard
810 method available to date.

811 (ii) *Bioaccessible phase and pitfalls*: For polyphenols, dialysis is often performed to remove
812 macromolecular-bound compounds⁹¹, but for carotenoids a combination of centrifugation
813 (e.g. 4,000×g for at least 30 minutes) and a filtration step (0.2 µm) has become the most

814 widely used method³¹ to separate the bioaccessible aqueous phase from larger lipid droplets
815 or crystals that would not be taken up by the enterocytes.

816 When combining *in vitro* digestion with cellular assays (e.g. cellular uptake/transport), the
817 toxicity of the bile salts must be accounted for, by including a clean-up step, e.g. solid phase
818 extraction⁹²⁻⁹⁴, or at least the sufficient dilution of samples (e.g. 4× dilution).

819 Finally, it should be considered that the colon may play an important role for the bioavailable
820 fraction. While it is well known that polyphenols can undergo many changes in the colon⁸⁸,
821 and may be absorbable in the colon, little is known for carotenoids, though a significant
822 fraction would be bioaccessible in the colon⁹⁵.

823 **On-going developments and future perspectives for *in vitro* food digestion**

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

829 The INFOGEST method is for adult digestion only. However, there is a strong need to apply
830 this method to specific human population groups, the most important being infants and the
831 elderly, but also adolescents and patients with cystic fibrosis or gastric bypass surgery, to
832 name but a few. A recent review⁹⁶ summarised the existing literature and provides some
833 recommendations on experimental digestion parameters, with the INFOGEST method being
834 the starting point for all other methods.

835 While static methods can be useful, they can be inadequate to simulate the dynamic
836 processes during digestion (e.g. pH gradients, gradual addition of enzymes and gastric fluid,
837 continuous gastric emptying, etc.). As mentioned earlier, various dynamic digestion
838 methods⁶⁻¹⁰ account for some of these factors. A low-cost semi-dynamic method was recently
839 developed⁴⁹ and described in detail⁵⁰, based on equivalent *in vivo* data from the digestion of
840 dairy products. International INFOGEST members are currently working on a consensus
841 method.

842 Enzymes from the small intestinal brush border membranes are recognised as playing a
843 major role in the activation of trypsinogen (enterokinase) and the further degradation of
844 proteins/peptides and carbohydrates as well as improving the bioaccessibility of
845 phytochemicals. The use of brush border enzymes falls into the grey area between
846 bioaccessibility (potentially absorbable) and bioavailability (available at the site of action) and
847 to date, it is not clear how they should be applied. BBM of animal origin have recently been
848 included in static digestion methods^{39,97,98} and can provide physiologically consistent
849 information⁹⁹. However, to date BBM enzymes are not commercially available and are

850 extracted from fresh animal intestines¹⁰⁰ or used as intestinal extracts. There is still a lack of
851 reliable information on the correct enzymatic activities, enzyme substrate ratio and diversity
852 of enzymes, which further limits the use of BBM in standardised digestion methods at the
853 moment. However, given the importance of BBM in the digestive process, further progress in
854 terms of defining digestive parameters is anticipated.

855

856 **TIMING**

857 Step 1, enzyme activity and bile assays: 4 to 5 days for all assays

858 Steps 2 and 3, preparation of solutions: 2 hours

859 Step 4, pH-adjustment experiment: 5 hours (time-lagged before the digestion experiment)

860 Steps 5 and 6, preparation of replicate tests as control: 20 min

861 Steps 7 to 32, whole digestion experiment: 5 to 8 hours, depending on number of food
862 samples and time points, for example: 1 food sample and 5 time points - approximately 5h; 2
863 food samples and 5 time points (2 gastric and 3 intestinal points) - approximately 8h

864 Steps 7 to 16, oral phase: 30 min

865 Steps 17 to 24, gastric phase: 3 hours

866 Steps 25 to 32, intestinal phase: 3 hours

867

868

869

870 **TROUBLESHOOTING**

871 Troubleshooting advice can be found in **Table 4**.

872

873

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986 AB, LE and IR wrote the article. MA, SB, TB, FC, AC, DD, CD, CE, SLF, UL, AdM, AIM, OM,
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 989 GEV, MSJW, WW and IR contributed to the definition of digestion parameters. RP wrote the

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990 online tools. RA and CM prepared the videos. MG, DJMcC and RPS contributed to the
991 manuscript by critical revision of digestion parameters and manuscript.

992

993 **Competing interests**

994 Rabbit lipase from rabbit gastric extract is available commercially from Lipolytech, a start-up
995 company founded by a researcher who had previously worked at the group of F. Carrière
996 (co-author of this manuscript). The laboratory of F. Carrière, a joint unit of Centre National de
997 la Recherche Scientifique (CNRS) and Aix Marseille University (AMU), has a research
998 collaboration contract with Lipolytech (CNRS reference number: 163451; signed on June
999 30th, 2017). However, the co-author F. Carrière does not financially benefit from this contract
1000 and, as an employee of CNRS and civil servant of the French state, is not allowed to have
1001 private consulting activity for a company contracting with his own laboratory.

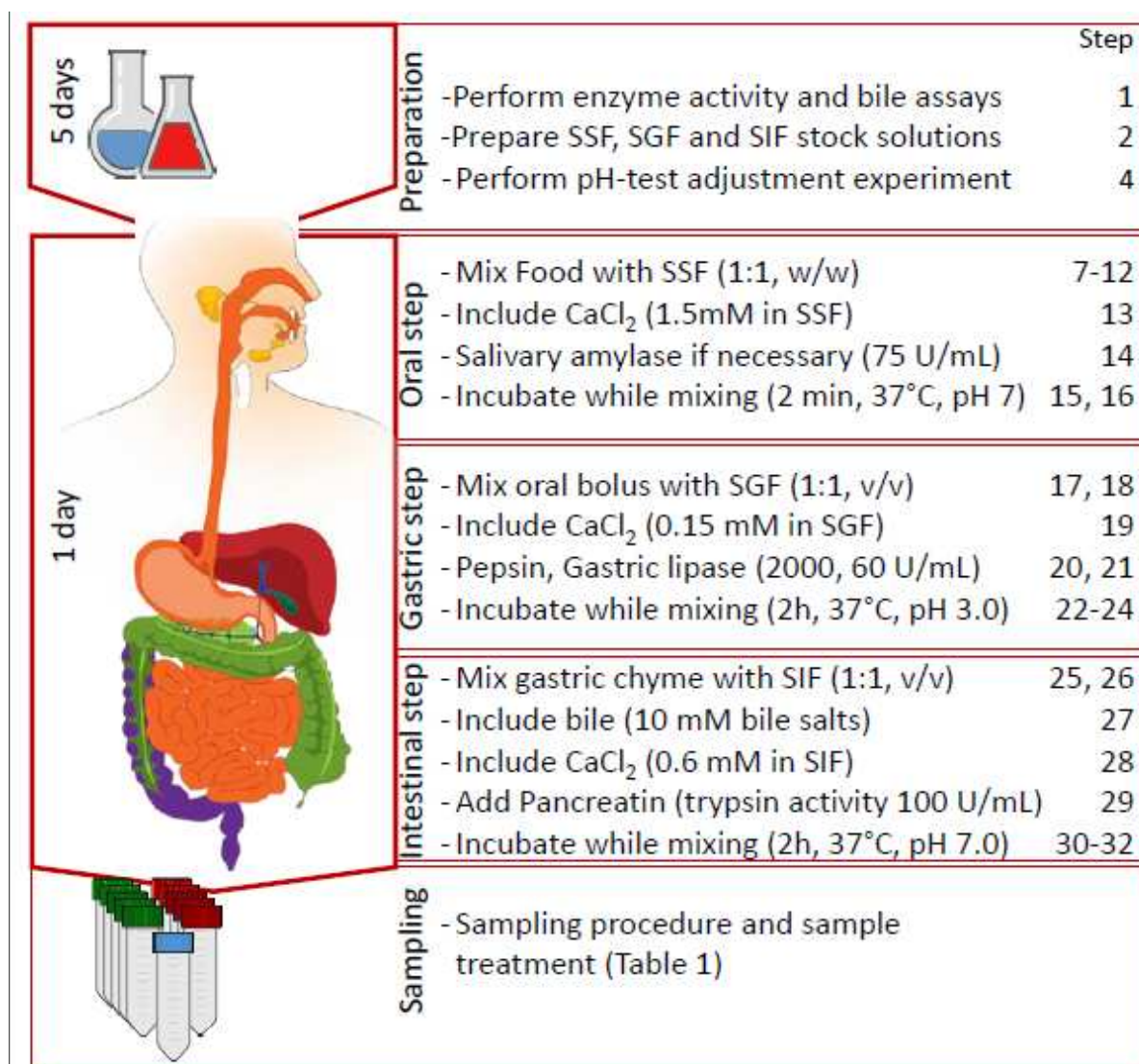
1002

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1007 **Figures**

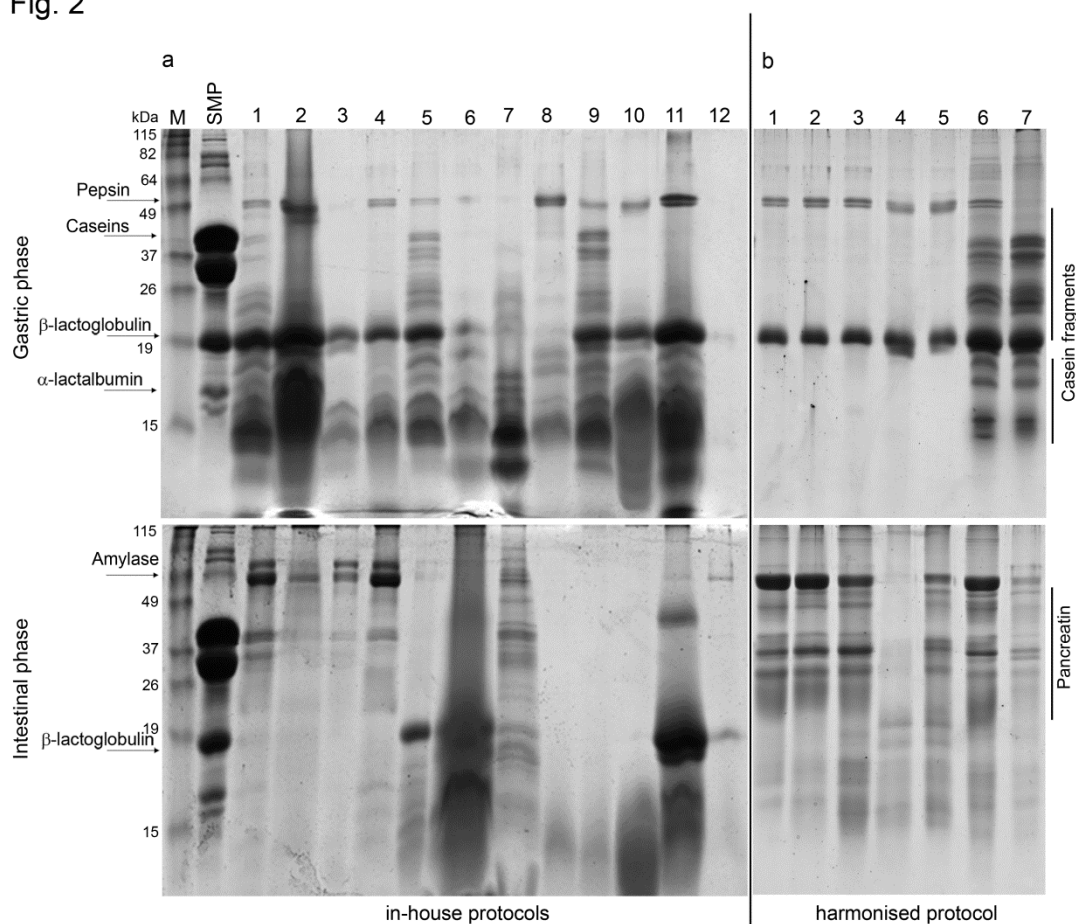
1008

1009 **Figure 1: Flow diagram of the INFOGEST 2.0 digestion method**

1010 Timing and flow diagram of the INFOGEST2.0 *in vitro* digestion method for food. SSF, SGF
 1011 and SIF stand for simulated salivary, gastric and intestinal fluid, respectively. Expected time
 1012 frame (left) and steps (right) corresponding to the step numbers in the Procedure section.

1013

Fig. 2

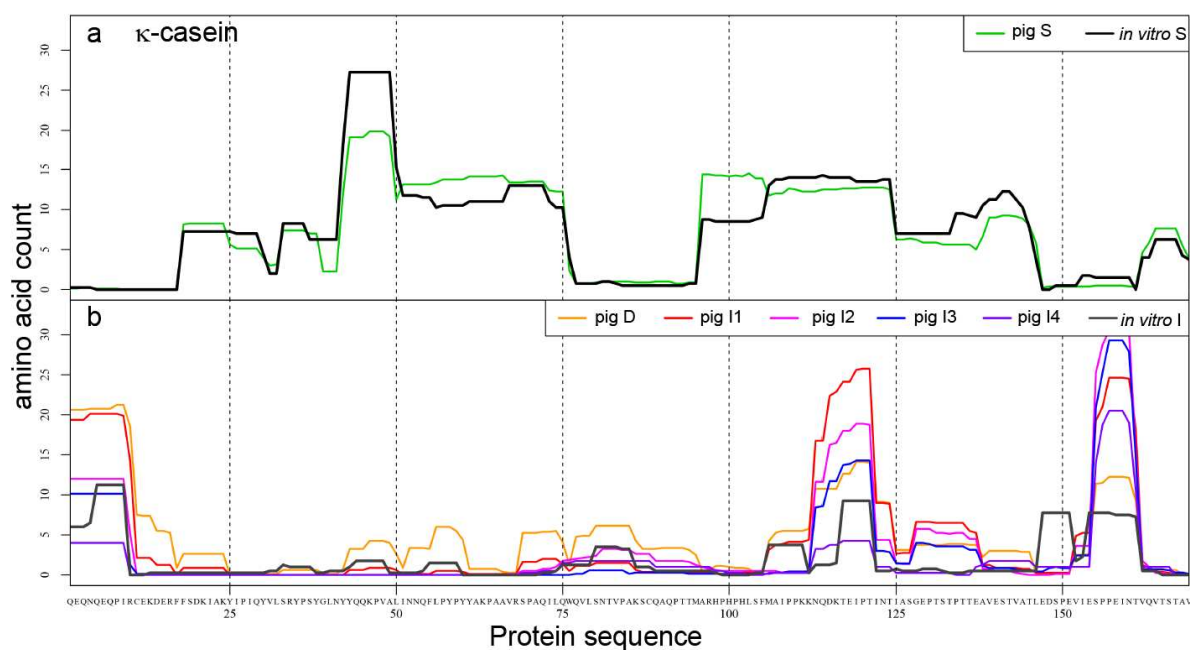


1014

1015 **Figure 2: Protein separation by gel electrophoresis of *in vitro* digested skim milk**
 1016 **powder (SMP)**

1017 Comparing results from in-house protocols performed in individual laboratories 1-12 (a), with
 1018 the harmonised protocol, performed in 7 different laboratories (b) after the gastric and the
 1019 intestinal phase of *in vitro* digestion. Undigested skim milk powder (SMP) is shown as a
 1020 control, specific protein bands are highlighted with arrows: casein fragments, partly
 1021 hydrolysed casein; pancreatin, bands originating from pancreatin. Figure adapted from Egger
 1022 et al.²⁸

1023

1024
1025

1026 **Figure 3: Comparison of *in vitro* digested skim milk powder (SMP) peptide patterns of**
1027 **κ -casein with *in vivo* (pig) digestion**

1028 (a) Gastric *in vitro* digestion samples (*in vitro* S) were compared to gastric pig samples (pig
1029 S, n = 8, as previously published by Egger et al.²⁹, approval number 2015_04_FR;26115). (b)
1030 Intestinal *in vitro* digestion samples were compared to pig sampling sections collected along
1031 the digestive tube from duodenum (D), proximal- (I1), median- (I2), distal jejunum (I3), and to
1032 ileum (I4)²⁹. The x-axis shows the amino acid (AA) sequence of κ -casein and the y-axis
1033 shows the number of times each amino acid was identified within a κ -casein peptide of ≥ 5
1034 AA in length.

1035
1036

1037

1038 **Related links**1039 **Key references using this protocol**1040 1. Egger, L. et al. *Food Res. Int.* **88**, 217–225 (2016):1041 <https://doi.org/10.1016/j.foodres.2015.12.006>1042 2. Egger, L. et al. *Food Res. Int.* **102**, 567–574 (2017):1043 <https://doi.org/10.1016/j.foodres.2017.09.047>1044 3. Sanchón, J. et al. *Food Chem.* 239, 486–494 (2018):1045 <https://doi.org/10.1016/j.foodchem.2017.06.134>

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Box 1 | Enzyme activity assays¹

PEPSIN ACTIVITY ASSAY

Principle: Haemoglobin + H₂O $\xrightarrow{\text{Pepsin}}$ TCA soluble tyrosine peptides

Unit definition: One unit produces a ΔA_{280} of 0.001 per minute at pH 2.0 and 37°C, measured as trichloroacetic acid (TCA)-soluble products

Substrate: 2 % w/v haemoglobin 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 10mM HCl at concentrations ranging 5-30 $\mu\text{g}/\text{mL}$

Mix 500 μL of haemoglobin with 100 μL of each pepsin solution (5-30 $\mu\text{g}/\text{mL}$) and incubate for 10 min at 37°C. To stop the reaction, add 1 mL of 5% w/v TCA. Centrifuge at 6,000 $\times g$ during 30 min and read the absorbance at 280 nm in quartz cuvettes.

LIPASE ACTIVITY ASSAY

Principle: Tributyrin + H₂O $\xrightarrow{\text{Lipase}}$ butyric acid + *sn*-2 monobutyrin

Unit definition: One unit releases 1 μmol butyric acid per minute at 37°C at the pH of the assay

Substrate: Tributyrin purity $\geq 99\%$

Enzyme solution: Lipase 1 mg/mL in H₂O

Assay solution for gastric lipase: 2mM Sodium taurodeoxycholate, 150 mM NaCl, 1 μM BSA

Assay solution for pancreatic lipase: 4mM Sodium taurodeoxycholate, 150 mM NaCl, 1.4mM $\mu\text{M}\text{CaCl}_2$

In a pH-stat at 37°C, mix 14.5 mL of assay solution with 0.5 mL of tributyrin, 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) or pH 5.5 (rabbit gastric lipase) or pH 8 (pancreatic lipase) for 5 min.

TRYPsin ACTIVITY ASSAY

Principle: TAME + H₂O $\xrightarrow{\text{Trypsin}}$ p-Toluene-Sulfonyl-L-Arginine + Methanol

Unit definition: One unit hydrolyses 1 μmol p-Toluene-Sulfonyl-L-arginine methyl ester (TAME) per minute at pH 8.1 and 25°C

Substrate: 10 mM TAME in H₂O

Enzyme solution: Trypsin in 1 mM HCl at concentrations ranging 10-20 $\mu\text{g}/\text{mL}$

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 + H₂O $\xrightarrow{\alpha\text{-Amylase}}$ Reducing Groups (e.g. Maltose)

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 % w/v Soluble potato starch in 20mM sodium phosphate buffer with 6.7 mM NaCl, adjusted to pH 6.9

Enzyme solution: 1 mg/mL Amylase in H₂O

Incubate 1 mL of substrate at 20°C, add the enzyme solution (0.5-1 mL, with estimated activity of 1 unit/mL) and incubate at 20°C for 3 min. Stop reaction with colour reagent (96 mM 3,5-dinitrosalicylic acid, 5.3 M sodium potassium tartrate). Complete enzyme volume with H₂O to 1 mL, cap the tube and boil it for 15 min. Add 9 mL of H₂O and read absorbance at 540 nm. Calculate the activity against a maltose standard curve.

¹Detailed assays for all enzymes in Supplementary Information

1395 **TABLES**

1396

1397 **Table 1:** Examples for the preservation and treatment of 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, drying).	74,101
Breakdown of nutrients: Proteins	Protein hydrolysis or resistant protein analysis	Stop gastric digestion (2 options)	Raise the pH to 7 for partial inactivation of pepsin; pH 8 for complete inactivation.	Addition of 1 M NaHCO ₃ or 1N NaOH	28
			Addition of pepstatin A for pepsin inhibition.	Add Pepstatin A at 0.5-1.0 µM final concentration.	102
		Stop intestinal digestion (3 options)	Addition of Pefabloc [®] SC (4-(2-aminoethyl)-benzolsulfonylfluorid-hydrochloride) for serine protease (trypsin and	Add 50 µl of Pefabloc (0.1 M) in water per mL of intestinal digesta. (5 mM final concentration).	28



			chymotrypsin) inhibition.		
			Addition of Bowman-Birk inhibitor (BBI) from soybean with ability to inhibit both trypsin and chymotrypsin.	Add 100µl of a BBI solution 0.05 g/L in water per mL of intestinal digesta.	¹⁰³
			Heat shock treatment	Sample treatment: 100°C, 5 min, but detrimental to food structure, especially protein and carbohydrate structures	⁴¹
Breakdown of nutrients: Lipids	Lipid hydrolysis	Stop lipase activity in the gastric phase (2 options)	Addition of Orlistat (tetrahydrolipstatin)	Add 10 µL/mL of a 100 mM Orlistat solution in ethanol (1 mM final concentration)	¹⁰⁴
			Raise the pH to 8		⁵⁹
	Lipid hydrolysis	Stop lipase activity in the intestinal phase (2 options)	Addition of lipase inhibitor (4-bromophenylboronic acid)	Add 5 µL/mL of a 1 M solution of 4-bromophenylboronic acid in methanol to 1 mL of digesta (5 mM final concentration).	¹⁰⁵
			Addition of methanol:chloroform	Addition of methanol: chloroform mixture used for Folch extraction	⁷⁶



Breakdown of nutrients: Carbohydrates	Starch hydrolysis	Stop amylase activity (4 options)	Addition of NaCO ₃	Dilute digesta in 2 volumes of 0.3 M NaCO ₃	¹⁰⁶
			Heat shock treatment	100°C for 5 min	
			TCA precipitation	Add 700 µL of 100% TCA to 5 mL digesta	¹⁰⁷
			Ethanol	Add sample to equal volume of ethanol	¹⁰⁸
Breakdown of oxygen sensitive phytochemicals	Degradation of polyphenols and carotenoids	Prevent contact with Oxygen	Flushing with Ar or N ₂ , pyrogallol addition (carotenoids) prior to small intestinal digestion	Flush sample 1 minute with Ar or N ₂	⁸⁷
Bioaccessibility	Bioaccessibility of digested nutrients	Stop pancreatic activities (see above Stop intestinal digestion)	Use of inhibitors e.g. Pefabloc. Test whether the use of enzyme inhibitors affect the results of the experiment.	See above <i>Stop intestinal digestion</i>	²⁸
			Use of dialysis membranes/ centrifugation tubes having		¹⁰⁹



			cut-off of 3 to 10kDa.		
			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 level (285-300 mOsm/kg H ₂ O).	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 bound to macromolecules or complexed form	Ultracentrifugation and filtration with certain cut-off filters (e.g. 0.2 µm)	110
			Cleavage of glucosides and esters	Addition of brush border vesicles	90
Probiotic survival	To determine the survival rates of probiotic bacteria to 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	Stop enzymatic activities	By heat shock	Heat treatment: 100 °C for 5 min but detrimental to food structure, especially protein and carbohydrate structures	114
			Immediate storage in ice before batch culture fermentation		115

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1400 **Table 2:** Volumes of electrolyte stock solutions of digestion fluids for a volume of 400 mL
 1401 diluted with water (1.25× concentrations).

			SSF (pH 7)		SGF (pH 3)		SIF (pH 7)	
Salt solution added	Stock concentrations		mL of Stock added to prepare 0.4 L (1.25x)	Final salt conc. in SSF	mL of Stock added to prepare 0.4 L (1.25x)	Final salt conc. in SGF	mL of Stock added to prepare 0.4 L (1.25x)	Final salt conc. 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 ₂ PO ₄	68	0.5	3.7	3.7	0.9	0.9	0.8	0.8
NaHCO ₃ *	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 ₂ O) ₆	30.5	0.15	0.5	0.15	0.4	0.12	1.1	0.33
(NH ₄) ₂ CO 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

Addition before use (volumes are indicated in **Table 3**, typical experiment of 5 mL of SSF):

CaCl ₂ (H ₂ O) ₂	44.1	0.3	0.025	1.5	0.005	0.15	0.04	0.6
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1402

1403

1404 **Table 3:** 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	8		8**	
CaCl ₂ (0.3 M) (mL)	0.025	0.005		0.04	
Enzymes	Salivary amylase	Pepsin	Gastric [#] Lipase	Trypsin in pancreatin	Bile salts
Enzyme activity (U/mL) or bile conc. (mM) in total digesta (final volume in mL at each digestion phase, see row below)	75 U/mL	2,000 U/mL	60 U/mL	100 U/mL	10 mM
Specific activity* (U/mg), Conc. (bile) mmole/g	100 U/mg	3,000 U/mg	25 U/mg	6 U/mg	0.667mmole/g
Conc. 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**	3**
H ₂ O (mL)	0.225	0.448		3.16	
HCl (5M) for pH adj. (mL)	-	0.4		-	
NaOH (5M) for pH adj. (mL)	-	-		0.8	
Final volume (mL)	10	20		40	
Remarks	- Use salivary amylase only for food containing starch - 1:1 (w/w) dilution with SSF should result in a paste-like consistency, add more water if necessary - Some foods may not be digested as expected due to high substrate to enzyme		#Rabbit gastric extract (RGE) contains gastric lipase and pepsin, i.e. the pepsin content needs to be accounted for in the total pepsin activity		

	ratio in the static digestion method and may need to be further diluted with water prior the oral phase, see Table 4 Troubleshooting		
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1405 *Specific enzyme activity or bile concentration: measured for each batch of enzymes or bile
 1406 extract according to standard assays (Supplemental Materials from Minekus et al. ²⁷), the
 1407 enzyme assays for gastric lipase and pepsin are described in the supplemental materials of
 1408 this manuscript

1409 **Total volume of SIF (1.25×): 16 mL including pancreatin and bile, both of which are
 1410 dissolved in SIF

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1412 **Table 4:** Troubleshooting

Procedure step (number)	Problem	Possible reason	Solution
Enzyme activity (1)	Pepsin activity results in lower activity units than specified	Enzyme activity measurement	Follow the standardised procedure using haemoglobin as substrate. Dissolve pepsin in 10 mM Tris, 150 mM NaCl, pH 6.5
Enzyme activity (1)	Amylase activity very low	DNS (3,5-dinitrosalicylic acid) does not react with product	DNS solution needs to be freshly prepared
Gastric phase (24)	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 <i>in vivo</i> digestion of cheese ²⁹ at the end of the gastric phase, the cheese has to be diluted with water at 1:2 (w/w) prior to the oral phase.
Gastric phase (24)	pH difficult to adjust during gastric digestion	Quick pH drift during gastric phase	Run a pH-test adjustment experiment with the same food to determine volumes and times for HCl addition
Gastric/intestinal	Difficulties taking a	Presence of different	Use individual sample tube



phase (24, 32)	homogeneous sample during digestion	phases (lipids, water, solids)	for each time point rather than withdrawal of samples from the digestion vessel.
Gastric/intestinal phase (24, 32)	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.
Intestinal phase (32)	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.
Intestinal phase (32)	Presence of insoluble material at the end of the intestinal phase	Non-digestible material	Use individual sample tube for each time point
Intestinal phase (32)	Poor lipid digestion at the end of digestion	Food contains 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.
Intestinal phase (32)	Starch digestion is too low	Incorrect method 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 activity of amylase.



Intestinal phase (32)	Starch digestion product concentration does not change over time	Starch digestion is finished before samples are collected.	Take more samples at earlier time points. Consider using less amylase to slow the reaction down. Check feasibility of results by expressing findings as % of starch digested.
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1416 Supplementary information

1417 The Supplementary Information (SI) consists of:

- 1418 1. Supplementary Figure 1
- 1419 2. Supplementary Methods: protocols of enzyme assays
- 1420 3. Supplementary videos
- 1421 4. Supplementary spreadsheets in Excel format

1422

1423 Supplementary Figure 1: Oral bolus hydration *in vivo*

1424 Bolus hydration (g of saliva / g of foods) *in vivo* just before swallowing, for various foods
1425 based on published data¹¹⁶⁻¹²³

1426

1427 Supplementary Methods

1428 Protocols of enzyme activity assays (summarised in **Box 1**) for α -amylase (EC 3.2.1.1),
1429 pepsin (EC 3.4.23.1), gastric lipase (EC 3.1.1.3), trypsin (EC 3.4.21.4), chymotrypsin (EC
1430 3.4.21.1), pancreatic lipase (EC 3.1.1.3) and bile salts (according to supplier's protocol)

1431

1432 Supplementary Videos:

1433 Supplementary Video 1

1434 INFOGEST 2.0 digestion procedure part 1

1435 Supplementary Video 2

1436 INFOGEST 2.0 digestion procedure part 2

1437 Supplementary Video 3

1438 Amylase activity assay

1439 Supplementary Video 4

1440 Pepsin activity assay

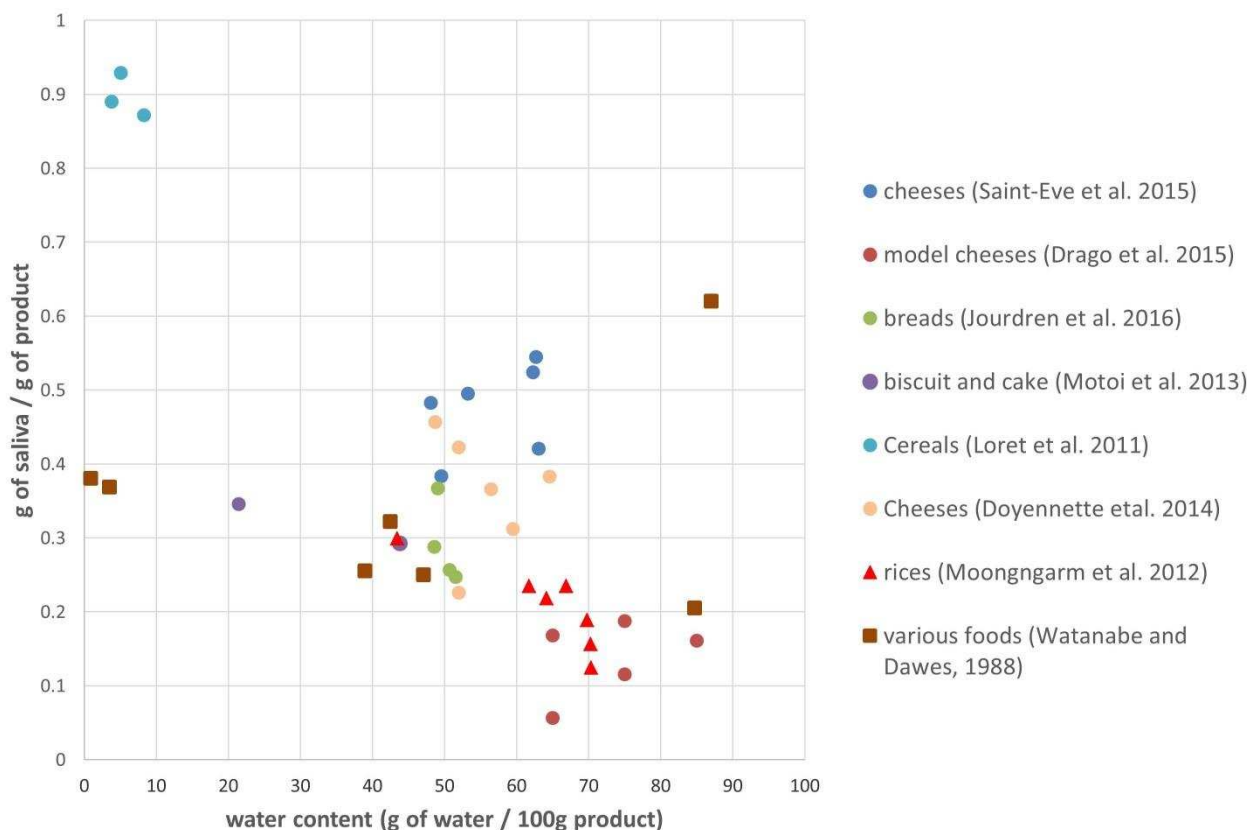
1441 Supplementary Video 5

1442 Lipase activity assay (both gastric and pancreatic)

- 1443 Supplementary Video 6
- 1444 Trypsin activity assay
- 1445 Supplementary Video 7
- 1446 Chymotrypsin activity assay
- 1447
- 1448 **Supplementary spreadsheets**
- 1449 Supplementary spreadsheets 1
- 1450 Excel spreadsheets to calculate the enzyme activities of all digestive
- 1451 enzymes.
- 1452 Supplementary spreadsheets 2
- 1453 Excel spreadsheets to calculate all volumes of simulated digestive fluids,
- 1454 enzyme and bile solutions based on the initial amount of digested food.
- 1455 In addition, the corresponding online spreadsheets and videos of the enzyme assays and
- 1456 digestion procedures are available here: www.proteomics.ch/IVD and on the INFOGEST
- 1457 website <https://www.cost-infogest.eu/>.
- 1458
- 1459



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Supplementary Figure 1

Oral bolus hydration *in vivo*Bolus hydration (g of saliva / g of foods) *in vivo* just before swallowing, for various foods based on published data¹⁻⁸

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1461

1462 **Supplementary Methods - Enzyme assays**

1463 Enzyme and bile assays are adapted from Minekus et al.¹, namely: α -amylase (EC 3.2.1.1),
 1464 pepsin (EC 3.4.23.1), trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), pancreatic lipase
 1465 (EC 3.1.13) and bile salts (according to supplier's protocol). The assay for gastric lipase has
 1466 been adapted from Carrière et al.² and merged with that for pancreatic lipase.

1467

1468 **α -Amylase Activity Assay (EC 3.2.1.1)**

1469 **References:** according to Bernfeld³

1470 **Method:** Spectrophotometric Stop Reaction

1471 **Principle:**

1472 Starch + H₂O $\xrightarrow{\alpha\text{-Amylase}}$ Reducing Groups (Maltose)

1473 **Unit definition:** One unit releases 1.0 mg of maltose from (potato) starch in 3 minutes at pH
 1474 6.9 and 20 °C.

1475 **Conditions:** T = 20 °C, pH = 6.9, A_{540nm}, light path = 1 cm

1476 **Procedure**

1477 Preparation of reagents

1478 **Substrate:** soluble potato starch (1.0% w/v)

1479 Preparation of substrate solution:

1480 Prepare 100 mL of a 20 mM sodium phosphate buffer containing 6.7 mM NaCl. Adjust the
 1481 pH to 6.9 at 20 °C with 1 M NaOH. Dissolve 0.25 g soluble potato starch (ref S2630 Sigma-
 1482 Aldrich) in 20 mL of the sodium phosphate buffer, pH 6.9. Heat the covered beaker while
 483 stirring and maintain the solution just below boiling temperature for 15 minutes. Cool to room
 484 temperature and complete the starch solution to the appropriate volume (25 mL) by addition
 485 of H₂O.

- 1486 **Standard Curve:** Prepare 10 mL of 0.2 % w/v maltose standard (M5885 Sigma-Aldrich).
- 1487 **Enzyme:** Shortly before the assay, prepare an enzyme solution of an estimated activity of 1
1488 unit/mL of α -amylase in purified H₂O
- 1489 **Assay solution:** Colour reagent solution 3,5-dinitrosalicylic acid (DNS)
- 1490 Prepare a 5.3 M sodium potassium tartrate solution in 2 M NaOH by dissolving 0.8 g NaOH
1491 in 10 mL H₂O and heating the solution at a temperature ranging between 50 to 70 °C. Add
1492 12.0 g of sodium potassium tartrate tetrahydrate (in 8.0 mL of warm 2 M NaOH solution,
1493 maintain the temperature constant while stirring to dissolve the tartrate but do not boil it.
- 1494 Prepare a 96 mM DNS solution by dissolving 438 mg of DNS in 20 mL of H₂O. Heat the
1495 solution at a temperature between 50 to 70 °C. Maintain at this temperature while constant
1496 stirring to dissolve DNS but do not boil it.
- 1497 Heat 12 mL of purified water to 60 °C and add slowly 8 mL of the 5.3 M the sodium potassium
1498 tartrate solution. Add 20 mL of the 96 mM 3,5-dinitrosalicylic acid solution and stir until
1499 complete dissolution. The solution can be stored in an amber flask at room temperature for
1500 one month.
- 1501 **Assay:**
- 1502 Set the spectrophotometer at 540 nm and 20 °C. Set a bench top shaking incubator fitted with
1503 a sample holder at 20 °C, a heating bath or block at 100 °C to stop the reaction, and an ice-
1504 bath to cool the sample.
- 1505 **Test:** Pipette 1 mL of substrate solution (potato starch) into cap covered tubes (15 mL), mix
1506 and incubate at 20 °C for 5 min to achieve temperature. Add 0.5 – 1 mL of enzyme solution
1507 (according to the scheme below), mix and incubate at 20 °C for exactly 3 minutes.
1508 Immediately thereafter, stop the reaction by addition of 1 mL of DNS solution. Complete the
1509 enzyme volume added to 1 mL, cap the tube, place it at 100 °C (heating bath or block) and
1510 boil it for exactly 15 minutes. Cool the tube for a few minutes on ice and add 9 mL of H₂O.
1511 Mix the reaction and pipette 3 mL in a cuvette and record the absorbance at 540 nm.
- 1512 **Blank:** For blank tests, follow the same procedure but no enzyme is added before the 3
1513 minutes incubation time.
- 514 **Pipetting scheme for three different enzyme concentrations:**

Volumes in mL	1 st enzyme	2 nd enzyme	3 rd enzyme	Blank
---------------	------------------------	------------------------	------------------------	-------

	concentration	concentration	concentration	
Substrate (potato starch)	1.00	1.00	1.00	1.00
Enzyme solution	0.50	0.70	1.00	-
DNS	1.00	1.00	1.00	1.00
2 nd addition of enzyme	0.50	0.30	-	1.00
H ₂ O	9.00	9.00	9.00	9.00

1515

1516 **Standard Curve with maltose:**1517 Dilute the maltose solution (0.2% w/v) according to the scheme in H₂O

Volumes in (mL)	D1	D2	D3	D4	D5	D6	D7	Std. Blank
Maltose solution	0.05	0.20	0.40	0.60	0.80	1.00	2.00	-
H ₂ O	1.95	1.80	1.60	1.40	1.20	1.00	-	2.00

1518

1519 1mL DNS reagent solution is added to each maltose standard, thereafter the tubes are boiled
1520 for 15 minutes, cooled on ice to room temperature and 9mL of H₂O are added.

1521 **Calculations**

1522 Standard Curve:

$$\Delta A_{540} \text{Standard} = \Delta A_{540} \text{Standard} - \Delta A_{540} \text{Std. Blank}$$

1523 Plot the $\Delta A_{540 \text{nm}}$ of the Standards versus the quantity of maltose [mg] and establish a linear
1524 regression:

$$\Delta A_{540} \text{Standard} = a \times [\text{maltose}] - b$$

1525 Enzyme activity:

$$\Delta A_{540} \text{Sample} = \Delta A_{540} \text{Test} - \Delta A_{540} \text{Test Blank}$$

$$\frac{\text{Units}}{\text{mg powder}} = \frac{[A_{540} \text{ Test} - A_{540} \text{ Test Blank}] - b}{(a \times X)}$$

1526

1527 a: slope of the linear regression for standards $\Delta A_{540 \text{nm}}$ vs the quantity of maltose (mg).1528 b: intercept of the linear regression for standards $\Delta A_{540 \text{nm}}$ vs the quantity of maltose (mg).

1529 X: quantity of amylase powder (mg) added before stopping the reaction.

1530

1531 **Pepsin Activity Assay (EC 3.4.23.1)**

1532 **References:** adapted from Anson *et al.* ^{4,5}

1533 **Method:** Spectrophotometric Stop Reaction

1534 **Principle:**

1535 Haemoglobin + H₂O $\xrightarrow{\text{pepsin}}$ TCA soluble tyrosine containing peptides

1536 **Unit definition:** One unit will produce a ΔA_{280} of 0.001 per minute at pH 2.0 and 37°C,
1537 measured as TCA-soluble products. These units are often referred to “Sigma” or “Anson”
1538 pepsin units.

1539 **Conditions:** T = 37°C, pH = 2.0, $A_{280\text{nm}}$, light path = 1 cm

1540 **Procedure:**

1541 Preparation of reagents

1542 **Substrate:** Prepare a haemoglobin solution by dispersing 0.5 g haemoglobin (bovine blood
1543 haemoglobin, ref H2500 Sigma-Aldrich) in 20 mL purified water, adjust to pH 2 with 300 mM
1544 HCl and complete the volume to 25 ml to obtain a solution at 2% w/v haemoglobin at pH 2.

1545 **Enzyme:** Prepare a stock solution of 1 mg/mL pepsin (porcine pepsin, ref. P6887 Sigma-
1546 Aldrich) in 10 mM Tris buffer, 150 mM NaCl at pH 6.5. The stock solution has to be stored on
1547 ice or refrigerated at 4°C. Just before the assay, a range of 5 to 10 concentrations of pepsin
1548 in 10 mM HCl has to be prepared. For instance, dilute the pepsin stock solution to prepare
1549 the following enzyme assay solutions: 5, 10, 15, 20, 25, 30 µg/mL.

1550 **Assay:**

1551 Set the spectrophotometer at 280 nm and 20°C. Set a bench top shaking incubator fitted with
1552 a sample holder at 37°C.

553 **Test:** Pipette 500 µL of haemoglobin solution into 2 mL Eppendorf tubes and incubate in a
554 shaking incubator at 37°C for 3-4 minutes to reach the assay temperature.

555 Add 100 µL of pepsin assay solutions for each concentration and incubate them for 10
556 minutes exactly. To stop the reaction, 1 mL of 5% w/v TCA (Trichloroacetic Acid) is added in

1557 each tube. In order to get a clear soluble phase available for absorbance measurement,
 1558 centrifuge the Eppendorf tubes at $6,000 \times g$ for 30 minutes to precipitate remaining
 1559 haemoglobin; remove the pellet.

1560 Place the soluble phase into quartz cuvettes and read the absorbance at 280 nm (A_{280} Test).

1561 **Blank:** For blank tests, the same procedure is followed but the pepsin is added after the
 1562 addition of TCA, which stops the reaction. The blank absorbance is noted A_{280} Blank.

1563 Because, the absorbance is a function of the pepsin concentration, a linear curve has to be
 1564 obtained. If no linear part is found, it can be due to a large amount of enzyme, and therefore
 1565 it is necessary to use more dilute enzyme assay solutions.

1566 **Calculations:**

$$\text{Units/mg} = \frac{[A_{280} \text{ Test} - A_{280} \text{ Blank}] \times 1,000}{(\Delta t \times X \times 0.001)}$$

1567 Δt : duration of the reaction, i.e. 10 minutes

1568 X = amount of pepsin powder (μg) in 1mL in the assay solution (i.e., 5, 10, 15, 20, 25, 30 μg)

1569 1,000 = dilution factor to convert μg to mg

1570 0.001 = ΔA_{280} per unit of pepsin

1571 Check that the activity obtained is the same for each tested concentration of pepsin, to make
 1572 sure that you are in the linear part of the pepsin concentration curve.

1573

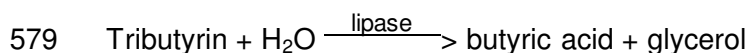
1574

1575 **Gastric and pancreatic lipase activity assay (EC 3.1.1.3)**

1576 **References:** Gargouri et al.⁶; Moreau et al.⁷; Carrière et al.^{2,8}, Erlanson and Borgström⁹

1577 **Method:** pH titration

1578 **Principle:**



580 The gastric and pancreatic lipase activity assay are conducted by pH titration and tributyrin
 581 as substrate. The free fatty acids released by the lipases are titrated at a constant pH by

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1582 sodium hydroxide (0.02 - 0.1 N) during at least 5 min. The concentration of NaOH is adjusted
1583 to allow the titrator to keep the pH as constant as possible during the titration.

1584 **Unit definition:** One unit releases 1 μmol of butyric acid per minute at 37°C at the pH of the
1585 assay: 6.0 for Human Gastric Lipase, 5.5 for Rabbit Gastric Lipase and 8 for Pancreatic
1586 Lipase. These units are often referred to International Units. Both, purified Human and Rabbit
1587 Gastric Lipases show a specific activity of approx. 1,200 U/mg protein on tributyrin^{7,10} and
1588 human Pancreatic Lipase has a specific activity of ca. 8,000 U/mg of protein on tributyrin²

1589 **Procedure:**

1590 Preparation of reagents:

1591 **Assay solution:** Prepare 200 mL of the following aqueous solutions which vary for gastric or
1592 pancreatic lipase:

1593

	Gastric Lipase		Pancreatic Lipase	
	Concentration [mg/L]	Corresponding weight [mg] for 200 mL	Concentration [mg/L]	Corresponding weight [mg] for 200 mL
NaCl	9,000 (150 mM)	1,800	9,000 (150 mM)	1,800
Sodium tauro-deoxycholate	1,000 (2 mM)	200	2,000 (4 mM)	400
BSA	100 (1 μM)	20	-	-
CaCl ₂	-	-	200	40
Tris-(hydroxymethyl)-aminomethane	-	-	36	7.20
pH	adjust with HCl (0.1M) at pH 5.5 (RGE) or pH 6 (HGL)		adjust with HCl (0.1 M) at pH 8	

1594

1595 **Titration Solution:** Prepare a solution of 0.1 N sodium hydroxide (NaOH) by dissolving 2 g
1596 NaOH in 500 mL of purified water. It is recommended to perform a back titration using 0.1 N
1597 HCl to confirm the precise molarity of the NaOH titration solution. Alternatively, commercial
1598 NaOH stock solutions can be used.



1599 **Enzyme:** Prepare a 1 mg/mL solution by dissolving 5 mg of lipase (e.g. rabbit gastric extract
 1600 powder, RGE25-100MG Lipolytech, France) in 5 mL of purified water. Store on ice. Perform
 1601 the assay with at least 2 different amounts of the enzyme solution, i.e. 50 and 100 μ L, at 1
 1602 mg/mL.

1603 **Substrate:** Use tributyrin of purity grade ($\geq 99\%$; ref T8626 Sigma-Aldrich)

1604 **Assay:**

1605 Set a thermo-regulated pH-stat device to 37°C fitted with a jacketed and capped reaction
 1606 vessel (20-70 mL) and mechanical stirrer, preferentially with a 3-pale propeller.

1607 Pour 14.5 mL of the assay solution and 0.5 mL of tributyrin into the titration vessel. Make
 1608 sure the volume of the assay is enough to ensure adequate pH-measurement, i.e., the pH
 1609 electrode is correctly immersed. By switching on the mechanical stirring of the apparatus,
 1610 tributyrin will get dispersed to form a fine oil-in-water emulsion after 3-5 min at 37°C.

1611
 1612 Switch on the automated delivery of titrant solution (0.1 N NaOH) to monitor the pH and
 1613 adjust it at the selected pH end-point of titration, i.e., pH 5.5 for rabbit gastric lipase, pH 6.0
 1614 for human gastric lipase or pH 8.0 for pancreatic lipase. Add 50 or 100 μ L of the enzyme
 1615 solution. Monitor the rate of titrant solution (NaOH) which is required to maintain the pH
 1616 constant at 37°C due to the release of free fatty acids. These conditions allow measuring
 1617 linear kinetics of free fatty release for at least 5 minutes.

1618 If pancreatic lipase does not contain colipase, add colipase at a molar excess (ratio of 2:1
 1619 colipase:lipase) before adding the enzyme.

1620 **Calculations:**

$$\frac{\text{Units}}{\text{mg powder}} = \frac{R(\text{NaOH}) \times 1000}{v \times [E]} \times F$$

1621
 1622 R(NaOH): Rate of NaOH delivery in μ mol NaOH per minute, i.e., μ mol free fatty acid titrated
 1623 per minute

1624 v: volume [μ L] of enzyme solution added in the pH-stat vessel

1625 [E]: concentration of the enzyme solution [mg powder/mL]

1626 F: correction factor to take into account the partial ionization (and titration) of fatty acids at
 1627 the pH of the assay. Only for the titration of butyric acid at pH 5.5, a correction factor F of
 1628 1.12 has to be applied.

1629 Check that the activity obtained is the same for each tested concentration of lipase, to make
 1630 sure that you are in the linear part of the enzyme concentration curve.

1631

1632 **Trypsin Activity Assay (EC 3.4.21.4)**

1633 **References:** adapted from Hummel¹¹ and following recommendations from the Worthington
 1634 laboratory

1635 **Method:** Kinetic spectrophotometric rate determination

1636 **Principle:**

1637 $\text{TAME} + \text{H}_2\text{O} \xrightarrow{\text{trypsin}} \text{p-Toluene-Sulfonyl-L-Arginine} + \text{Methanol}$

1638 **Unit definition:** One unit hydrolyses 1 μmol of p-toluene-sulfonyl-L-arginine methyl ester
 1639 (TAME) per minute at 25°C and pH 8.1

1640 Unit conversion: 1 TAME Unit = 19.2 USP/NF Units = 57.5 BAEE Units

1641 **Conditions:** T = 25°C, pH = 8.1, $A_{247\text{-nm}}$, Light path = 1 cm

1642 Preparation of reagents

1643 **Substrate:** TAME (ref. T4626 Sigma-Aldrich) at 10 mM is prepared and dissolved in purified
 1644 water.

1645 **Enzyme:** Prepare at least 2 concentrations of trypsin (porcine trypsin, ref. T0303 Sigma-
 1646 Aldrich) ranging between 10-20 $\mu\text{g/mL}$ in 1 mM HCl.

1647 **Assay solution:** 46 mM Tris/HCl buffer, containing 11.5 mM CaCl_2 at pH at 8.1 and 25°C.

1648 **Assay:**

1649 Set the spectrophotometer at 247 nm and 25°C.

650 **Test:** Pipette 2.6 mL of assay solution and 0.3 mL of the substrate (10 mM TAME) into
 651 quartz cuvettes, mix by inversion and incubate in spectrophotometer at 25°C for 3-4 minutes
 652 to achieve the temperature.



1653 Add 100 µl of each concentration of trypsin solutions and record in continuum the
 1654 absorbance increase at 247 nm (ΔA_{247}) during 10 min, until levelling off. Determine the slope
 1655 ΔA_{247} from the initial linear portion of the curve. If no linear part is found, repeat the test with
 1656 a lower or higher amount of enzyme.

1657 **Blank:** For blank assays, follow the same protocol by replacing the enzyme with buffer
 1658 (equilibration is usually reached faster, 5 min). The blank slope, ΔA_{247} , should be close to
 1659 zero.

1660 **Calculations:**

1661 The slopes ΔA_{247} [unit absorbance/minute] are established for both the blank and the test
 1662 reactions by using the maximum linear rate over at least 5 minutes:

$$\text{Units/mg} = \frac{[\Delta A_{247} \text{ Test} - \Delta A_{247} \text{ Blank}] \times 1000 \times 3}{(540 \times X)}$$

1663 ΔA_{247} : slope of the initial linear portion of the curve, [unit absorbance/minute] for the Test
 1664 (with enzyme) and ΔA_{247} Blank without enzyme

1665 540: molar extinction coefficient (L/(mol × cm)) of TAME at 247 nm.

1666 3: Volume (in millilitres) of reaction mix

1667 X: quantity of trypsin in the final reaction mixture (quartz cuvette) [mg]

1668 Check that the activity obtained is the same for each tested concentration of trypsin, to make
 1669 sure that you are in the linear part of the enzyme concentration curve.

1670

1671 **Chymotrypsin activity assay (EC 3.4.21.1)**

1672 **References:** adapted from Hummel¹¹ and Rick¹²

1673 **Method:** Kinetic spectrophotometric rate determination

1674 **Principle:**

1675 BTEE + H₂O $\xrightarrow{\text{chymotrypsin}}$ N - Benzoyl - L - Tyrosine + Ethanol

1676 **Unit Definition:** One unit of chymotrypsin hydrolyses 1.0 µmol of N-Benzoyl-L-Tyrosine
 1677 Ethyl Ester (BTEE) per minute at pH 7.8 and 25°C.

1678 Conditions: T = 25°C, pH = 7.8, $A_{256\text{nm}}$, Light path = 1 cm



1679 Preparation of reagents:

1680 **Substrate:** Dissolve the substrate, BTEE (ref. B6125 Sigma-Aldrich), at a concentration of
 1681 1.18 mM in methanol/purified water. Weigh 18.5 mg of BTEE, dissolve it in 31.7 mL of
 1682 absolute methanol and complete to 50 mL with deionized water in a 50 mL volumetric flask.

1683 **Enzyme:** The enzyme is dissolved in 1 mM HCl. Prepare at least 2 concentrations of
 1684 chymotrypsin (porcine chymotrypsin, ref. C7762 Sigma-Aldrich) ranging between 10-30
 1685 µg/mL in 1 mM HCl.

1686 **Assay solution:** 80 mM Tris/HCl buffer, containing 100 mM CaCl₂ at pH at 7.8 and 25°C.

1687 **Assay:**

1688 Set the spectrophotometer at 256 nm and 25°C.

1689 **Test:** Mix 1.5 mL of the assay solution and 0.3 mL of the substrate (1.18 mM BTEE) into
 1690 quartz cuvette, mix by inversion and incubate in spectrophotometer at 25°C for 3-4 minutes
 1691 to achieve temperature equilibration. Add 100 µl of each concentration of the chymotrypsin
 1692 solutions and record the absorbance increase ΔA at 256 nm (ΔA₂₅₆) during 10 min in
 1693 continuum, until levelling off. Determine the slope ΔA₂₅₆ from the initial linear portion of the
 1694 curve. If no linear part is found repeat the test with a lower or higher amount of enzyme.

1695 **Blank:** For blank assays, follow the same protocol by replacing the enzyme with buffer only
 1696 (equilibration is usually reached faster, 5 min). The blank slope ΔA₂₅₆ Blank should be close
 1697 to zero.

1698 **Calculations:**

1699 The slopes ΔA₂₅₆ [unit absorbance/minute] are established for both the blank and the test
 1700 reactions by using the maximum linear rate over at least 5 minutes:

$$\text{Units/mg} = \frac{[\Delta A_{256} \text{ Test} - A_{256} \text{ Blank}] \times 1000 \times 3}{(964 \times X)}$$

1701 ΔA₂₅₆: slope of the initial linear portion of the curve, [unit absorbance/minute] for the Test
 1702 (with enzyme) and ΔA₂₅₆ Blank without enzyme

703 964: molar extinction coefficient L/(mol × cm) of BTEE at 256 nm.

704 3: Volume (in millilitres) of reaction mix

705 X: quantity (mg) of chymotrypsin in the final reaction mixture (quartz cuvette)

1706 Check that the activity obtained is the same for each tested concentration of chymotrypsin, to
1707 make sure that you are in the linear part of the enzyme concentration curve.

1708

1709 **Pancreatin**

1710 The amount of pancreatin is normalized to the trypsin activity. However, to digest fat
1711 containing food, the lipase activity should be recorded as well. Therefore, to measure the
1712 enzyme activities of the pancreatin (porcine pancreatin 8 x USP specifications, ref P7545
1713 Sigma-Aldrich), the protocols are the same as described above. For trypsin (or chymotrypsin)
1714 Pancreatin is dissolved in 1 mM HCl (pH 3). Pancreatin is difficult to dissolve, mix during 10
1715 minutes using a magnetic stirrer and then keep the solution on ice or at refrigerated
1716 temperature 4°C to prevent loss of activity. Dilute the pancreatin to a concentration ranging
1717 between 0.1 to 1 mg/mL and measure at least 3 different dilutions. Vortex pancreatin before
1718 pipetting it to the enzyme reaction vessel. To measure the lipase activity in pancreatin,
1719 dissolve it in 150 mM NaCl at pH 6.8 (pancreatic lipase is degraded at low pH), and follow
1720 the above procedure to record lipase activity.

1721

1722 **Bile salts in bile**

1723 The concentration of bile salts in the bile (fresh or commercial) can be measured with a
1724 commercial kit (bile acid kit, 1 2212 99 90 313, DiaSys Diagnostic System GmbH, Germany,
1725 MAK309-1KT, Merck or similar) according the supplier's protocol. Measure the bile at
1726 different concentrations bearing in mind the linearity range of the kit.

1727

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