



Digestion of milk proteins: Comparing static and dynamic *in vitro* digestion systems with *in vivo* data



Lotti Egger^{a,*}, Olivia Ménard^b, Christian Baumann^a, Desirée Duerr^a, Patrick Schlegel^c, Peter Stoll^c, Guy Vergères^a, Didier Dupont^b, Reto Portmann^a

^a Agroscope, Schwarzenburgstr. 161, 3003 Bern, Switzerland

^b STLO, UMR 1253, INRA, Agrocampus Ouest, 35000 Rennes, France

^c Agroscope, Tiroleire 4, 1725 Posieux, Switzerland

ARTICLE INFO

Keywords:

Static *in vitro* digestion
Dynamic *in vitro* digestion
Dairy proteins
Peptides
Mass spectrometry
Free amino acids

ABSTRACT

In the frame of the COST action INFOGEST, a static *in vitro* digestion protocol has been elaborated aiming at the improvement of data comparability by harmonizing the experimental conditions. The success in harmonization was confirmed with inter-laboratory trials using skim milk powder as a standardized model food. Moreover, the physiological relevance of the gastric and intestinal endpoints of the static digestion protocol was demonstrated in a pig *in vivo* trial, with the same skim milk powder and samples collected from different sections of the digestive tract, as well as in a human study with from jejunal effluents. *In vivo*, digestion is a dynamic process influenced by peristalsis and by the gradual secretion of enzymes and juices and the dwell time of the food. To mimic these physiological mechanisms, dynamic *in vitro* digestion protocols are widely used. Until now, the differences of protein hydrolysis taking place during dynamic and static *in vitro* digestion have not been investigated. In this study, the gradual hydrolysis of the main milk proteins present in skim milk powder was digested with the dynamic DIDGI®-system using adult digestion protocol and the static harmonized INFOGEST method. Protein hydrolysis was analyzed by gel electrophoresis, peptide patterns were measured with mass spectrometry, and free amino acids with high pressure liquid chromatography. The peptide patterns at the gastric and intestinal endpoints of *in vitro* digestion showed a good approximation to the *in vivo* results from pigs. Moreover, gradual peptide generation was comparable in both *in vitro* digestion conditions. However, the dynamic protocol reflected the physiological situation better at the level of free amino acid release. Nonetheless, in both *in vitro* digestion protocols, absorption of free amino acids is not simulated, and they are therefore limited in reflecting the *in vivo* situation at this level.

1. Introduction

Recently, a simple static *in vitro* digestion (IVD) model was elaborated by the INFOGEST network within the COST Action FA 1005 (Minekus et al., 2014). Inter-laboratory experiments have shown that the protocol improved the comparability of experimental data (Egger et al., 2016). The comparison of protein hydrolysis between this harmonized *in vitro* digestion protocol with the *in vivo* pig digestion of skim milk powder (SMP) was assessed at the level of proteins (SDS-PAGE), peptides (mass spectrometry), and total FAA (HPLC) and has shown that the gastric and intestinal endpoints were similar in the two systems (Egger et al., 2017). The harmonized static digestion protocol is now widely applied and has been cited over 300 times. However, static digestion models aim to simplify a highly complex and dynamic

physiological process in which peristalsis, enzyme secretion, gastric emptying, and absorption processes, to name the most obvious, have a major influence on the fate of food disintegration. Moreover, milk is unique in its digestion kinetics due to the content of proteins with completely different properties. The coagulation of caseins in the gastric compartment induced by pepsin and the acidic conditions leads to a prolonged gastric digestion. Soluble whey proteins in contrast have been shown to reach the intestinal compartment much faster (Boirie et al., 1997). To obtain a better approximation to these physiological processes, dynamic IVD protocols have been developed, such as the DIDGI® system (Ménard, Picque, & Dupont, 2015). This system allows the continuous addition of digestion juices and enzymes, the progressive adjustment of the pH, and gastric emptying at predetermined rates. This study aimed first at the comparison of protein hydrolysis in

Abbreviations: FAA, free amino acids; IVD, *in vitro* digestion; SMP, skim milk powder

* Corresponding author.

E-mail address: charlotte.egger@agroscope.admin.ch (L. Egger).

<https://doi.org/10.1016/j.foodres.2017.12.049>

Received 27 October 2017; Received in revised form 13 December 2017; Accepted 16 December 2017

Available online 18 December 2017

0963-9969/ © 2017 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license

(<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

skim milk powder (SMP) between the static INFOGEST digestion protocol and the dynamic DIDGI® protocol at the gastric and intestinal endpoints. In addition, time-resolved experiments were performed to visualize the kinetic of protein hydrolysis in the two systems and to display possible differences and limitations of static IVD. Second, protein hydrolysis during IVD was compared to pig *in vivo* digestion samples, which were collected along the digestive tract after the ingestion of the same SMP (Egger et al., 2017). Thus, protein digestion was analyzed in samples collected during time-resolved IVD at different levels of hydrolysis, analyzing whole proteins by SDS-PAGE, peptides by mass spectrometry, and total and FAA by HPLC.

2. Materials and methods

2.1. Chemicals and reagents

SMP from Fonterra, NZ, low-heat organic composed of 42.34% protein, 0.89% fat, and 49.8% lactose (w/w). All chemicals used for the simulated fluids were purchased from Sigma Aldrich as previously described (Minekus et al., 2014). Enzymes were from Sigma Aldrich; for static IVD: Porcine pepsin (P7012), porcine pancreatin (P7545), and bile extract (B8631); for dynamic IVD: porcine pepsin (P6887), porcine pancreatin (P7545), and bovine bile extract (B3883).

2.2. Static *in vitro* digestion with the harmonized protocol

SMP was digested *in vitro* according to the harmonized protocol (INFOGEST, 2014; Minekus et al., 2014). Briefly, SMP (5 mL of a 1/10 dilution of SMP in H₂O, w/v) was mixed with 5 mL of simulated saliva without amylase for 2 min and then mixed with 10 mL of simulated gastric juice containing pepsin (2000 U/mL of digesta) for different time points (0.1, 0.5, 1, 3, 5, 10, 20, 30, 60, 120 min) with a maximal gastric endpoint of 120 min. For the intestinal phase, 20 mL of simulated intestinal juice containing pancreatin (100 U trypsin activity/mL of digesta) and bile (10 mmol/L in the total digesta) were added after the completed gastric phase and incubated for different time points for (0.1, 0.5, 1, 3, 5, 10, 20, 30, 60, 120 min) with the maximal intestinal endpoint at 120 min. All incubations were performed at 37 °C on a rotating wheel. The enzyme activities and the bile concentration were measured according to the assays described in the harmonized protocol (Minekus et al., 2014). Pepsin activity in samples collected during the gastric phase was stopped by the addition of NaOH until reaching a pH of 7 followed by immediate snap freezing in liquid nitrogen. Reactions in the intestinal phase were stopped by the addition of Pefabloc® (5 mM) at the end of the time points of sample collection, prior to the snap freezing.

2.3. Dynamic *in vitro* digestion

SMP, after rehydration at 10% (w/v) in water, was submitted to gastrointestinal digestion using the dynamic *in vitro* digestion system DIDGI®. The system was set up to simulate the adult gastro-intestinal digestion conditions. Table 1 summarizes the parameters used in the digestion experiments. To mimic the fasted gastric conditions, 24 mL of simulated gastric fluid adjusted to pH = 2.2 (Lentner, 1973) was added in the gastric bowl before milk ingestion.

Gastric and intestinal emptying rates followed the mathematical model described by (Elashoff, Reedy, & Meyer, 1982) and were determined after milk consumption in adults. The gastric and intestinal emptying half-times ($t_{1/2}$) 35 and 85 min, respectively were taken from (Minekus, Marteau, Havenaar, & Huis in T Veld, 1995) and were assessed during consumption of milk in human volunteers. The compilation of data measuring gastric pH in adults from (Malagelada, Longstreth, Summerskill, & Go, 1976; Minekus et al., 1995) allowed the determination of a linear regression describing the gastric acidification curve ($\text{pH} = 1.68 + 3.82^{(-t/42)}$). The gastric pH was controlled using

Table 1

Gastrointestinal conditions for the *in vitro* dynamic digestion of skim milk simulating adult conditions.

Simulated gastric and intestinal fluid (stock solution adjusted at pH 6.5)	NaCl	100 mmol/L
Gastric conditions (37 °C)	CaCl ₂	1 mmol/L
Fasted state/initial conditions	SGF	24 mL
Milk	pH	2.2
	Total volume	100 mL
	Flow rate	0–10 min
		10 mL/min
Gastric pH (acidification curve)	$\text{pH} = 1.68 + 3.82^{(-t/42)}$	
With t = time after ingestion in min		
Pepsin (porcine)	Flow rate	
	0–10 min	1 mL/min
	10 min - end gastric	0.5 mL/min
Gastric emptying (Elashoff fitting)	$t_{1/2}$	35 min
Intestinal conditions (37 °C)	β	1.15
Intestinal pH	pH	6.5
Bile (bovine)	Flow rate	4%
	0–30 min	0.5 mL/min
		2%
	30–180 min	0.5 mL/min
Pancreatin (porcine)		7%
	Flow rate	
	0–180 min	0.25 mL/min
Intestinal emptying (Elashoff fitting)	$t_{1/2}$	85 min
	β	1.4

HCl 1 M. The pepsin amount was based on the activity of the powder and fixed to cover 2000 units/mL of gastric content (Minekus et al., 2014). Gastric and intestinal simulated fluids were made of sodium and calcium chloride (Lindahl, Ungell, Knutson, & Lennernäs, 1997) and adjusted to pH = 6.5 to avoid enzymes autolysis.

The intestinal conditions were set up as reported by (Minekus et al., 1995). Intestinal pH was maintained at 6.5 by the addition of NaHCO₃ 1 M.

SMP digestion experiments were performed in triplicate. Digesta were sampled after 0.5, 3, 6, 8, 10, 14, 20, 30, 60, and 90 min of gastric digestion and after 11, 14, 17, 20, 30, 60, 90, 120, and 180 min of intestinal digestion. Samples were frozen and freeze-dried after the addition of protease inhibitors, e.g., Pepstatin A (0.72 µmol/L) for gastric digesta and Pefabloc® (5 mmol/L) for intestinal digesta.

2.4. *In vivo* pig trial

The pig experiment was approved by the animal welfare department of the government authority (approval number 2015_04_FR; 26115) and has been previously described in detail (Egger et al., 2017). Animals received the same batch of SMP as used for the dynamic and static *in vitro* digestions. The pigs received three meals at T - 6 h, T - 3 h, and T - 1.5 h prior to sacrifice, each consisting of 250 g of SMP dissolved in 500 mL of H₂O giving a ratio of 250/750: w/w, reaching a concentration of 33% of SMP. In addition drinking water was given *ad libitum*. The higher concentration of SMP compared to the IVD was necessary, because after weaning the pigs refused the uptake of milk in a liquid form but accepted it as a slurry of 33% concentration. All samples (gastric liquid and solid, intestinal I1-4) were collected after 1.5 h after the last of three SMP ingestions.

2.5. Sample preparation and gel electrophoresis

The samples were normalized according to the input of SMP, that is, the dilution of SMP during the digestion steps was considered in order to load the samples with equal amount of SMP. Samples were mixed with sample buffer (Tris-HCl 350 mM, pH = 6.8, SDS 10%, DTT 100 mM, glycerol 50%) prior to separation by SDS-PAGE (15% polyacrylamide). A molecular weight marker (Benchmark™, Invitrogen) was

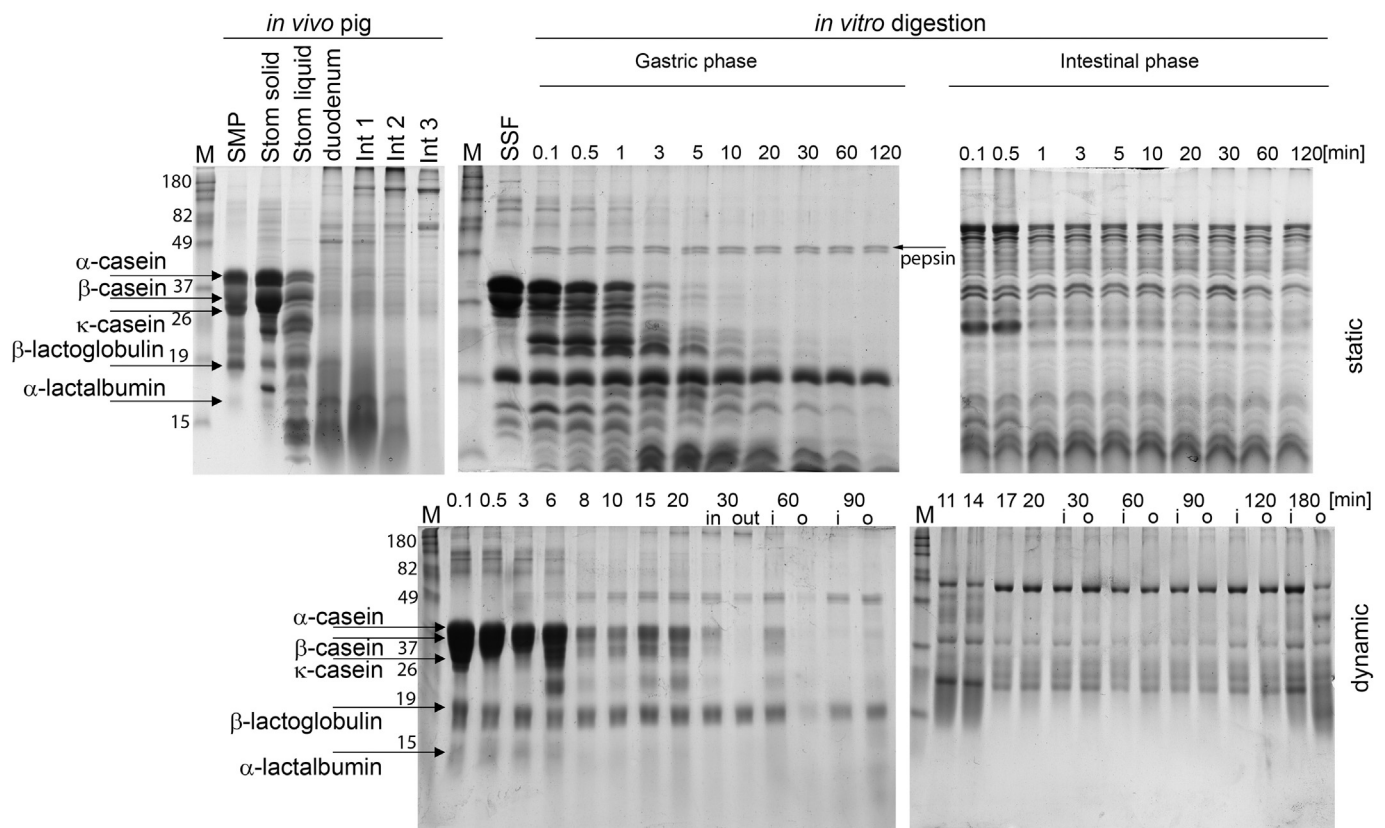


Fig. 1. Protein hydrolysis after gastric and intestinal phases of static and dynamic IVD. Representative Coomassie blue-stained SDS-PAGE gels of the gastric and intestinal phases of static and dynamic IVD with time-resolved sampling, aside from the pig *in vivo* protein patterns from different sampling sections. In: sampling at the gastric/intestinal inlet, out: sampling at the gastric/intestinal outlet. SSF: simulated salivary fluid.

included. Thereafter, the gels were stained with colloidal Coomassie Blue (Kang, Gho, Suh, & Kang, 2002).

2.6. LC-MS peptide identification and length analysis

MS analysis was performed as previously described (Egger et al., 2016; Egger et al., 2017; Kopf-Bolan et al., 2014). Briefly, after sample filtration through Microcon columns (Ultracel YM-30, Millipore), the peptides were separated on a Rheos 2200 HPLC (Flux Instruments, Switzerland) equipped with an XTerra MS C18 column (3.5 μ m, 1.0 mm $\times$ 150 mm, Waters). The HPLC was directly coupled to a linear ion trap mass spectrometer (LTQ, Thermo Scientific, Switzerland) using an ESI interface. The signals of an m/z^{-1} between 100 and 1300 were measured in multiple overlapping mass windows. The MSMS spectra of the overlapping mass windows were merged and peptides were identified with a search in Mascot (Matrix Science) using a home built database containing all typical milk proteins of different species. Peptides from the five main milk proteins (β -casein, α_{s1} -casein, α_{s2} -casein, κ -casein, and β -lactoglobulin) were monitored. The minimal criteria for peptide identification was an ion score of 20 and being on first place of identification. If questionable, the results were manually verified by checking the fragmentation pattern (peptide coverage and fragmentation pattern). To visualize the peptide abundance, individual amino acids within the identified peptides from the abovementioned dairy proteins were summed up and a color code was applied. The amino acid counting was performed with a home built Perl script extending Mascot search engine, the color codes were adjusted using Microsoft Excel. The colors range from green to red, indicating low and high recurrence of specific amino acids, respectively. Unidentified protein sequences are shown as white stretches. The colors were normalized to the maximal number of identifications within the corresponding protein and

digestion phase, e.g., red in α_{s1} -casein can correspond to a different number than red in β -lactoglobulin.

2.7. HPLC analysis

To determine FAA, the samples were normalized to SMP input, as described for gel electrophoresis, and equal volumes containing equal amounts of SMP were analyzed by HPLC according to the method published by Bidlingmeyer and coworkers (Bidlingmeyer, Cohen, & Tarvin, 1984).

2.8. Spearman correlation

Spearman correlations were calculated for the gastric and intestinal phase considering all five investigated proteins (β -casein, α_{s1} -casein, α_{s2} -casein, κ -casein, and β -lactoglobulin) based on the data of the peptide profiles shown in Fig. 2 and Supplemental Fig. 3.

2.9. Replication of experiments, statistical analysis and normalization between digestion models

Static and dynamic *in vitro* digestion time course experiments were performed three times independently ($n = 3$). Samples were analyzed in triplicate by mass spectrometry and in duplicate by HPLC. All samples were separated by SDS-PAGE, and representative gels are shown. The Spearman correlation matrix was calculated with R software using rank order data (Spearman). Normalization between static and dynamic IVD models was performed in order to obtain equal amounts of SMP input. Dilutions of SMP during the different digestion phases were considered for each model. The pig experiment was designed to obtain typical digestion patterns for each sampling section along the digestive

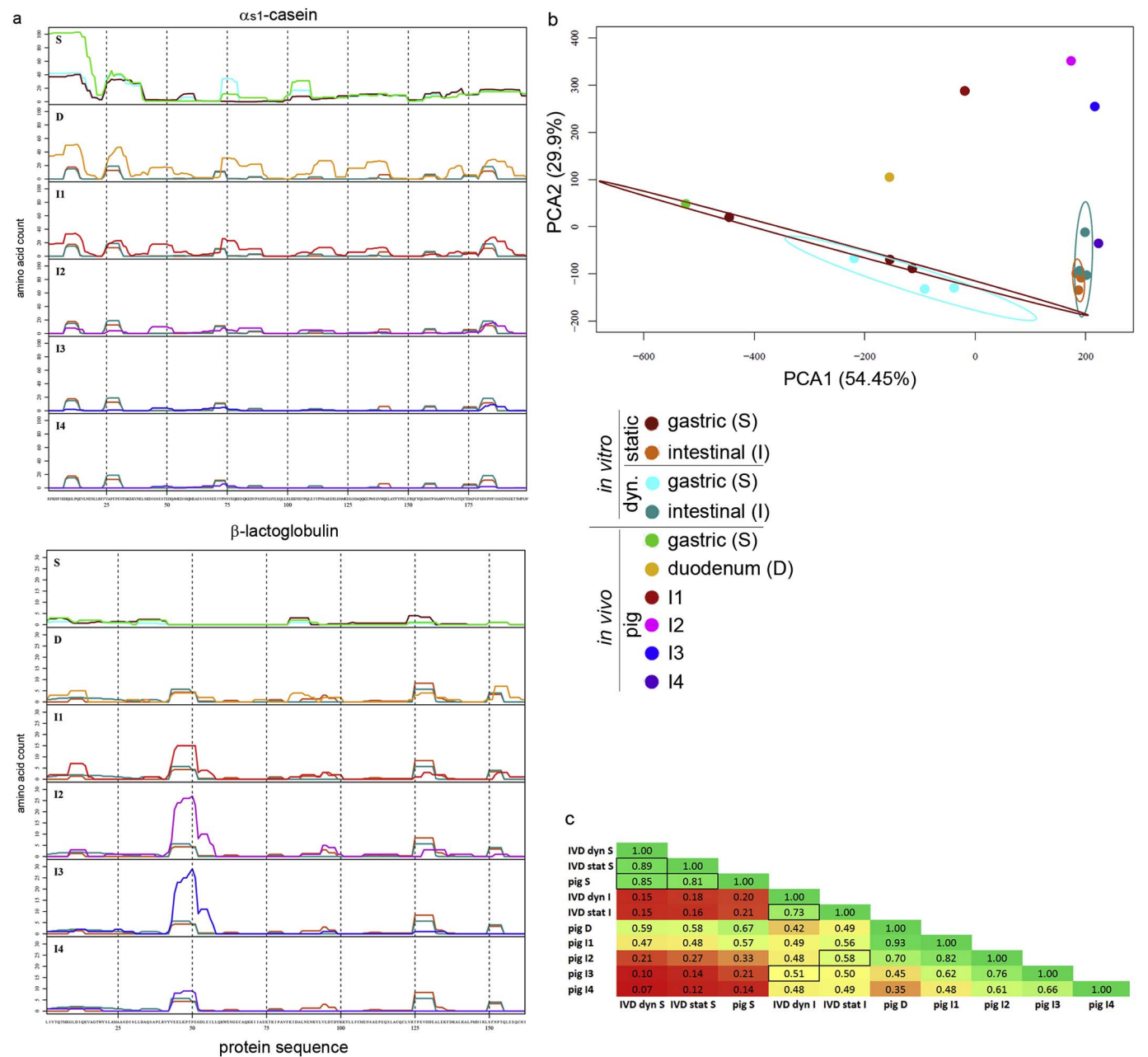


Fig. 2. Peptide patterns showing the average endpoints after gastric and intestinal static and dynamic IVD. Comparison of average peptide patterns between dynamic and static IVD with pig sampling sections. Gastric (S) endpoints after static (brown) and dynamic (turquoise) IVD were compared with pig stomach (green). Intestinal endpoints after static (petrol) and dynamic (orange) IVD of α s1-casein and β -lactoglobulin were compared with the other sampling zones of pig digestion; D: duodenum (yellow); I1: proximal jejunum (red); I2: median jejunum (pink); I3: distal jejunum (blue); I4: ileum (purple) (a). PCA analysis performed with the peptides from the five major milk proteins considering the three replicates of static and dynamic IVD and an average sample from each pig sampling section (b). Spearman correlation matrix calculated with the peptides considering all five major proteins. Best correlations between gastric samples (S) and intestinal samples (I) between all three models have a black border (c). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tract. Therefore, the pigs were fed three times with SMP, with the inconvenience that the calculation of a precise amount of SMP present in each sampling section at the time of sacrifice is not possible. As a consequence, the quantitative normalization between IVD and the pig assay was calculated relative to the maximal amount of peptides per digestion system for each protein.

3. Results and discussion

SMP was digested *in vitro* with the harmonized INFOGEST protocol (Minekus et al., 2014) and with the dynamic model DIDGI® (Menard et al., 2014). Samples were collected at different time points, and the

enzyme activities were stopped immediately by adjusting the pH to 7 in the gastric phase or by protease inhibitors, prior to snap freezing. The time course experiments of both dynamic and static digestion were performed three times independently and either the representative results (SDS-PAGE) or the average results of the three independent experiments are shown.

3.1. Protein hydrolysis during static and dynamic IVD

The analysis of proteins by SDS-PAGE are shown on representative gels (Fig. 1) and the replicates with identified enzymes from gastric and intestinal fluids are shown in Supplemental Fig. 2; in general, the

protein hydrolysis showed a similar kinetic behavior in the static and dynamic digestion (Fig. 1). Intact caseins disappeared during the first 30 min of digestion in the static system and after 60 min of dynamic digestion, whereas β -lactoglobulin was persistent in both systems during the whole gastric phase, which is in agreement with previous studies (Egger et al., 2016; Kopf-Bolan et al., 2014; Mandalari et al., 2009). In both systems, no β -lactoglobulin was observed as soon as the SMP was incubated with the intestinal fluid; the remaining bands on the gels corresponded to enzymes present in the pancreatin (Fig. 1, intestinal phase), confirming earlier results (Egger et al., 2016; Egger et al., 2017; Kopf-Bolan et al., 2012). Next, protein hydrolysis during IVD was compared qualitatively to the pig experiment, where all the samples were collected 1.5 h after the last of three SMP ingestions. In the pig, the gastric content consisted of a firm coagulum, surrounded by a liquid phase and therefore, a liquid and a solid gastric sample were collected and analyzed. In the solid sample, caseins were mainly intact, whereas in the liquid sample casein hydrolysis was ongoing and a weak band of β -lactoglobulin was visible (Fig. 1, Stom solid, Stom liquid; (Egger et al., 2017)). This β -lactoglobulin band was almost completely hydrolyzed in the duodenal phase, and no intact caseins were identified in this sampling section (Fig. 1, duodenum; (Egger et al., 2017)). Although the *in vivo* experiment had no time resolution, it allows two main conclusions that were reproduced at the gastric endpoint by both IVD models: i) β -lactoglobulin was resistant to gastric digestion, visible as intact band in the intestinal phase and ii) no intact caseins were identified in the intestinal samples. These results are in agreement with results obtained during gastric digestion of raw milk with a human gastric simulator (HGS) (Ye, Cui, Dalgleish, & Singh, 2016). On the other side, the formation of a firm coagulum that occurred in the stomach of the pigs (Dupont & Tome, 2014) was not observed in the two investigated IVD models. In dynamic conditions, a coagulum was formed in the stomach with an intense syneresis (Supplemental Fig. 1). The difference in firmness of the coagulums formed *in vivo* and *in vitro* is certainly the result of differences in protein concentration between the two experiments. Indeed, 33% of SMP was used for *in vivo* trial whereas *in vitro* experiments were performed at 10% SMP. Due to a slower hydrolysis of proteins within a coagulum (Ye et al., 2016), it is difficult to simulate the exact kinetics of protein hydrolysis occurring *in vivo* with an IVD model and should therefore be considered as a qualitative analysis. However, the gastric and intestinal endpoints of both IVD systems showed a good approximation to the pig *in vivo* samples.

3.2. Peptide generation during IVD

Peptides were analyzed by mass spectrometry in three independent experiments of static and dynamic time course experiments. Peptide identifications were performed for the five most abundant milk proteins (β -casein, β -lactoglobulin, α_{s1} -casein, α_{s2} -casein, and κ -casein), considering a minimal length of five amino acids. At first, the peptide patterns of the gastric and intestinal endpoints were compared to the peptide patterns from the pig *in vivo* data (Egger et al., 2017), by representing the data in a line plot where the protein sequence is shown on the x-axis and the number of times an amino acid was identified within a peptide of the corresponding protein on the y-axis (Fig. 2a). In the gastric phase, the peptide patterns were very similar, correlation between static and dynamic IVD calculated for α_{s1} -casein achieved a factor of 0.77 and correlated with a factor of 0.71 (static) and 0.88 (dynamic) with the *in vivo* experiment. Peptide patterns were generated for all five major proteins (Supplemental Fig. 4a–c), as well as the individual correlation coefficients between IVD systems and with the *in vivo* experiment (Supplemental Fig. 5). The highest correlation for β -casein and κ -casein IVD was with the *in vivo* section I4. However, with this late sampling section, the correlation is not reliable because it is only based on the few peptides identified in this section of pig intestine. Moreover, the total correlation calculated over all proteins was 0.89 between IVD experiments, 0.81 (static-*in vivo*), and 0.85 (dynamic-*in vivo*), as highlighted with a black border (Fig. 2c). The intestinal end-points of IVD correlated with a factor of 0.73 (Fig. 2c) and were compared with the different intestinal sampling sections of the pigs. The highest correlation coefficient of 0.51 was observed in the distal jejunum for the dynamic protocol (Fig. 2a, I3) and of 0.58 for the static IVD in the median jejunum of the pigs (Egger et al., 2017). The later sampling sections (I3, I4) contained fewer peptides due to absorption processes, which are not mimicked by IVD and therefore are less comparable. Although the peptide patterns were qualitatively very similar, some peptides were identified with a higher incidence in the *in vivo* experiment, such as the N-terminal regions of α_{s1} -casein and κ -casein in the gastric phase or to a lesser extent the region of amino acids 125–140 of β -casein (Fig. 2a (S), Supplemental Fig. 4a, c (S)). These differences might be due to unprecise normalization between IVD samples and the *in vivo* experiment, where three meals were given at different time points and the total amount of SMP in the corresponding sampling section could not be determined. A principle components analysis (PCA) was performed with the three individual values for static and dynamic endpoints and the average values of the pig sampling sections ($n = 8$). The gastric phases of the three models were close together and the intestinal endpoint of the IVD co-localized with the median and distal pig samples (Fig. 2b). The physiological comparability of peptide patterns between IVD and *in vivo* is in agreement with a previous study on human jejunal digests of casein and whey proteins (Sanchón et al., 2018). The evolution of protein hydrolysis over time was visualized with heatmaps of the five proteins, generated with the average values of the three experiments for both IVD methods and for each sampling time point, as previously described (Egger et al., 2016). The color code indicates low peptide abundance in green, medium abundance in yellow, and high abundance in red. Stretches where no peptides were identified were left white. The color intensities are normalized within the corresponding digestion model. IVD time course experiments of dynamic and static digestions were aligned with the average peptide patterns obtained from the different sampling sections of the pigs (Egger et al., 2017) (Fig. 3, Supplemental Fig. 3). In all three models, the kinetic of protein hydrolysis was visible for all the proteins in the gastric and intestinal phases; however, the evolution seemed more pronounced in the dynamic model. In both IVD models, in the case of α_{s1} -casein, being a pepsin susceptible protein, a high number of peptides was present at the very beginning of the gastric phase, which decreased over time (Fig. 3, upper panel). In contrast to this progression, only a few peptides from β -lactoglobulin were generated during the whole gastric phase, with increasing numbers throughout the intestinal digestion process (Fig. 3, lower panel). In both IVD systems, a clear-cut difference in peptide patterns occurred between the gastric and intestinal phases, although the digestion was immediately stopped after addition of simulated intestinal fluid in the first sample of the static digestion (Fig. 3, static, intestinal time 0.1 min). This observation confirmed the results from SDS-PAGE (Fig. 1), where no intact β -lactoglobulin was visible in the same sample at time 0.1 min (Fig. 1, *in vitro* digestion, intestinal phase). The peptide patterns in both gastric phase of IVD were comparable to the *in vivo* sample collected from the pig stomach (Fig. 3, *in vivo*, S) and the patterns in the IVD intestinal phase were comparable to the *in vivo* sample from the medium jejunum (Fig. 3, *in vivo*, I2). Moreover, some digestion resistant sequences such as e.g. the regions from amino acid 8–12 (HQGLP) or 25–30 (VAPFPE) in α_{s1} -casein (Fig. 3), that were still present at the late stages of digestion, e.g., after 120–180 min of dynamic, after 120 min of static, or in pig samples I2–I3, were overlapping. The sample I4, collected from the ileum contained only very few peptides, because most of the peptides or amino acids were most probably absorbed at this distal part of the intestine (Egger et al., 2017). This section therefore does not reflect the digestion-resistant regions.

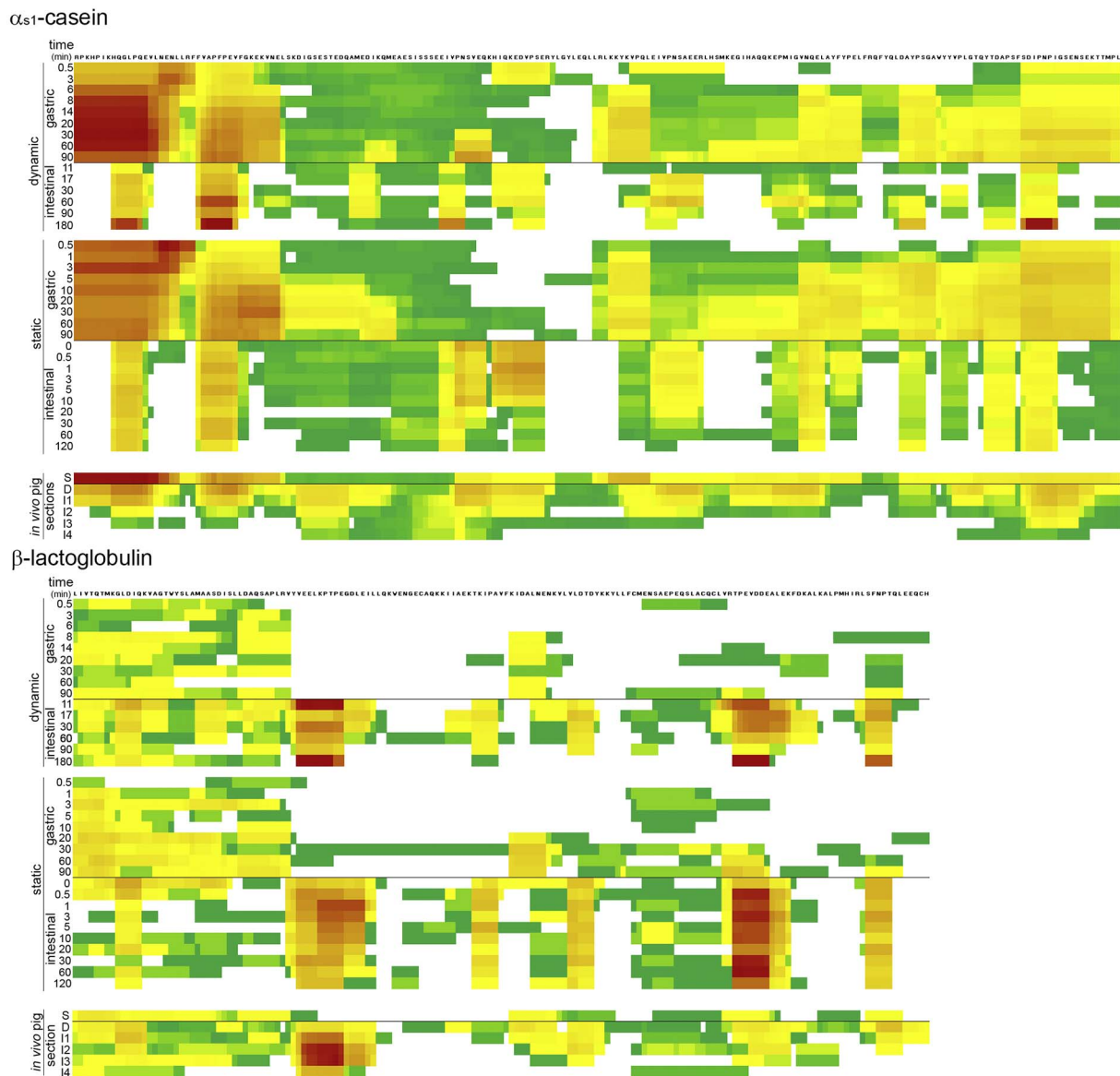


Fig. 3. Peptide patterns showing the gradual protein hydrolysis during static and dynamic IVD.

Average peptide patterns for α_{s1} -casein and β -lactoglobulin from each time point of IVD ($n = 3$) or pig sampling section ($n = 8$) were aligned according to the protein sequence. A high number of identifications in red over yellow to a low number of identifications in green. Unidentified stretches are left white. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Release of FAA after static and dynamic IVD

FAA was analyzed by HPLC (Bidlingmeyer et al., 1984) at each collected time point of static and dynamic IVD. The average values represent three independent digestions and are represented as the relative amount of the maximal FAA present in the corresponding digestion protocol considering the ratio of food to secretion at each time point for both IVD systems (Fig. 4a, b). During the gastric phase, a clear progression in the release of FAA is visible in the dynamic system, whereas in the static protocol, the values are only minimally increasing. This difference in the dynamic evolution can be explained by the continuous addition of pepsin in the dynamic protocol and in parallel with the inactivation of pepsin in the static protocol, possibly due product inhibition or autolysis of pepsin (Cornish-Bowden & Knowles, 1969; Galea, Dalrymple, Kuypers, & Blakeley, 2000). The autolysis of pepsin might be marginal, as there is no decrease visible on the SDS gel throughout the gastric phase (Fig. 1, arrow). Although there is a progression visible in FAA release during the dynamic IVD, in both *in vitro*

systems, only a very small amount of total FAA is released during the gastric phase: 0.38% for static (120 min) and 2.7% for dynamic (90 min) digestion. The release of very low amounts of FAA during the gastric phase is in agreement with results from *in vivo* experiments in human (Boutrou et al., 2013), as well as in the pig assay, where only 3.5% of the maximal FAA release was observed in the gastric phase (Egger et al., 2017). As already observed at the level of peptides, there is again a clear-cut difference between the end of gastric and the start of the intestinal digestion in both IVD systems in the amount of FAA release. The addition of pancreatin in the intestinal phase of IVD leads to an increase of FAA from 0.38% to 50.2% in the static and from 2.7% to 29% in the dynamic protocol (Fig. 4b). During the intestinal phase, the evolution in FAA release was 15% with the static and more pronounced with the dynamic protocol (36.2%). At the end of both IVD intestinal phases, the values converged to 65.1% (static) and 65.5% (dynamic) of the total FAA. This amount is in accordance with previous results from *in vitro* digestion (Picariello et al., 2015). It is difficult to extrapolate the amount of released amino acids between IVD and the *in vivo* situation,

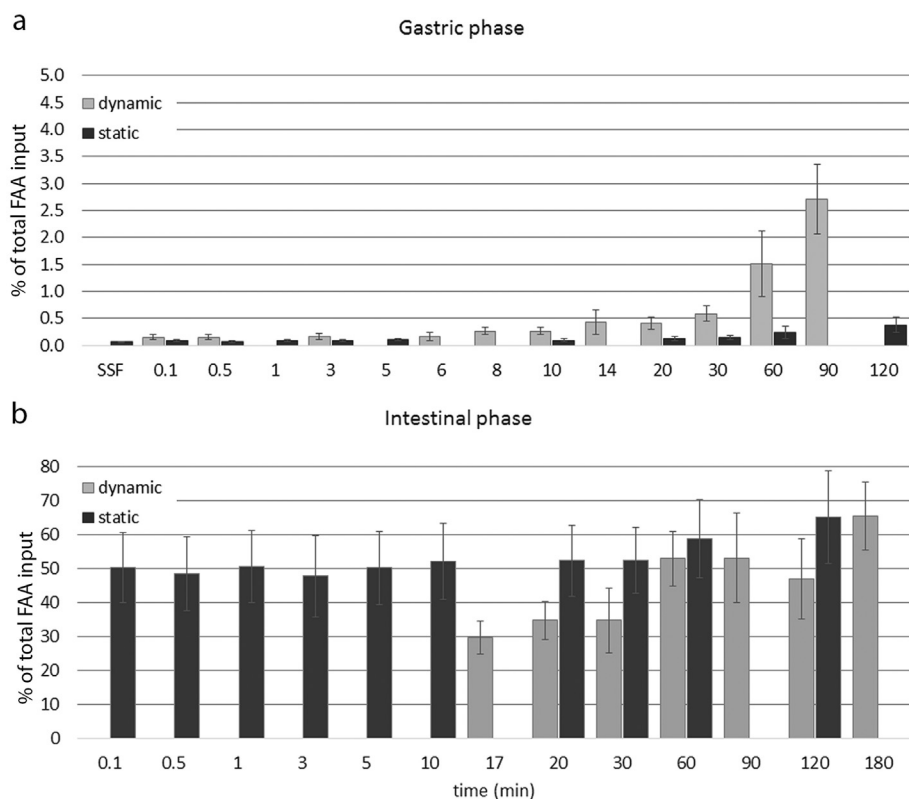


Fig. 4. Release of FAA during static and dynamic IVD. Relative release (normalized to SMP input for each protocol) of FAA during dynamic (grey) and static (black) IVD in the gastric (a) and intestinal (b) digestion phases. Error bars indicate the average deviation of the triplicates.

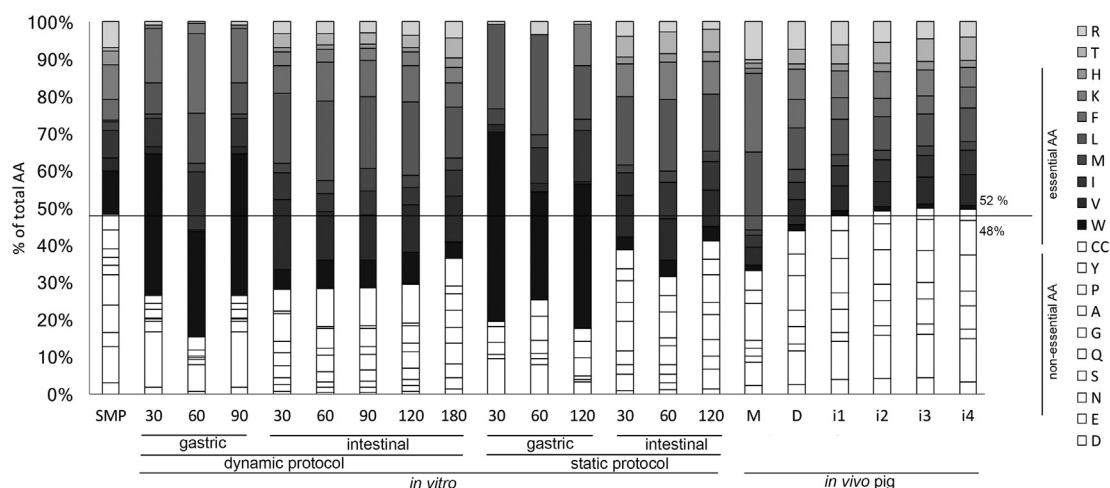


Fig. 5. Distribution of essential and non-essential amino acids during static and dynamic IVD.

Comparison of the ratio of essential and non-essential AA between the calculated AA present in the undigested SMP, the released FAA during the static and dynamic IVD, and FAA in the pig sampling sections. S: Stomach; D: duodenum; i1: proximal jejunum; i2: median jejunum; i3: distal jejunum; i4: ileum.

as in the latter model, absorption processes are taking place and further protein hydrolysis occurs by enzymes of the brush border membrane, which are absent in both IVD models. Moreover, it is unknown to what extent all proteins are degraded to FAA and difficult to estimate how much of the total ingested protein are absorbed as peptides. However, it has been shown that quantifiable amounts of peptides could be identified in the jejunum in healthy humans (Boutroun et al., 2013), and such peptides were even found in the blood circulation afterwards (Caira et al., 2016).

3.4. Preferential release of essential AA after static and dynamic IVD

The release of essential *versus* non-essential AA was assessed and compared to the ratio present in the original product, which was

calculated at 52:48% for SMP. Interestingly, this ratio increased for both protocols, being highest in the gastric phase with 85:15% after 60 min of dynamic digestion and 83:17% after 120 min of static digestion. In the pig *in vivo* situation, this shift occurred as well, however to a lesser extent, 67:33% in the stomach (S). This can be explained by the absorption processes and maybe even the preferential absorption of essential AA in the pig intestines. At later intestinal stages, this ratio decreased again in all digestion models (Fig. 5). This observation is of interest for nutritional purposes and would require further investigations to understand the physiological mechanisms.

4. Conclusion

In this work, SMP was digested with the standardized INFOGEST *in*

in vitro digestion protocol and the dynamic protocol DIDGI®. Protein hydrolysis in the two tested IVD models were compared between each other and to the *in vivo* pig digestion of the same SMP. The gastric and intestinal endpoints of both IVD systems showed a good approximation to the pig *in vivo* digestion. The kinetic in protein hydrolysis, especially the release of FAA during the gastric phase, showed a dynamic evolution and was closer to the *in vivo* situation in the dynamic digestion protocol, due to the constant addition of active enzymes. However, it must be kept in mind that these IVD protocols are confined to the intestinal phase without mimicking the enzymes of the brush border membrane and the absorption of nutrients at the end. Therefore, the direct comparison with the *in vivo* situation at this final digestion step is difficult.

Acknowledgements

COST Action FA1005 INFOGEST (<http://www.cost-INFOGEST.eu/>) is acknowledged for providing funding for travel, meetings, and conferences. We thank Urs Kämpfer from the Department of Chemistry and Biochemistry, University of Berne for performing the HPLC analyses. The authors declare that they have no conflict of interest. Contributors: E. L., M. O., D. D., V. G., and P. R., designed research; E. L., M. O., P. R., Sch. P., B. Ch., and D. D. conducted research; E. L., P. R., M. O., and B. Ch., analyzed data and performed statistical analysis; E. L., P. R., M. O. wrote the paper; E. L., M. O., and P. R. had primary responsibility for final content. St. P. was responsible for animal facility. All authors have read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodres.2017.12.049>.

References

- Bidlingmeyer, B. A., Cohen, S. A., & Tarvin, T. L. (1984). Rapid analysis of amino acids using pre-column derivatization. *Journal of Chromatography*, 336(1), 93–104.
- Boirie, Y., Dangin, M., Gachon, P., Vasson, M. P., Maubois, J. L., & Beaufre, B. (1997). Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proceedings of the National Academy of Sciences of the United States of America*, 94(26), 14930–14935.
- Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., ... Leonil, J. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *The American Journal of Clinical Nutrition*, 97(6), 1314–1323.
- Caira, S., Pinto, G., Vitaglione, P., Dal Piaz, F., Ferranti, P., & Addeo, F. (2016). Identification of casein peptides in plasma of subjects after a cheese-enriched diet. *Food Research International*, 84, 108–112.
- Cornish-Bowden, A. J., & Knowles, J. R. (1969). The pH-dependence of pepsin-catalysed reactions. *The Biochemical Journal*, 113(2), 353–362.
- Dupont, D., & Tome, D. (2014). Chapter 20 - Milk proteins: Digestion and absorption in the gastrointestinal tract. *Milk proteins* (pp. 557–569). (2nd ed.). San Diego: Academic Press.
- Egger, L., Ménard, O., Delgado-Andrade, C., Alvito, P., Assunção, R., Balance, S., ... Portmann, R. (2016). The harmonized INFOGEST *in vitro* digestion method: From knowledge to action. *Food Research International*, 88, 217–225.
- Egger, L., Schlegel, P., Baumann, C., Stoffers, H., Guggisberg, D., Brügger, C., ... Portmann, R. (2017). Physiological comparability of the harmonized INFOGEST *in vitro* digestion method to *in vivo* pig digestion. *Food Research International*, 102, 567–574. <http://dx.doi.org/10.1016/j.foodres.2017.09.047M>.
- Elashoff, J. D., Reedy, T. J., & Meyer, J. H. (1982). Analysis of gastric emptying data. *Gastroenterology*, 83(6), 1306–1312.
- Galea, C. A., Dalrymple, B. P., Kuypers, R., & Blakeley, R. (2000). Modification of the substrate specificity of porcine pepsin for the enzymatic production of bovine hide gelatin. *Protein Science*, 9(10), 1947–1959.
- INFOGEST. *Static in vitro digestion method for food*. (2014). <https://www.youtube.com/watch?v=LNSlIb-OJGc> (In YouTube).
- Kang, D., Gho, Y., Suh, M., & Kang, C. (2002). Highly sensitive and fast protein detection with Coomassie brilliant blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Bulletin of the Korean Chemical Society*, 2.
- Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergères, G., Portmann, R., & Egger, L. (2012). Validation of an *in vitro* digestive system for studying macronutrient decomposition in humans. *The Journal of Nutrition*, 142(2), 245–250.
- Kopf-Bolanz, K. A., Schwander, F., Gijs, M., Vergères, G., Portmann, R., & Egger, L. (2014). Impact of milk processing on the generation of peptides during digestion. *International Dairy Journal*, 35(2), 130–138.
- Lentner, C. (1973). *Geigy scientific tables*. Vol. 1.
- Lindahl, A., Ungell, A.-L., Knutson, L., & Lennernäs, H. (1997). Characterization of fluids from the stomach and proximal jejunum in men and women. *Pharmaceutical Research*, 14(4), 497–502.
- Malagelada, J. R., Longstreth, G. F., Summerskill, W. H., & Go, V. L. (1976). Measurement of gastric functions during digestion of ordinary solid meals in man. *Gastroenterology*, 70(2), 203–210.
- Mandalari, G., Adel-Patient, K., Barkholt, V., Baro, C., Bennett, L., Bublin, M., ... Mills, E. N. (2009). *In vitro* digestibility of beta-casein and beta-lactoglobulin under simulated human gastric and duodenal conditions: A multi-laboratory evaluation. *Regulatory Toxicology and Pharmacology*, 55(3), 372–381.
- Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., & Picque, D. (2014). Validation of a new *in vitro* dynamic system to simulate infant digestion. *Food Chemistry*, 145, 1039–1045.
- Ménard, O., Picque, D., & Dupont, D. (2015). The DIDGI® system. In K. Verhoeckx, P. Cotter, I. López-Expósito, C. Kleiveland, T. Lea, A. Mackie, T. Requena, D. Swiatecka, & H. Wichers (Eds.). *The impact of food bioactives on health: In vitro and ex vivo models* (pp. 73–81). Cham: Springer International Publishing.
- Minekus, M., Alming, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., ... Brodtkorb, A. (2014). A standardised static *in vitro* digestion method suitable for food - An international consensus. *Food & Function*, 5(6), 1113–1124.
- Minekus, M., Marteau, P., Havenaar, R., & Huis in T Veld, J. H. J. (1995). A multi-compartmental dynamic computer-controlled model simulating the stomach and small intestine. *Fund for the replacement of animals in medical experiments*. Vol. 23. Nottingham: ROYAUME-UNI.
- Picariello, G., Miralles, B., Mamone, G., Sanchez-Rivera, L., Recio, I., Addeo, F., & Ferranti, P. (2015). Role of intestinal brush border peptidases in the simulated digestion of milk proteins. *Molecular Nutrition & Food Research*, 59(5), 948–956.
- Sanchón, J., Fernández-Tomé, S., Miralles, B., Hernández-Ledesma, B., Tomé, D., Gaudichon, C., & Recio, I. (2018). Protein degradation and peptide release from milk proteins in human jejunum. Comparison with *in vitro* gastrointestinal simulation. *Food Chemistry*, 239, 486–494.
- Ye, A., Cui, J., Dalgleish, D., & Singh, H. (2016). Formation of a structured clot during the gastric digestion of milk: Impact on the rate of protein hydrolysis. *Food Hydrocolloids*, 52, 478–486.