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Food-dependent set-up of the DiDGI® dynamic *in vitro* system: Correlation with the porcine model for protein digestion of soya-based food

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ABSTRACT

The present study compared *in vivo* protein digestion in a miniature pig model with the dynamic *in vitro* system DiDGI®, using three digestive compartments (stomach, duodenum, and jejunum + ileum). Two soya-based meals—commercial soya milk and tofu—were studied, each with the same macronutrient content but different macrostructures. Our aim was to first deduce from the *in vivo* experiments in pigs key digestive parameters such as gastric pH, stomach emptying kinetics, and intestinal transit time, in order to design a relevant set-up for the dynamic *in vitro* system. Then, we compared digestive samples collected at fixed sampling times from both *in vivo* and *in vitro* models regarding different values related to proteolysis. We observed similar evolutions of gastric peptide distribution and duodenal proteolysis between models. Overall, apparent ileal digestibility of nitrogen was similar *in vitro* and *in vivo* and the differences between the two meals were conserved between models.

1. Introduction

Animal experiments are increasingly difficult to perform due to the economic resources they require and the ethical concerns that must be addressed. Despite this, it is still standard—indeed, recommended—for assessments of protein and amino acid digestibility to be conducted using human or pig models (FAO, 2013). *In vitro* systems could present suitable alternatives, but they first must be shown to mimic as much as possible real physiological conditions and must be validated using *in vivo* data. For example, Rozan et al. (1997) examined quantitative correlations between digestion end-points obtained *in vivo* and *in vitro*, specifically by comparing true digestibility assessed in rats with the final degree of hydrolysis measured using the pH-stat method. Other studies have gone further by comparing different *in vitro* protocols and methods; for example, the true digestibility of chickpea quantified in rats was found to correlate better ($R^2 = 0.6785$, $p = 0.0640$) with the degree of hydrolysis determined using O-phthalaldehyde after incubation with pepsin/pancreatin (Tavano, Neves and da Silva Júnior, 2016). Similarly, evaluations of *in vitro* static nitrogen or amino acid digestibility—in which samples were either filtered with a 1.2-μm filter (Millipore) (Saunders, Connor and Booth, 1973) or a 1 kDa-cut off dialysis bag (Vachon et al., 1987; Rozan et al., 1997)—have found favourable correlations with different nutritional quality indicators of proteins measured in rats. Instead, correlations between the apparent ileal digestibility of amino acids obtained from pigs and *in vitro* static amino acid digestibility (after filtration of the samples on a glass filter, pore size 40–90 pm; Cho & Kim, 2011) were mixed, with the coefficient of correlation variable from one amino acid to another. Proteolysis has also been examined *in vitro* using enzyme-linked immunosorbent assays (ELISA; Ménard et al., 2014) or by LC-MS/MS identification of released peptides (Egger et al., 2015). In these studies, though, comparisons with *in vivo* results were globally affected by a high degree of inter-individual variability, which forced the authors to differentiate each animal in the presentation of the results. This complicated the analysis, because it was difficult to determine if a strong correlation was due to the quality of the simulation or an artefact of the high *in vivo* variability (Walther et al., 2019). In general, the number of possible set-ups to replicate digestion *in vitro* is large, and a major remaining challenge is to identify those that are most physiologically relevant (Bohn et al., 2018). To address this, the INFOGEST network recently published standardised

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approaches for static protocols, i.e. those that do not take into consideration the kinetics of digestion (Brodkorb et al., 2019). However, no such consensus has yet emerged for dynamic systems of digestion. Indeed, these models are even more complex than their static counterparts, and require extensive knowledge of how digestive physiology changes over time (*e.g.*, gastric emptying kinetics or pH changes). To address this challenge, different teams have designed multiple systems that aim to replicate these dynamic conditions *in vitro*. Of these, the Vatier (Nunez, Bichot and Paris, 1998) and the Mainville (Mainville, Arcand and Farnworth, 2005) models consist of a succession of reactors, each connected to the next, which mimic the stomach and other compartments of the small intestine. More recently, the DiDGI® (Digesteur Dynamique Gastro-Intestinale; Ménard et al., 2014 or the ESIN (Engineered Stomach and small Intestine; Guerra et al., 2016) systems were developed on the same basis. The most well-known system is the TIM-1 (Minekus, 2015), which also simulates contractile aspects of digestion. However, only limited data exist that assess the correlation of these *in vitro* systems with *in vivo* models, and most of these data focus on the digestion of milk proteins. To our knowledge, correlations between *in vivo* and *in vitro* digestion of plant protein have only been performed using static *in vitro* models and *in vivo* data from the rat model. However, the rat model has several drawbacks with respect to mimicking human digestion, as the eating behaviour of rats and their physiological parameters of digestion are not directly comparable to those of humans (Davies and Morris, 1993). Instead, the gastrointestinal conditions in pigs are much more similar to those of humans, both anatomically and physiologically (Henze et al., 2018) and for this reason, the porcine model should be preferred for studies of protein digestion and digestibility (FAO, 2013).

The aim of this study was to examine the correlation between a porcine model of food-protein digestion and a dynamic *in vitro* gastrointestinal system, the DiDGI®. Specifically, we used data from three sets of experiments on cannulated mini-pigs (gastric, duodenal, and ileal) to (i) determine optimal parameters for the set-up of the *in vitro* system (pH, chyme transfer) and (ii) compare the evolution of digestion in both *in vivo* and *in vitro* models, specifically with respect to dry matter content, local apparent degree of proteolysis, and molecular weight distributions of peptides. This latter goal was accomplished by comparing the digestion of two soya-based foods (soya milk and tofu) in the DiDGI® and in mini-pigs. Ileal digestibilities were also obtained from both models and compared.

2. Materials and methods

2.1. Test meals

Two soya-based food matrices were tested: commercial tofu and commercial soya milk. Soya milk was UHT- (Ultra-High Temperature) packed. Compositions of foodstuffs have been previously published (Reynaud, Lopez, Riaublanc, Souchon, & Dupont, 2020; Binnerts, Van't Klooster, & Frens, 1968). Food matrices were supplemented with maltodextrin, sugar, and soya oil (Emile Noël, France) in order to standardise the amounts of protein (30.0 g), fat (23.1 g), and calories (980 kcal) in each diet (Table S1). The meals used for the DiDGI® experiments had the same nutrient proportions (7.5 g of protein, 5.8 g of fat and carbohydrates for a total of 245 kcal).

Dry matter content (DM) was determined by oven-drying at 105 °C overnight. Crude fat content was determined using the Randall method after 12 h incubation in 1 M HCl (ISO, 2008). Total nitrogen content was measured using the Kjeldahl method according to Thiex, Manson, Anderson, and Persson (2002). To calculate crude protein content, conversion factors of 5.36 for pea proteins, 5.50 for soya, and 5.50 for wheat proteins were used according to (Mariotti, Tomé and Mirand, 2008). Starch content was measured using a polarimeter (European Commission, 2009). Gross energy was calculated from macronutrient content using standard conversion factors.

2.2. In vivo digestion study on mini-pigs

All procedures were conducted in accordance with European guidelines (Directive 2010/63/EU) and were approved by the Auvergne Animal Experimentation Ethics Committee (CEMEAA) and the French Government. Yucatan mini-pigs were housed in subject pens in a ventilated room with controlled temperature (21 °C). Between sampling days, they received 500 g/d of a standard diet for minipigs, distributed in two equal portions at 8:00 and 16:00, and had free access to water. They were divided into three groups.

2.2.1. Gastric group

The gastric test included four mini-pigs (weight = 24.9 ± 1.2 kg, 8 months old). Three weeks before the trials began, mini-pigs were surgically fitted with a cannula (silicone rubber; 12-mm internal diameter, 17-mm outer diameter) at the greater curvature in the lateroventral region of the corpus. Digesta were sampled a minimum of 5 min before ingestion, then 20, 40, 60, 90, 120, 180, 270, 360, and 450 min after ingestion of the meal. To each sample, 10 µl of pepstatin A solution (P5318, #027M4005V, Sigma-Aldrich, USA) were added per ml of digesta, at a concentration of 0.5 $mg.ml^{-1}$ in methanol/acetic acid (9:1). Samples were then placed in liquid nitrogen and stored at −20 °C.

2.2.2. Duodenal group

The duodenal test involved six mini-pigs (weight = 24.7 ± 0.4 kg, 8 months old). Three weeks before the experiment, a cannula (silicone rubber; 12-mm internal diameter, 17-mm outer diameter) was fitted on mini-pigs 5–10 cm under the pyloric sphincter, as described previously by Bauchart et al. (2007). Each meal contained a Cr-EDTA solution (30 ml, 2.77 $mg_{Cr}.ml^{-1}$) as a liquid marker (Binnerts et al., 1968), and 450 mg Ytterbium-acetate as a solid-phase marker (Siddons et al., 1985).

Duodenal sampling was performed a minimum of 5 min before ingestion and then 20, 40, 60, 90, 120, 180, 270, 360, and 450 min after ingestion of the meal. The pH of the collected duodenal content (15 ml maximum) was measured with an IFSET probe (1001–004 ISFET, Sentron, Holland), then the sample was divided into two parts. One part was freeze-dried (Pilot Freeze Dryer, Cryotec, France) for marker quantification; the other part was blocked with 1 mM final concentration of Pefabloc SC (AEBSF) (24305500, Roche, USA) diluted in distilled water. Then, the blocked mixture was vortexed for 5 s, placed in liquid nitrogen and stored at −20 °C until analysis.

The freeze-dried portions of the collected duodenal contents were also used to determine the dry matter content (DM) of duodenal samples (based on the weight difference before and after freeze-drying).

Freeze-dried duodenal contents were then ground and prepared for microwave plasma-atomic emission spectrometry (MP-AES) with successive steps of mineralisation (550 °C, 6 h), nitric acid dissolution (3 min boiling in 5% HNO_3^- (w/w)), and filtration (Whatman filter 4–7 µm). The amount of chromium-labelled indigestible content was then determined with a 4210 MP-AES apparatus (Agilent). The percentage of markers recovered was calculated by multiplying the measured content of the marker by the fixed volume sampled from the cannula (12 ml) and dividing this by the initial amount of the marker that was present in the meal.

2.2.3. Ileal group

The ileal test involved six mini-pigs (weight = 20.2 ± 1.5 kg, 8 months old). Three weeks before the experiment, mini-pigs were surgically fitted with a cannula (silicone rubber; 12-mm internal diameter, 17-mm outer diameter) at their distal ileum (10 cm before the ileocecal valve). Chromium oxide (Cr_2O_3) was used as an indigestible transit marker, and was added up to the amount of 0.3% of meal dry matter content.

Contents were collected between 1 and 9 h after the distribution of

meals in pre-weighed plastic bags (Sachet Whirl-Pak, 120 ml, 7.5 × 18.5 cm) attached to the ileal cannula. Full bags were replaced with new ones as necessary. Content was immediately transferred to aluminium dishes (previously weighed) and stored at −18 °C. Samples were pooled in 1-h pools and stored at −20 °C.

Ileal samples underwent the same treatments as the freeze-dried duodenal samples, i.e. freeze-drying, grinding, and mineralisation, to enable chromium quantification in samples using microwave plasma-atomic emission spectrometry (4210 MP-AES, Agilent). Then, a representative sample of the entire postprandial period (per animal-meal combination) was constituted using a fixed percentage (DM basis) from each batch of collected effluent (hourly), starting from the first detection of chromium.

2.3. Determination of operating parameters for the DiDGI® dynamic in vitro system

2.3.1. Gastric emptying equation

Gastric emptying was modelled according to Elashoff's equation (Elashoff, Reedy and Meyer, 1982) and parameters were determined for each meal from mineral data collected through the duodenal cannula.

For each timepoint, the total amount of marker collected up to this time was summed. This function is thought to follow the equation:

$$f(t)=A(1-2^{-(\frac{t}{t_{1/2}})^{\beta}} \quad (1)$$

where A is the maximum percentage of marker collected compared to initial marker intake; β is the shape of the curve; and $t_{1/2}$ is the time at which 50% of the meal has been emptied from the compartment (here the stomach). The parameters of interest are $t_{1/2}$ and β, from Elashoff's equation. Our model assumed that the flux of chyme was continuous and homogeneous. To avoid aberrant regression, samples that did not demonstrate a plateau in their kinetics were removed from the analysis; after this filtering step, at least five animals per meal and per marker were retained for analysis. Regression parameters are presented in Fig. 1A.

2.3.2. Intestinal transit flow

Transit of the bolus from the mouth to the ileal cannula was estimated with formula (1). In initial tests, the plateau of recovered chromium was not reached (Fig. 1B), so we set A (the maximum percentage of marker collected compared to initial marker intake) to 0.5. This can be interpreted as the hypothesis that the maximum content that could be recovered through the ileal cannula was 50%.

2.3.3. pH in the stomach and duodenum

We used food-dependent gastric acidification equations from a previous study (Reynaud, Buffière, et al., 2020), using the mean data of pH catheter probes. Duodenal kinetics of pH were deduced from a non-linear regression of the pH measured in duodenal samples (Fig. S1).

2.3.4. Endogenous secretions

We simulated the saliva and basal gastric fluid present in the stomach when the chyme arrived by adding 12 ml of simulated saliva fluid (SSF) and 40 ml of simulated gastric fluid (SGF) to the initial meal. These solutions were prepared according to (Minekus et al., 2014) and quantities were determined based on euthanasia data (data not shown).

These amounts of endogenous secretions came from the recommendations of INFOGEST (Minekus et al., 2014). Therefore, in order to maintain the same global ratio of protein:protease from one meal to another, enzymes were injected as follows:

In the stomach, the total amount of pepsin units (P6887, #SLBV3035, Sigma-Aldrich, USA) was 80 000 per gram of food protein and the speed of injection was 0.5 ml.min^{-1} (concentration of the stock solution: 3333 U_{pepsin}.ml^{-1}). In the duodenum, the total amount of trypsin units from pancreatin (P7545, #SLBV6830, Sigma-Aldrich, USA) was 4 000 per gram of food protein and the speed of injection was 0.25 ml.min^{-1} (concentration of the stock solution: 333 $U_{trypsin}$.ml^{-1}). Biliary acids solution (B8631, Sigma-Aldrich, USA) was secreted at 0.5 ml.min^{-1}. For the first 30 min, a 40 mmol.l^{-1} solution of biliary acids was used, then the concentration was reduced to 20 mmol.l^{-1}.

2.4. In vitro digestion study using DiDGI®

2.4.1. The dynamic three-compartment model of the gastrointestinal tract

This study used a three-compartment system instead of the original two-compartment system described in (Ménard et al., 2014). In the original system, the two compartments represent the stomach and the small intestine, respectively; to this set-up, Adouard and colleagues (2016) added a third compartment to split the small intestine into 2 independent sections i.e. the duodenum and the jejunum + ileum (Adouard et al., 2016). In our experiment, the third compartment emptied into a receptacle, the contents of which were analogous to those collected though the ileal cannula of mini-pigs.

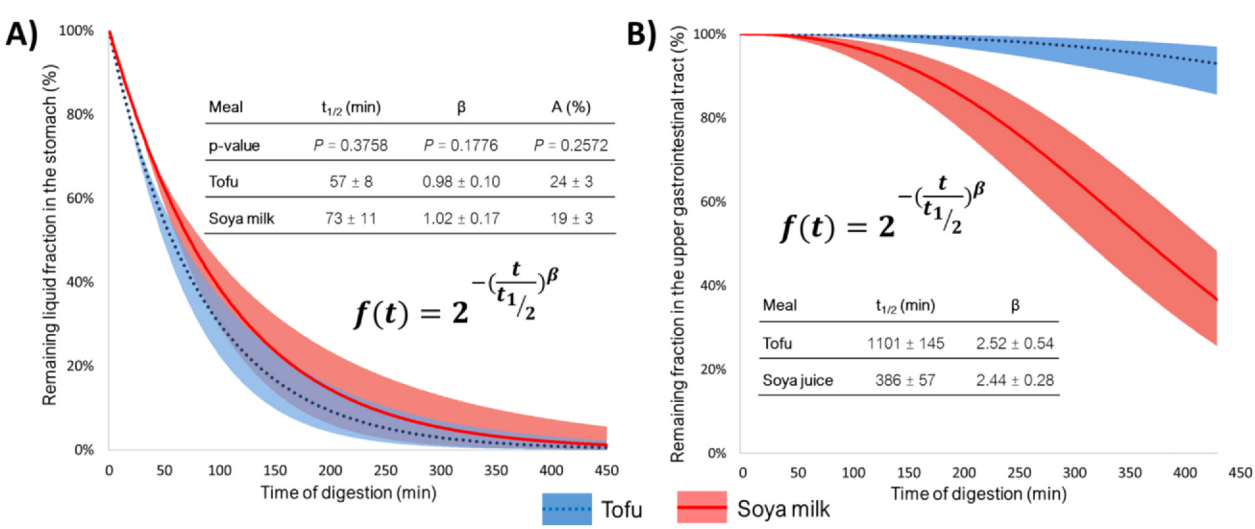

Fig. 1. A) Parameters of gastric emptying (MEAN ± SEM; n = 5–6). Mixed model ANOVA was performed on regression parameters (β, t1/2, A), with animal as random effect and meal as fixed effect. No significant difference was observed between soya milk and tofu meals. B) Parameters of the intestinal flow after tofu meal (broken blue curve) or soya milk meal (solid red line) ingestion (MEAN ± SEM; n = 5–6). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Parameters of the DiDGI® digestion.

Parameters	Soya milk	Tofu
Time of digestion	360 min	
Temperature	37 °C	
Gastric pH equation	$1.8 + \frac{5.5}{1+exp(0.40(2-t))} + exp(0.04(t-174))$	$1.5 + \frac{4.7}{1+exp(0.40(3-t))+\exp(0.02(t-9))} + \frac{1.5\times\ln(1+\exp(-t/300))}{\ln(2)}$
Duodenal pH equation	$2.86 + \frac{3.41}{(1+191.26)^{0.89}}$	
Gastric emptying equation	$2^{(\frac{-t}{73})^{1.02}}$	$2^{(\frac{-t}{57})^{0.98}}$
Fixed volume of the duodenum	50 ml	
Intestinal flow equation	$2^{(\frac{-t}{386})^{2.44}}$	$2^{(\frac{-t}{1101})^{2.52}}$
Pepsin secretion	600 000 U_{pepsin} over 360 min (0.5 ml.min^{-1}) (P6887)	
Pancreatin secretion	30 000 $U_{trypsin}$ over 360 min (0.25 ml.min^{-1}) (P7545)	
Bile secretion	40 mmol.l^{-1} until 30 min, then 20 mmol.l^{-1} (0.5 ml.min^{-1}) (B8631)	

2.4.2. Digestion programme

The digestion programme was designed following consultation with available *in vivo* data and existing literature. It was food-dependent and is summarised in Table 1. pH was regulated with 0.2 N HCl and 1 N NaOH solutions.

2.4.3. Post-hoc treatment of ileal content for the simulation of intestinal absorption

All contents collected from the third DiDGI® compartment corresponded to the *in vivo* effluent arriving at the ileal cannula; in both cases, ileal contents were collected in aluminium plates on the same sampling schedule. At least once per hour of digestion, once the chyme reached the *in vitro* "ileum", contents were manually stirred and a 2-ml sample was collected and blocked with 1 mM final concentration of Pefabloc SC (76307, #117M4033V, Sigma-Aldrich, USA) diluted in distilled water. Samples were vortexed for 5 s and put on ice before being stored at −20 °C. The remainder of the ileal contents was frozen at −20 °C before being freeze-dried.

All of the frozen samples from a given digestion were pooled, homogenised, and centrifuged (5 000 G, 10 min, 20 °C). Pellets and supernatants were weighed, and total nitrogen content was determined (Kjeldahl method). Supernatants were then subjected to different methods for simulating intestinal absorption, described below. From those processes, we generated values for simulated-absorbed and simulated-non-absorbed fractions of ileal content.

2.5. Values to be correlated between in vivo and in vitro experiments

2.5.1. Dry matter content (DM) in duodenal and ileal sections

Dry matter content was determined from the difference between sample weights before and after freeze-drying, with the exception of the duodenal contents sampled from the DiDGI®, for which the oven method (105 °C, overnight) was used.

2.5.2. Proteolysis in gastric and duodenal sections

Determination of the concentration of α-amino groups, an indicator of proteolysis in the stomach and the duodenum, was carried out using the ninhydrin reaction described by (Moore and Stein, 1954). This technique was applied to gastric and duodenal samples that had been blocked immediately after they had been taken. The total nitrogen content of each sample was measured (Kjeldahl method) to calculate the "local apparent degree of proteolysis" (LADP) with the following equation, where the density of contents was considered to be 1:

$$LADP(\%) = \frac{AG\ in\ supernatant\,(mg(N).\ l^{-1})}{Ntot\ in\ sample\,(gN.\ 100g^{-1})} \times 10^{-2} \quad (2)$$

2.5.3. Molecular weight determination by size-exclusion HPLC (HPSEC) of gastric samples

Gastric samples taken from mini-pigs and from the DiDGI® prior to ingestion and at 20, 60, 90, and 180 min post-ingestion were analysed with respect to the molecular weight distribution of peptides. Samples were centrifuged (5 000 G, 10 min, 20 °C), diluted 1:10 in an trifluoroacetic acid (TFA):acetonitrile:water buffer (0.01:2:10 v:v:v), and then filtered through a 0.40-µm pore-size filter. Peptides were separated according to their molecular weight with a column (Shodex Asahipak GF-310HQ, Interchyme, France) that was connected to a Waters e2695 separation module equipped with a Waters 2998 UV/Visible detector with diode array (Waters Inc., USA). Elution was performed under an isocratic 0.2 ml.min^{-1} flow of TFA:acetonitrile:water buffer at 37 °C. Detection was performed at 214 nm. Sample injection volume was 30 µl. The column was calibrated by injecting 30 µl of five markers of known molecular weight, each at a concentration of 2 mg.ml^{-1}: blue dextran (2000 kDa) and carbonic anhydrase (30 kDa), cytochrome *C* (12.384 kDa), vitamin B12 (1.855 kDa), and tripeptide GlyGlyGly (189 Da). The resulting calibration curve (log molecular weight vs retention time) enabled us to distinguish among 13 ranges of molecular weight: > 35 kDa, 35–18 kDa, 18–10 kDa, 10–6 kDa, 6–3 kDa, 3–2.5 kDa, 2.5–2 kDa, 2–1.5 kDa, 1.5–1 kDa, 1–0.8 kDa, 800–500 Da, 500–204 Da, < 204 Da.

2.5.4. Protein digestibility

Using the data obtained from the mini-pigs fitted with ileal cannulas, the apparent and true ileal digestibility of crude protein and amino acids were determined (Reynaud et al., 2021). These reported values were used here to study the correlation between *in vivo*- and DiDGI®-generated data.

Total nitrogen content (elemental analyser, vario ISOTOPE cube, Elementar) of both the simulated–absorbed and simulated–non-absorbed fractions of the DiDGI® ileal content were determined.

Estimates of digestibility obtained from the DiDGI® system are hence expressed as the ratio $X_{simulated\ absorbed\ fraction}/(X_{simulated\ absorbed\ fraction} + X_{simulated\ non\text{-}absorbed\ fraction})$

2.5.5. Data & statistical analysis

Data are disclosed as mean ± SD (standard deviation) for *in vitro* data and as mean ± SEM (standard error of the mean = SD / √number of replicates) for *in vivo* data or To present the data, we chose to use the SEM for *in vivo* data because n = 4 − 6 and the variability is high. Bars on the graphics are reduce and are more readable. For *in vitro* data, we chose the SD because n = 3 and variability between replicates is moderate. Repeated measures ANOVA was performed on dry matter content (DM), local apparent degree of proteolysis (LADP), and molecular weight distributions to detect significant differences; the model included the fixed effects of model, food, time, model*time, model*food, food*time, and food*model*time. A post-hoc LSMEANS test

was performed when interactions were significant. Differences were considered statistically significant if the p-values were < 0.05. All statistical analyses were performed using SAS (SAS University Edition, Release 3.71; SAS Institute Inc., Cary, NC, USA). Modelling of marker data was performed with XLSTAT (XLSTAT Quality 18.07, Addinsoft, France). Gastric data were analysed with a principal component analysis (PCA) carried out in XLSTAT.

3. Results

3.1. Effect of the model on dry matter content

Dry matter content (DM) in the duodenum compartment of the DiDGI® was compared to the DM of samples taken from mini-pigs fitted with a duodenal cannula. Data are presented in Fig. S2. In both the *in vivo* and *in vitro* models, DM values globally decreased after the intake of soya milk and tofu (from 13% to 5% and from 20% to 5%, respectively). For both soya milk and tofu, there was a significant model*time interaction: for the digestion of soya milk, DM values were significantly different between *in vivo* and *in vitro* models at 270 min, and the same was true for the digestion of tofu at 120 and 180 min. Beside this, no significant differences were detected for DM values throughout the duodenal digestion. Generally speaking, observations made from the different models at a given time point were consistent with each other, with the exception of the instances noted above.

Next, we examined the kinetic evolution of DM values between samples taken from the ileal group of mini-pigs and those taken from the output of the DiDGI® (Fig. 1B). Because of the way the DiDGI® was set up, the tofu chyme arrived in the last compartment more slowly than the soya milk chyme, and the tofu-derived ileal content was collectable only starting from 300 min. A significant model*time interaction was detected for both soya milk ($p = 0.0113$) and tofu ($p = 0.0329$). The DM values of *in vivo* ileal samples were always lower than their corresponding *in vitro* ileal samples from the DiDGI®, but for the soya milk meal, these differences were statistically significant only for the samples taken at 120 min ($p = 0.0006$) and 180 min ($p = 0.0282$). For the tofu meal, instead, *in vitro* DM was significantly higher than *in vivo* DM for both sampling times available (300 min and 360 min, $p < 0.0028$). This result was expected, as *in vivo* digestion also includes absorption by the small intestine, which was not simulated during the *in vitro* dynamic digestion.

3.2. Effect of the model on local apparent degree of proteolysis

In the gastric phase (Fig. 2A & A'), the local apparent degree of proteolysis (LADP) was similar between *in vivo* and *in vitro* models for both meals; the only exception to this was found with the tofu meal sampled at 20 min, for which LADP differed *in vitro* and *in vivo* ($p = 0.0031$). Moreover, the effect of time was not significant, meaning that the kinetics were constant and LADP did not vary significantly as a function of time.

At the duodenal step of the soya milk digestion (Fig. S2B), significantly different LADP values were obtained from the DiDGI® and from mini-pigs only at the end of the assay, at 360 min ($p < 0.0001$). For the tofu meal (Fig. S2B), instead, the kinetics of the evolution of LADP were unchanged between *in vivo* and *in vitro* models (interaction effect: $p = 0.5954$). The LADP in the duodenum was determined for each *in vitro* replicate and animal/food combination between 20 and 180 min of digestion, and these values were plotted as a function of the dry matter content (DM) of the samples (Fig. 3). There was significantly more variation in duodenal DM (interaction food*time, $p < 0.0001$) with the tofu meal (5–23%) than with the soya milk meal (6–18%). For this reason, plotted values associated with the tofu meal were highly dispersed on the graphic, with a particularly visible distinction between DiDGI® replicates and the corresponding animal trials. Interestingly, at 20 and 60 min, tofu DM was similar between *in vivo* and *in vitro* assays, whereas LADP values obtained from the DiDGI® were lower than those from mini-pigs. At 120 and 180 min, the DiDGI® generated a tofu chyme that was less hydrolysed, and with a lower DM, than that found in mini-pig samples. For digestions of the soya milk meal, the largest differences between *in vivo* and *in vitro* tests were found at 20 and 180 min, at which time DM values were lower in the DiDGI® than in the mini-pig duodenum. At the end of digestion, the soya milk meal seemed to be more hydrolysed in the DiDGI® (8–11%) than *in vivo* (5– 8%). Overall, the two meals followed two different patterns of digestion in the duodenum (interaction food*time of LADP: $p < 0.0001$, and of DM: $p < 0.0001$): for soya milk, LADP increased as digestion progressed and the DM decreased, while for tofu, LADP decreased with DM as digestion progressed.

3.3. Effect of the model on the molecular weight distribution of gastric peptides

Table S2 presents the results of statistical analyses that examined the relative proportions of gastric peptides, as determined by their molecular weight, in the outputs of the *in vivo* and *in vitro* systems at 20, 60, 120 and 180 min after tofu or soya milk meals ingestion. Fig. 4 is a graphical representation of a principal component analysis (PCA) of the proportions of gastric peptides grouped by molecular weight plus the gastric LADP. Only fractions for which at least one significant interaction was detected were included; thus, data for the > 35 kDa, 500–800 Da, and 204–500 Da fractions were removed from the PCA. The x-axis, the first principal component, labelled F1, contributed 46.8% of the observed variability. It was positively correlated with the proportions of low-molecular-weight fractions (< 2.5 kDa) and negatively correlated with the proportions of higher-molecular-weight fractions (> 3 kDa) (Fig. 4A). The y-axis, the second principal component, labelled F2, contributed 21.5% of the observed variability and was positively correlated with the proportion of the 2.5–3 kDa fraction (Fig. 4A). In Fig. 4B to 4E, each sample is plotted on this plane in order to observe the evolution of the *in vivo* or *in vitro* digestions between 20 and 180 min. The first observation to note is that the variability among animals was higher than the variability between *in vitro* assays. Indeed, for each food and at each time, the DiDGI® replicates (of tofu and soya milk digestions in Fig. 4B and Fig. 4C respectively) group together, whereas the replicates of digestion in each pig were quite distinct (Fig. 4D and 4E). At 20 min of digestion, *in vivo* and *in vivo* data were on different side of the factorial plan: gastric peptides observed *in vivo* were larger (> 3 kDa) than those observed *in vitro* (< 2.5 kDa). Then, the distribution of gastric peptides tended to become similar along the digestion. As gastric digestion progressed, the proportion of 2–3 kDa peptides increased globally, so that most assays reached the same area on the factorial plane at 180 min. Exceptions were observed in two animals who ingested the soya milk meal. A high degree of divergence was observed at 20 min for *in vivo* data, but starting from 120 min, proteolysis patterns from most animals were similar to each other. No clear difference between meals was observed *in vivo* whereas in the *in vitro* digestion of the tofu and the soya milk meals, the relative proportion of the 2–3 kDa fraction appeared to increase more slowly for the soya milk meal than for the tofu meal.

In particular, proportions of three fractions—1.5–2 kDa ($p = 0.0070$), 1–1.5 kDa ($p = 0.0003$), and 0.8–1 kDa ($p < 0.0001$)—evolved differently over time in the two models (Fig. S4 C, D, and E). In each case, the DiDGI® yielded decreasing or more-stable values than mini-pigs. In analysing the fractions, we also noted the following interesting results:

- In the 10–18 kDa fraction (Fig. S4 A), there were significant effects of the interactions model*food ($p = 0.0347$) and model*food*time ($p = 0.0499$), but not of the interactions food*time ($p = 0.4915$) and model*time ($p = 0.1323$). Indeed, the proportion of this fraction increased over time in the DiDGI® but remained constant in the

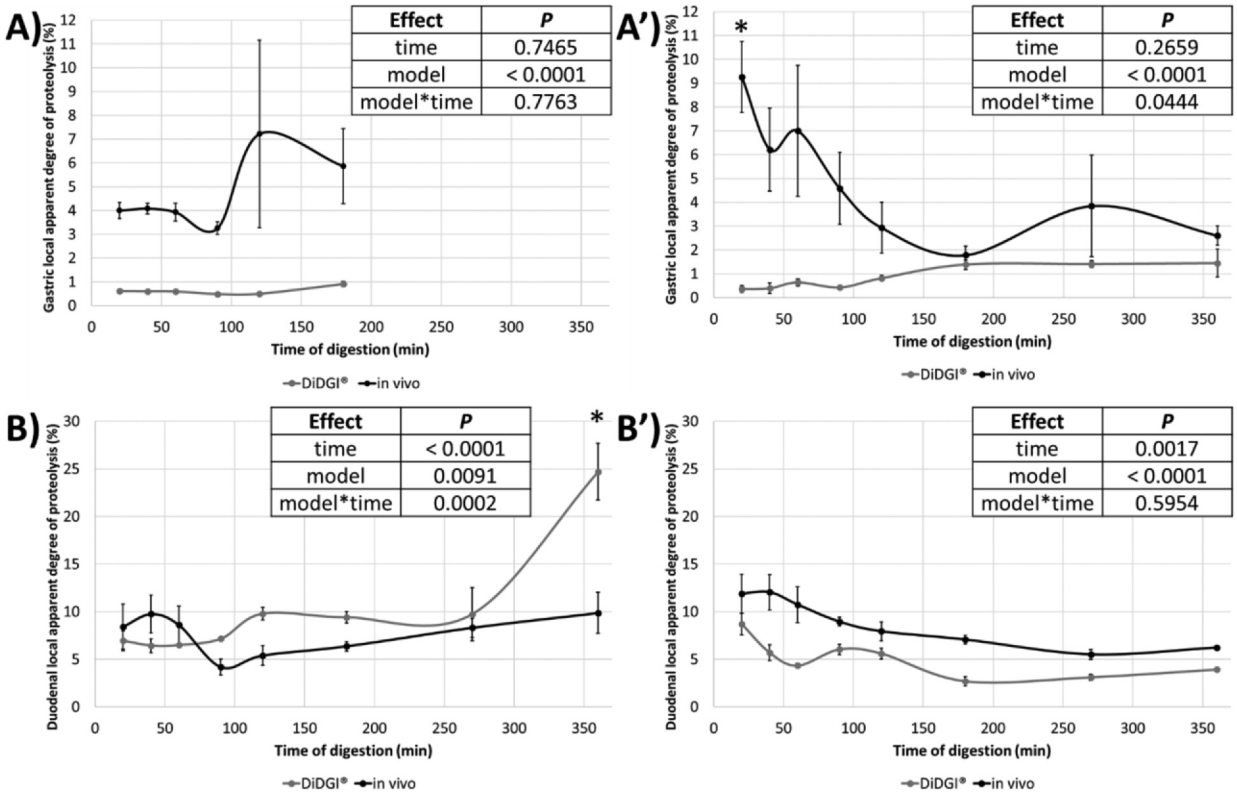

Fig. 2. Kinetics of proteolysis in the stomach and the duodenum, as measured by the evolution of the local apparent degree of proteolysis (LADP) in gastric (A: soya milk meal and A': tofu meal) and duodenal samples (B: soya milk and B': tofu). Data are MEAN ± SEM; n = 4 animals in gastric assays; n = 6 animals in duodenal assays. Repeated measures ANOVA was performed, with model, time, and model*time as fixed effects. Significant interactions were found for gastric LADP following tofu input ($p = 0.0444$) and duodenal LADP following soya milk input ($p = 0.0002$); a post-hoc LSMEANS test was therefore performed. Stars above curves indicate a significant difference between models at the given sampling time ($p < 0.05$).

mini-pig model.

- In the 2.5–3 kDa fraction (Fig. S4 B), there were significant effects of the interactions model*time ($p = 0.0232$) and model*food*time ($p = 0.0439$), but not the interactions food*time ($p = 0.1674$) and model*food ($p = 0.9487$). Proportions of the 2.5–3 kDa fraction evolved differently between models, and the effect of the model differed between the tofu meal and the soya milk meal.
- In the 1–1.5 kDa fraction (Fig. S4 D), we detected the significant interactions model*time ($p = 0.0003$) and food*time ($p = 0.0015$), but the third-order interaction, model*food*time, was not significant ($p = 0.5582$). A compensation phenomenon occurred, in which different kinetics were observed between the tofu and the

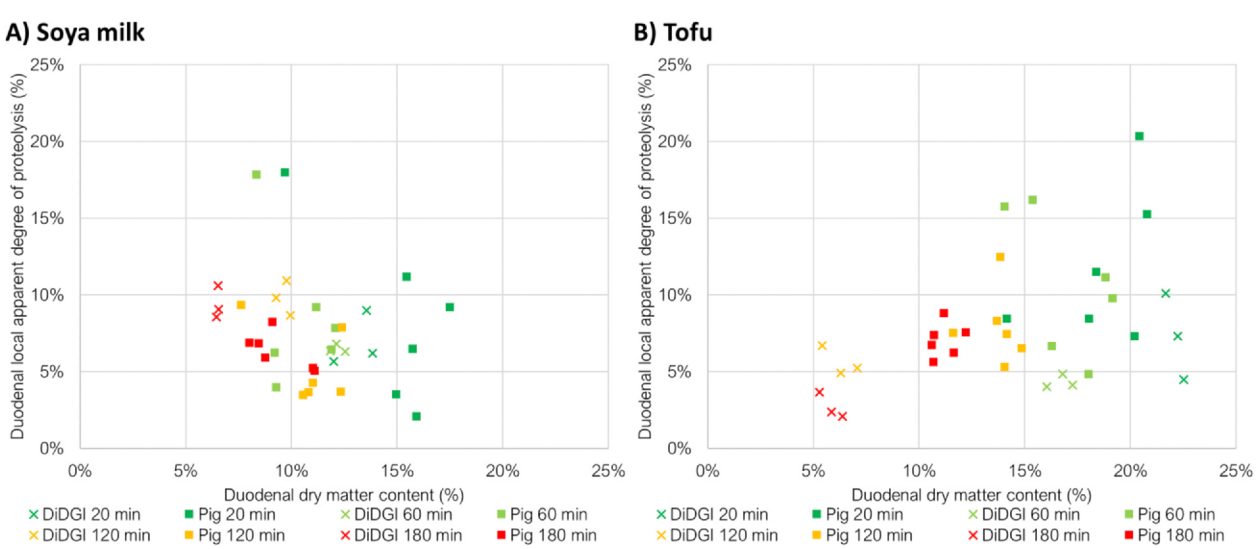

Fig. 3. Values for local apparent degree of proteolysis in the duodenum were plotted against duodenal dry matter content following ingestion of A) the soya milk meal or B) the tofu meal. DiDGI® data (3 repetitions) are represented by crosses and mini-pig data (6 animals) are represented by solid squares. Colours indicate the time elapsed since ingestion (dark green: 20 min; light green: 60 min; orange: 120 min; red: 180 min). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

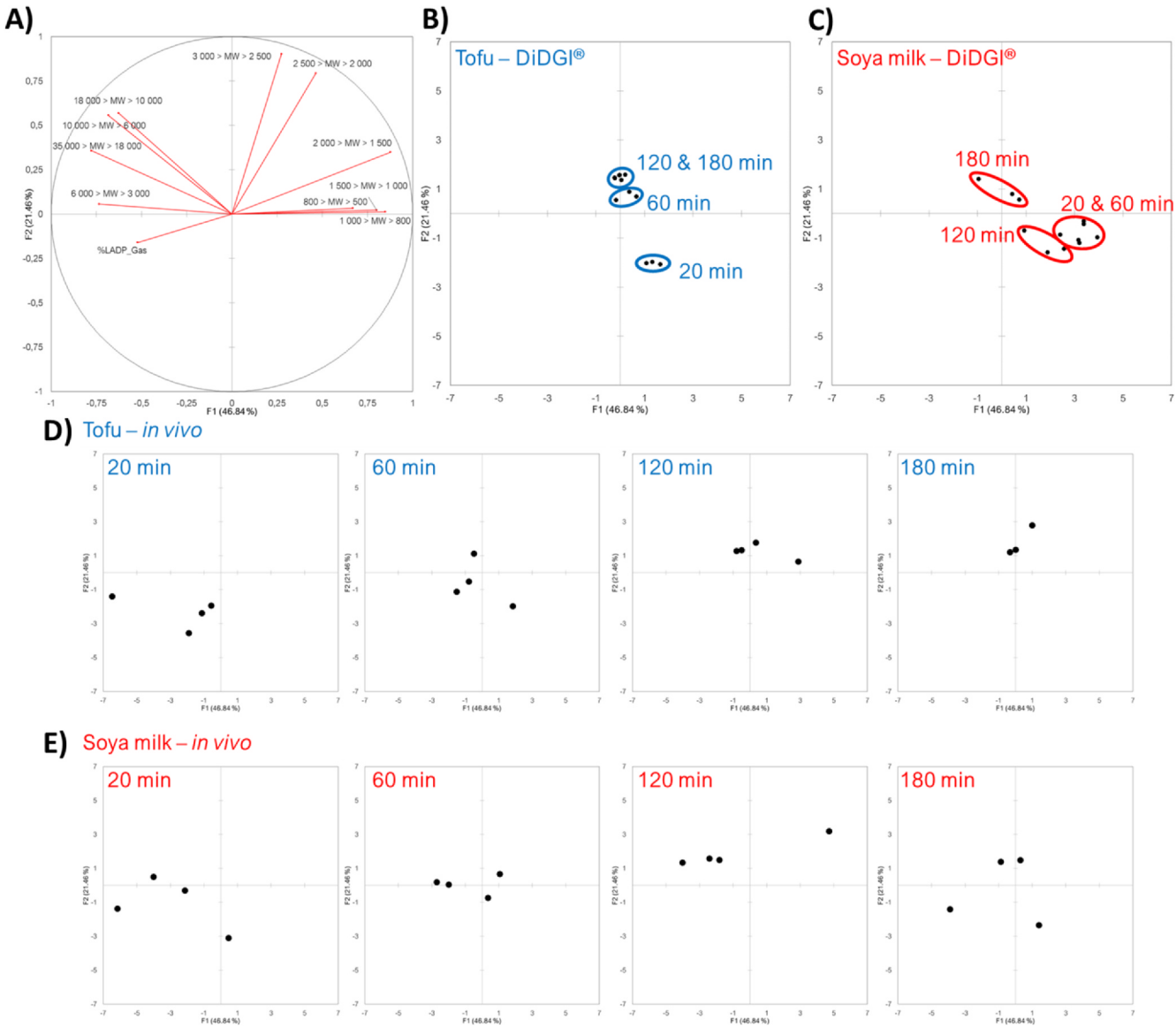

Fig. 4. Results from animal/*in vitro* trials visualised using a Principal Components Analysis (PCA) of the molecular weight distributions of peptide fractions and the local apparent degree of proteolysis in the stomach. A) Graphical representation depicting the circle of correlation and contribution of each variable to the x- and y-axes. B) Graphical representation of the tofu meal digestion at different times in the first compartment of the DiDGI®. C) Graphical representation of the soya milk meal digestion at different times in the first compartment of the DiDGI®. D) Graphical representations of the tofu meal digestion at different times in the stomach of mini-pigs E) Graphical representations of the soya milk meal digestion at different times in the stomach of mini-pigs. n = 4 animals (except for the tofu *in vivo* at 180 min where n = 3) and n = 3 *in vitro* assays.

soya milk meals in the DiDGI®, but not in mini-pigs. Furthermore, until 120 min, higher proportions of this fraction were detected in the soya milk meal in mini-pigs than in the DiDGI® or from the tofu meal in either model.

3.4. Effect of the model on ileal digestibility

Table 2 shows the *in vivo* and *in vitro* digestibility of the tofu and soya milk meals. For the *in vitro* calculations, the difference between endogenous and dietary nitrogen was not considered. Moreover, because no significant difference was observed between the two meals with respect to the true ileal digestibility *in vivo* (soya milk meal: 99.4 ± 2.4%; tofu meal:101.8 ± 8.9%), only the *in vivo* apparent ileal digestibility was compared with the *in vitro* digestibility of nitrogen. Although a significant effect was detected for food ($p = 0.0365$), there was no effect of model ($p = 0.4207$) or of the model*food interaction ($p = 0.5805$).

Table 2
Comparison of crude protein (N × 6.25) digestibility values obtained *in vivo* and *in vitro* for meals based on tofu and on soya milk. Values indicate percentage of crude protein (%) (MEAN ± SEM). A two-way ANOVA was performed on the *in vitro* and *in vivo* apparent digestibilities. p-values of effects were: model: $p = 0.4207$; food: $p = 0.0365$; model*food: $p = 0.5805$. Different letters (a and b) indicate significant differences ($p < 0.05$).

Model	Type of digestibility	Soya Milk	Tofu
in vivo	True	99.4 ± 2.2%	97.0 ± 3.9%
	Apparent	71.3 ± 2.5%[a]	56.5 ± 6.4%[b]
in vitro	Centrifugation	72.7 ± 1.4%[a]	63.7 ± 3.5%[b]

Indeed, when we performed a simple centrifugation of ileum content from the DiDGI®, and considered the supernatant as absorbed and the pellet as undigested, we obtained the same range of values for nitrogen digestibility as those obtained *in vivo*.

The difference in the apparent ileal digestibility of nitrogen measured *in vivo* between the soya milk meal (71.3 ± 2.5%) and the tofu meal (56.5 ± 7.8%) was also found using the DiDGI® system, with the *in vitro* digestibility of nitrogen from soya milk and tofu equal to 72.7 ± 1.4% and 63.7 ± 3.5%, respectively.

As the soya milk and the tofu meals were totally digestible when we examined the *in vivo* true ileal digestibility, we expect that the nitrogen measured in the pellet of *in vitro* ileal content likely came from endogenous secretions.

4. Discussion

Other studies have compared *in vivo* data from pig experiments with the nitrogen balance resulting from *in vitro* digestions that included a filtration/dialysis step to simulate intestinal absorption (Babinszky et al., 1990; Boisen and Fernández, 1995; Minekus, 1998; Cho and Kim, 2011). There have also been investigations focused on individual amino acids, which have yielded contrasting results depending on the amino acid under consideration (Boisen and Fernández, 1995; Pujol and Torrallardona, 2007; Jezierny et al., 2010; Cho and Kim, 2011). In the literature, most of the recent examinations of the correlation between *in vivo* and *in vitro* protein digestion have used milk proteins, which are almost completely digested at the ileum stage (Minekus, 1998). For this reason, efforts have been made to compare the evolution of proteolysis throughout digestion and not only the end results (Egger et al., 2015). Here, we aimed to build on these previous works by investigating the condition of proteins throughout the digestive process.

In the ileum, proportions of *in vivo* digestible nitrogen and *in vitro* simulated-absorbed nitrogen were similar, and the difference between the soya milk and tofu meals was conserved from one model to another. Early studies correlating measures of pH drop *in vitro* with the apparent or true fecal digestibility of protein *in vivo* were performed on a large spectrum of foodstuffs, including plant-based foods (Hsu et al., 1977; Perdersen and Eggum, 1981), but these results were shown to be highly dependent on the individual buffering capacity of a food created by its mineral and protein content (Moughan et al., 1989). Later, Ihekoronye (1986) compared the final degree of hydrolysis *in vitro*, measured with TNBS, with measures of apparent fecal digestibility obtained from rats (Satterlee, Kendrick and Miller, 1977) and in doing so found similar values for textured soya protein (*in vivo*: 83.3%, *in vitro*: 83.7%) and solubilised soya flour (*in vivo*: 78.9%, *in vitro*: 80.0%). Here, instead, the apparent digestibility of our two soya-based food products was markedly lower than those previously published values: 10 points lower for soya milk, 20 points lower for tofu. Such a difference could originate from numerous factors, such as differences between the models used or in the food items studied.

In the duodenum, differences in DM kinetics might have arisen from different sources, such as inappropriate patterns of gastric emptying and/or dilution of the chyme in endogenous secretions (basal and postprandial, gastric and duodenal). *In vivo*, we expected a higher amount of endogenous secretion because of the contributions of mucus and hormone secretions, as well as cell desquamation, that were not considered in *in vitro* assays. The differences observed in LADP may have been due in part to variations in the quantity of digestive enzymes secreted (pepsin and pancreatin) and the dynamic of bolus transfer from one compartment to another. The most important factor, though, may have been changes in the local ratio of protease to protein. We had expected that the concentration of endogenous nitrogen compounds would be higher in the mini-pig duodenum than in the second compartment of the DiDGI®; however, no significant difference was observed between models with respect to the total nitrogen content of gastric samples (0.1–0.7 gN.100 g^{-1}) or duodenal samples (0.1–0.4 gN.100 g^{-1}). Moreover, in mini-pig experiments, the position of the duodenal cannula was set between the pylorus and the common bile/pancreatic ducts. This meant that, in *in vivo* duodenal samples, pancreatic enzymes were perhaps not even present at their maximal levels.

Some studies have investigated correlations of protein digestion between an *in vivo* model and a dynamic *in vitro* system by comparing transfers and pH modification through the digestive process (Dupont et al., 2019). Minekus (1998) examined the delivery and the absorption of dietary nitrogen in the different compartments of the system and compared this with marker data obtained from cannulated animals. In that study, though, protein hydrolysis was investigated only indirectly, by analysing the absorption of nitrogen (Minekus, 1998). Instead, Chiang and colleagues (2008) investigated the gastric digestibility of protein by TCA precipitation *in vivo* and in a dynamic *in vitro* system, and found a strong correlation between the two models ($r = 0.97$; $p = 0.0001$). TCA precipitation is a good first step to evaluate the state of proteins in a mixture, but here we went further by looking at the degree of hydrolysis of proteins and at the distribution of peptides by their molecular weights.

In the stomach, we were unable to precisely track the evolution of the local apparent degree of proteolysis of the tofu meal due to the high inter-individual variability in animals, the low number of mini-pigs involved, and the nature of the sampling scheme. Thus, the main comparisons that we were able to make between the tofu and soya milk meals with respect to gastric digestion were based on the molecular weight distributions of peptides. Here, the interaction food*time had a significant effect on the proportions of the 1–1.5 kDa and free amino acid fractions (< 204 kDa). Moreover, the 0.8–1 kDa and 204–500 Da fractions; *in vivo*, were more abundant in the soya milk meal than in the tofu meal. As final point of discussion, the tofu left the stomach more rapidly than the soya milk. It is because of the difference regarding the gastric emptying equations. However, after soya milk intake, gastric content was collectable from the DiDGI® for only 180 min, while *in vivo*, this content was collectable for 450 min. According to the recovered markers, at 180 min most of the soya milk chyme had emptied from the pig stomach, and content sampled after that time originated mostly from endogenous secretions.

5. Conclusion

In the present study, we elaborated a food-dependent digestion programme for the DiDGI® based on experimental data obtained from mini-pigs. We used this to then investigate the correlation between these two models with respect to the dry matter content in the duodenum and the ileum, the local apparent degree of proteolysis in the stomach and in the duodenum, and the molecular weight distribution of gastric peptides. Globally, no clear correlation was found at the gastric step because of the inter-individual variability of pigs and an inadequate release of pepsin in the DiDGI®. However, at a later stage of digestion, when the stomach was almost emptied, proteolysis patterns were largely consistent between models, especially for the tofu digestion. Differences were found in individual parameters in the duodenal phase, but the global kinetics were consistent from one model to another. Finally, from the ileal phase, we obtained similar values for nitrogen digestibility in both *in vivo* and *in vitro* models.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author Contributions

Conceptualization: YR, DD, DR, IS, DP and ML, Data curation: YR, Formal analysis: YR, Funding acquisition: DD, DR, IS, DP and ML, Investigation: YR, AC, DF, and AM, Methodology: DD, IS, DP, ML, AC and YR, Supervision: DD, DR, IS, DP and ML, Visualization: YR, Writing - original draft: YR, Writing - review & editing: All authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128276.

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