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Myriam M.-L. Grundy, Julien Labarre, Frederique Mayeur-Nickel, Jaap J. van Milgen, David Renaudeau. An in vitro and in vivo approach to characterise digesta from pigs fed different forms of pea flour. *Journal of Animal Science*, 2023, 101, pp.skad037. 10.1093/jas/skad037 . hal-03977449

**HAL Id: hal-03977449**

**<https://hal.science/hal-03977449v1>**

Submitted on 16 Mar 2023

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# An in vitro and in vivo approach to characterize digesta from pigs fed different forms of pea flour

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## Abstract

In vitro models of digestion are useful tools to explore the behavior of dietary fiber sources in gastrointestinal conditions. To evaluate the validity of our digestion model, digesta obtained in vivo and in vitro were characterized and the impact of cell wall integrity on protein bioaccessibility and digestibility evaluated. Six cannulated barrows [Pietrain × (Large White × Landrace)] were included in a 2 × 2 Latin square design where they were fed two diets identical in chemical composition but differing in nutrient bioaccessibility. Pea was given either as flour (R1, most proteins encapsulated by intact cell walls) or reconstituted flour (R2, mixture of proteins and purified, broken cell walls). Digesta were collected at the duodenal and ileal cannulas at regular interval and after slaughtering, following ingestion of either R1 or R2. The two diets were also digested in vitro using a static gastrointestinal model. The original pea ingredients as well as the digesta collected in vivo and in vitro were characterized (i.e., particle size measurement, microscopy observations and gel electrophoresis) and then compared with each other. The degradation of the pea ingredients differed greatly between the two forms of flour, where particles filled with nutrients were recovered at the latest stage of R1 intestinal digestion as observed with the particle size distribution and the microscopy images. These results were consistent with the in vivo and in vitro digestibility analysis that showed lower protein hydrolysis for R1 than that for R2 (about 19% difference in protein digestion regardless of the method). Overall, great similarities were found between the digesta collected in vivo and in vitro, especially regarding the particle size measurements. To summarize, a substantial proportion of the proteins contained in R1 was retained within the pea cells following gastrointestinal digestion. These encapsulated proteins reduced the amount of amino acids and small peptides available for absorption. This mechanism will have consequences on postprandial metabolism of amino acids and bacterial population based on the delivery form of the dietary fiber.

## Lay Summary

Although dietary fiber plays an essential role in the gastrointestinal health of pigs, it can also compromise the digestion and absorption of nutrients, especially of proteins. New ingredients such as pulses can be both good sources of protein and fiber, with no harmful effect for the animal or the environment, provided they are given in an adequate form (more or less structured). The objective of this work was to investigate how the dietary fibers (intact or broken down, encapsulation mechanism) of a pulse, pea, influenced the digestion of proteins. The approach of this study consisted in combining in vitro and in vivo studies with biochemical and biophysical techniques to determine how dietary fiber affected protein digestibility in pea flour. The results of this study showed good agreement between in vivo and in vitro data. Overall, breaking down of the dietary fibers led to 19% increase in protein digestion. These findings demonstrated that the form of ingestion of dietary fibers is crucial to optimize protein digestion. Moreover, our in vitro model of gastrointestinal digestion was capable of simulating pea degradation in pig during digestion and provide a good estimate of protein hydrolysis.

**Key words:** cell wall, encapsulation, in vitro model, pea, pig, protein digestibility.

**Abbreviations:** ADF, acid detergent fibre; ADL, acid detergent lignin; CP, crude protein; DM, dry matter; GB, gastric phase without enzyme; GD, gastric phase with enzymes; IB, intestinal phase without enzyme; ID, intestinal phase with enzymes; IDF, insoluble dietary fibre; OPA, o-phthalaldehyde; PSD, particle size distribution; R1, “regular” pea flour; R2, reconstituted pea flour; SDF, soluble dietary fiber; SDS-PAGE, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; TCA, trichloroacetic acid.

## Introduction

The use of alternatives of soybean as a sustainable source of protein in animal diets is a subject of increasing attention. Indeed, the reluctance towards genetically modified plants, the dependency of European Union (EU) on imported soybean from non-EU countries and environmental concerns, including greenhouse gas emission and deforestation, leads to search for alternative, local sources of protein while maintaining growth performance and carcass traits (Wilkinson and Young, 2020; Djuragic et al., 2021). In this context, legumes,

such as pea, is known to be an attractive alternative to soybean meal in pig feed given their protein content (White et al., 2015). However, pea and its by-products contain dietary fibers that can have a negative impact on nutrient digestibility and energy uptake from the feed by the pig (Buraczewska, 2001; Noblet and Le Goff, 2001).

The mechanisms by which fibers that compose pea cell walls influence protein digestion are not yet fully understood. Because pea cell walls are mainly composed of insoluble polysaccharides (i.e., insoluble pectin and hemicellulose), in an aqueous medium such as the lumen of the digestive tract,

Received October 15, 2022 Accepted January 25, 2023.

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they could encapsulate nutrients such as proteins (Talbot and Ray, 1992; Leterme et al., 1996). This encapsulation phenomenon influences the quantity of proteins released from the plant matrix (bioaccessibility), the rate at which they are hydrolyzed by digestive enzymes, the transit time, and the site of absorption of the products of digestion (Bach Knudsen et al., 2012; Grundy et al., 2016). Pea is, therefore, an interesting biological model for understanding how insoluble fibers interact with proteins and thus reduce their digestibility.

In vitro models of digestion could potentially be an alternative to experiments performed in vivo, the latter being costly, time consuming, and sometimes problematic from an ethical and animal welfare point of view. However, in vitro models have to reflect as closely as possible the events occurring in the gastrointestinal tract of the animals and thereby be able to predict accurately the digestibility of nutrients such as proteins.

This study aimed at investigating the impact of protein encapsulation by cell walls on their release from the pea matrix (bioaccessibility) and their hydrolysis by proteases using both in vitro and in vivo methods. Another objective was to validate our in vitro digestion model and our characterization approach to in vivo data. We hypothesized that using a diet with pea with the highest degree of intact cell walls (“regular” pea flour) would result in the lowest protein bioaccessibility and hydrolysis.

## Materials and Methods

### Animal ethics

The pigs came from the herd of the INRAE UE 3P experimental facility (INRAE, Saint-Gilles, France). All procedures used in this study were in compliance with the ethical standards of the European Community (Directive 2010/63/EU) and was approved by the regional ethical committee (Comité Rennais d’Ethique en matière d’Expérimentation Animale; authorization number: APAFIS#31859-2021060209568651 v3).

### In vivo experimental design

Six barrows [Pietrain × (Large White × Landrace)] were included in a 2 × 2 Latin square design study, replicated over two consecutive 14-day periods. At approximately 40 kg of body weight (BW; 38.4 ± 2.9 kg), the pigs were surgically fitted with two T-cannulas, at the proximal duodenum (20 cm after the pylorus) and terminal ileum (15 cm before the ileocecal valve) according to procedures adapted from Sauer et al. (1983). Following a week of recovery, pigs were housed in individual pens and offered the experimental diet six times a day (every 4 hours; R1 or R2, see description below). After a 10-day adaptation period to the diet, the duodenal and ileal digesta were collected at regular intervals for up to 12 h (two collection days per treatment or four collection days in total per pig). At the end of the experiment, the animals were sacrificed and the content of their digestive system collected (three animals per diet).

### Pea materials, experimental diets, and reagents

Pea (*Pisum sativum* L.) seeds, protein, starch, and fibers were provided by Roquette (Vic-sur-Aisne, France), see Table 1 for the chemical composition of the ingredients. The pea seeds were ground using a hammer mill fitted with a 2.5 mm grid to produce pea flour. The pea ingredients were included into two feed formulations of identical chemical composition but

of two different forms (degrees of encapsulation by cell wall): “regular” pea flour (R1, proteins encapsulated by mostly intact cell walls) and reconstituted pea flour (R2, proteins and purified, broken cell walls). The description of the two diets, including their chemical composition, can be found in Table 2. Briefly, R1 contained pea flour, pea starch (to match R2 composition), vitamins and minerals; whereas R2 contained pea fiber (broken cell walls), pea protein, pea starch, vitamins and minerals. R1 and R2 were given as flour to the animals and used in the same form during the in vitro experiments. Regular pea flour (i.e., R1 without vitamins, minerals, and marker) was also analyzed in vitro to evaluate the effect of including other compounds to the pea flour on protein hydrolysis.

Titanium dioxide (TiO<sub>2</sub>; 0.3%) was included as an indigestible marker for the digestibility calculations (see Eq. 2). The meals and digesta were prepared as described by Tsanatsidou and Zachariadis (2020); the nitric and sulfuric acid dilutions were applied. The TiO<sub>2</sub> present in the samples was then measured by inductively coupled plasma-atomic emission spectrometry.

Pepsin from porcine gastric mucosa (#P6887, 2145 U/mg of solid), bovine bile extract (#B3883), pancreatin from porcine pancreas (#P7545, 3.4 U/mg of solid based on trypsin activity) were purchased from Sigma (Saint Quentin Fallavier, France). All other chemicals, solvents, and reagents were from Sigma (Saint Quentin Fallavier, France) or Thermo Fisher Scientific (Illkirch-Graffenstaden, France).

### Simulated in vitro digestion

The pea flour and the two diets (R1 and R2) were digested using an adapted version of the standardized in vitro static digestion protocol developed by the Infogest consortium to simulate protein digestion in pigs (Brodkorb et al., 2019). After 2 min of incubation at 39 °C and pH 7 to simulate the oral phase, the samples were exposed to gastric (2 h at pH 3) and intestinal (4 h at pH 7) digestion. A set of digestion experiments without enzymes was also conducted to follow the release of proteins from the pea matrices (here defined as bioaccessibility). Details about the composition of the simulated fluids for each phase can be found elsewhere (Brodkorb et al., 2019). The protease reaction was stopped by raising the

**Table 1.** Chemical composition of the pea ingredients presented on a dry mass basis (DM)

	Flour	Protein	Starch	Fiber
Gross energy, MJ/kg DM	18.17	23.38	16.99	17.09
DM, %	87.68	95.80	89.26	95.65
N, %	3.85	13.25	0.11	1.25
CP, %, N × 5.4	20.79	71.53	0.57	6.75
Starch, %	49.04	0.10	92.26	36.14
TDF, %	19.58	19.32	–	52.13
IDE, %	13.75	13.29	–	47.99
SDE, %	5.83	6.03	–	4.14
NDF, %	7.24	3.69	–	23.35
ADF, %	1.75	0.09	–	8.29
ADL, %	0.06	0.03	–	0.03
Ash, %	3.71	4.30	0.13	3.06

**Table 2.** Ingredients (% as feed-basis) and chemical (% dry mass basis, DM) composition of the two diets<sup>1</sup>

Formulation	R1	R2
Ingredients %		
Pea	82.0	
Pea fiber		24.8
Pea protein		20.2
Pea starch	14.7	51.7
Dicalcium phosphate	1.1	1.1
Calcium carbonate	1.0	1.0
Premix 0.5%	0.5	0.5
Salt	0.4	0.4
Titanium dioxide	0.3	0.3
Total	100.0	100.0
Measured chemical composition, %DM		
GE, MJ/kg	17.3	17.8
Ash	3.0	1.8
Total nitrogen	3.15	3.15
Protein (N x 5.4)	17.0	17.0
Starch	53.6	55.3
TDF	18.2	18.2
NDF	5.9	6.8
ADF	1.4	2.2
ADL	0.04	0.03

<sup>1</sup> R1 = “regular” pea flour with mostly intact cell walls; R2 = reconstituted pea flour with broken cell walls.  
GE = gross energy

pH to 9 at the end of the gastric or intestinal phase. The samples were then centrifuged (2,500 × g at 4 °C for 10 min), the pellet washed, and filtered using a cell strainer (Falcon, 40 µm aperture). The washing step permitted to remove the enzymes and other proteins from the pepsin, pancreatin, and bile salt preparations. The washed pellets were then dried at 60 °C until constant weight and analyzed for protein (nitrogen) content using the Dumas method (see below). The supernatant was used for the analysis of the products of proteolysis with the *o*-phthalaldehyde (OPA) method (see section below, “Protein and proteolysis products assessment”). A separate set of digestion were carried out to characterize the digesta (particle size and microscopy) on fresh samples, after centrifugation. Each digestion was performed in triplicate.

### Chemical analysis

For chemical composition assessment of the in vivo samples, the digesta were frozen after collection and then freeze-dried. The crude protein (CP, measured as nitrogen) content of the ingredients, diets, and digesta was determined with the Dumas method (LECO, FP828 Carbon/Nitrogen/Protein Determinator, Villepente, France, AOAC 968.06); a nitrogen conversion factor of 5.4 was used (Mariotti et al., 2008). The ingredients and diets were also analyzed for starch (AOAC Method 2014.10), dry matter (AOAC 935.29), and ash (AOAC 942.05). Gross energy was determined with an adiabatic oxygen bomb calorimeter (C 6000, IKA-Werke GmbH & Co, Stauffen, Germany). Soluble and insoluble dietary fiber were measured to estimate total dietary fiber content (method AOAC 991.43; Megazyme kit assay, Megazyme, Product

Code: K-TDFR). Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) contents were determined using fiber bags (Model F57, Ankom Technology, Macedon, NY, USA) and a fiber analyzer (ANKOM DELTA Automated Fiber Analyzer, Ankom Technology, Macedon, NY, USA) using a modified procedure of the Van Soest’s method (Van Soest et al., 1991). Each set of samples was analyzed in triplicate.

### Characterization of the in vivo and in vitro digesta

Samples of the in vivo and in vitro digesta were kept on ice after collection and until analysis (within 1 h of collection).

### Particle size measurement

The particle size distribution (PSD) of the baseline materials (pea materials and the two diets, R1 and R2) as well as the in vivo and in vitro digesta were measured with a Malvern laser diffraction particle sizer 3000 coupled to a dispersant unit (Hydro LV) filled with distilled water (Malvern Instruments Ltd., Palaiseau, France). The refractive index of water was 1.330 and 1.530 for the pea samples, and the absorbance value was 0.001. The PSD, as average volume percentage, is presented as the means of at least three replicates.

### Microscopy

The samples were mounted on microscopy slides, and then visualized with an apotome microscope and the Zen software (Apotome, Zeiss, France). Images were captured using 10× and 20× objective lenses.

### Protein and proteolysis products assessment

The extent of protein hydrolysis (digestion) was estimated from the crude protein measurement of the recovered in vitro (washed pellet) and in vivo digesta by the Dumas method as described above (see Chemical Analysis section). The supernatant of the in vitro samples were precipitated in trichloroacetic acid (TCA; 0.83 mL of 5% TCA added to 0.5 mL of supernatant) and analyzed using the standardized OPA spectrophotometric assay in microplates (Mulet-Cabero et al., 2017). After preparation of the OPA solution (0.1 M sodium tetraborate, 5.7 mM dithiothreitol, 3.5 mM sodium dodecyl sulfate, and 6 mM OPA), 10 µL of sample was incubated at room temperature for 20 min, and the absorbance was read at 340 nm. Leucine was used for the standard curve with concentrations ranging from 0 to 10 mM. This method permitted to determine the amount, in millimolar (mM), of primary amino groups (amino acids and small peptides).

The proteins and proteolytic products of the in vitro samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE Bis–Tris gels (4%–12% Bis–Tris, Thermo Fisher Scientific, Illkirch-Graffenstaden, France #NP0322Box). For each sample, the supernatant (65 µL of the liquid phase of the collected digesta) was dissolved in 10 µL of reducing agent (DL-dithiothreitol, DTT; stock concentration of 77.5 mg/mL in H<sub>2</sub>O) and 25 µL of NUPAGE LDS sample buffer (4×, Thermo Fisher Scientific, Illkirch-Graffenstaden, France #NP0007). Then, 15 µL of each mixture were loaded into gels, and after migration (70 V for 30 min and 110 V for 1 h) the gels were stained with Coomassie Blue. PageRuler Prestained Protein (Thermo Fisher Scientific, Illkirch-Graffenstaden, France #26616) was used as a molecular weight marker. The gels were scanned with an ImageQuant LAS 4000 system (GE Healthcare).

## Calculation and statistical analysis

Bioaccessible (incubated samples without enzymes; quantity of protein released from the food matrix before hydrolysis) and hydrolyzed (incubated samples with enzymes) proteins, expressed in %, were determined as follows:

$$\text{Bioaccessible or hydrolyzed protein} = \left( \frac{m_{\text{Total original proteins}} - m_{\text{Recovered proteins}}}{m_{\text{Total original proteins}}} \right) \times 100 \quad (1)$$

where  $m_{\text{Recovered proteins}}$  is the mass in g of protein recovered after *in vitro* incubation and  $m_{\text{Total original proteins}}$  is the mass in g of protein originally present in the flour or the meal (R1 or R2).

The apparent ileal digestibility of crude protein (measured as nitrogen) was calculated according to the following equation:

$$\text{Apparent digestibility (\%)} = \left[ 1 - \left( \frac{N_{\text{Digesta}}}{N_{\text{Meal}}} \right) \times \left( \frac{\text{TiO}_2_{\text{Meal}}}{\text{TiO}_2_{\text{Digesta}}} \right) \right] \times 100 \quad (2)$$

where  $N_{\text{Digesta}}$  and  $N_{\text{Meal}}$  are the quantity, in percentage (dry mass basis), of nitrogen present in the digesta and meal, respectively.  $\text{TiO}_2_{\text{Meal}}$  and  $\text{TiO}_2_{\text{Digesta}}$  are the quantity, in percentage (g/kg on a dry mass basis), of titanium dioxide present in the meal and digesta, respectively.

The data were analyzed using R studio (version 4.1.2) by a Kruskal–Wallis test followed by Dunn's post hoc test as a nonparametric method. For all tests, the significance level was set at  $P < 0.05$  (two-tailed) and the data are expressed as means of replicate  $\pm$  standard deviation (SD).

## Results

### Characterization of the pea ingredients and diets

The composition of the ingredients and diets are shown in Tables 1 and 2, respectively. The pea ingredients were of high purity; the extracted starch and fiber containing little protein. However, some starch was still present in the extracted fiber. Overall, the pea fiber sample contained, as expected (Leterme et al., 1996), predominantly insoluble fiber (Table 1) and no intact cells were visible (Figure 1.3). Indeed, no cell could be distinguished on the images (Figure 1.3A and 3B) while starch granules (transparent round structure of about 25–30  $\mu\text{m}$ ) and proteins (pink structures of about 90–95  $\mu\text{m}$ ) could be clearly seen. In contrast, the pea flour, on its own (Figure 1.1A and B) or as part of R1 (Figure 1.2A and B), contained particles with intact cell walls encapsulating nutrients (center of the particles) as well as broken cells (edge of the particles), and this regardless of the size. The pea cells (about 120  $\mu\text{m}$  length) within the plant tissue were thus delimited by a cell wall that appears intact, which is confirmed by the presence of starch granules and other nutrients inside the cell (Figures 1.1B, 2A and 2B). The pea flour (including for R1) had particles of two main sizes,  $\sim 1$  mm and  $\sim 30$   $\mu\text{m}$  (50% of the samples had a size above 530  $\mu\text{m}$ ), as demonstrated by the two peaks observed in Figure 2; while R2 contained particles of more evenly spread sizes (90% of the sample had a size below 430  $\mu\text{m}$ ). The latter particles corresponded to cell fragments from the pea fiber and protein aggregates as shown by the PSD (Figure 2A).

### Characterization of the *in vivo* digesta

Digesta were collected at two sites, duodenum and ileum, and at different intervals following the test meal ingestion (1 and 4 h Duo; and 3, 8 and 12 h Ileal). Overall, Figure 3 shows the presence of pea particles even at the latest stage of R1 digestion (ileal digesta at 12 h). It also revealed that the smallest particles, most possibly free starch granules (about 30  $\mu\text{m}$  in size according to Figure 3B), were digested in the upper gut and were not visible at the terminal ileum. Intact cells were still visible within the collected particles however the edge of the particles appear more and more damage as the digestion progressed. The inside of the cells (darker after longer digestion time) seem to lose their integrity (initiation of nutrients digestion leading in particular to loss in starch crystallin structure).

At the early stage of digestion of R2 (1 and 4 h Duo), some proteins and starch granules were still clearly visible (Figure 4). The content of the digesta recovered at the ileum, even after only 3 h of ingestion, appeared very different from the original meal (Figure 1.3). It seems to be made of cell fragments (possibly from the animal but also from the pea fiber) and agglomerates of undigested material (i.e., complexes formed from undigested nutrients and polysaccharides).

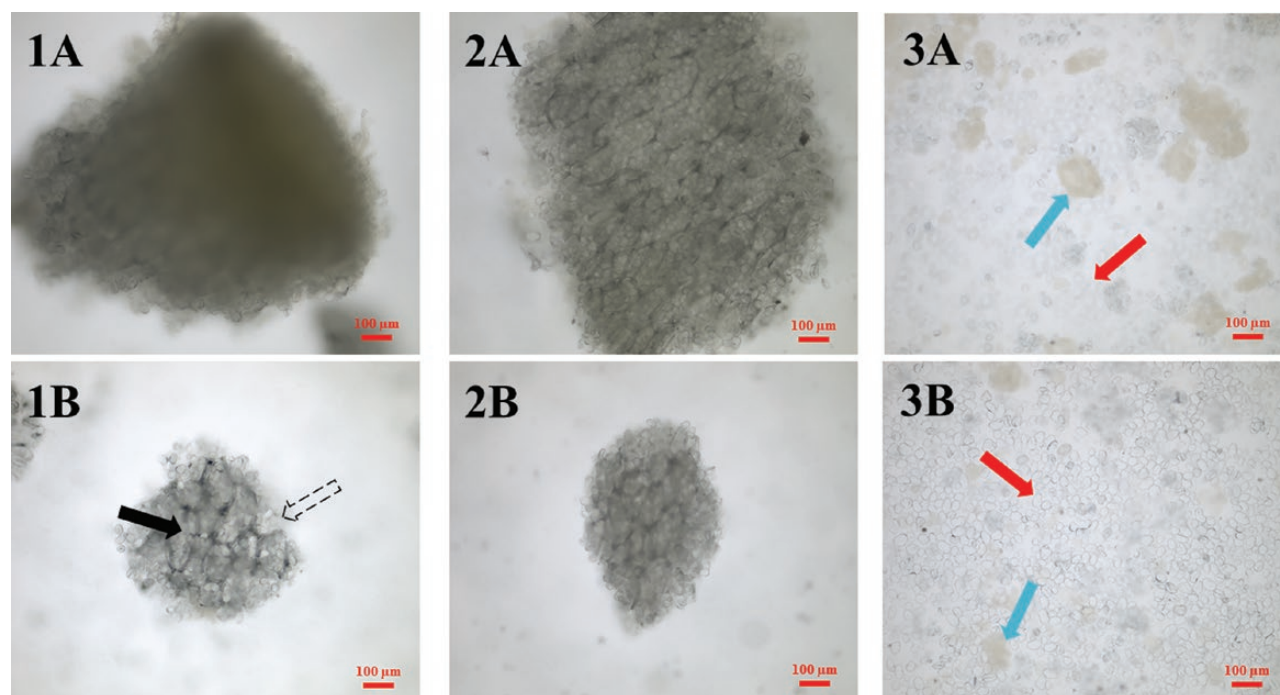
### Characterization of the *in vitro* digesta

The mass of material recovered following incubation in the gastric and intestinal phases decreased with incubation time and in the presence of enzymes (Figure 5). Regardless of the treatment, the amount of material left after incubation diminished drastically compared with the starting ingredients suggesting that an important fraction of the nutrients solubilized in the aqueous phase. After the 6 h of digestion, about 13% of the original weight of R2 was recovered, whereas the values for the flour and R1 were higher, i.e., 35% and 31%, respectively.

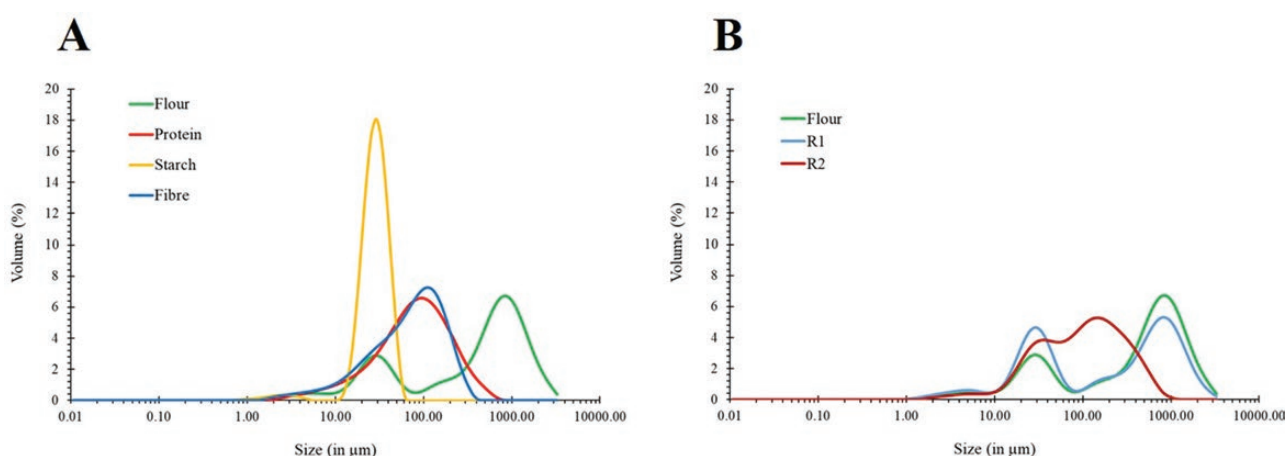
The average PSD of the pea ingredients following gastrointestinal incubation are depicted in Figure 6. Similar to the *in vivo* data, there was a shift in the distributions of the flour and R1 towards the right, indicating a disappearance of the small particles (i.e., solubilized proteins and starch granules) and swelling of the some of the large particles (Figure 6A and B). Regarding R2, an opposite trend was observed with the particles of largest size being broken down, suggesting that the protein aggregates were dissolved and eventually hydrolyzed (see Figure 7.3D).

Examples of typical microstructure of the samples recovered at the different stages of digestion are shown in Figure 7. Examination of the digesta recovered after gastric incubation, revealed that some proteins and starch granules escaped digestion. However, most of the remnant material from R2 intestinal digestion was composed of cell wall fragments (i.e., dietary fiber) without any visible protein (Figure 7.3D). On the other hand, the flour and R1 digesta contained particles with some cells still intact and full of nutrients (Figure 7.1D and 2D). The cells situated at the edge of the particles appeared to be depleted of their content; this phenomenon is all the truer than the particles are small. This implies that the enzymes are able to diffuse through the pea cell wall. These observations are consistent with the *in vivo* data described above (Figures 3 and 4).

Great similarities could be distinguished between the PSD and the visual aspect of the particles recovered *in vivo* and *in vitro* (Supplementary Figure S1). In particular, the gastric phase matched the digesta collected at the duodenal cannula



**Figure 1.** Light microscopy images of the pea flour (1A and 1B), R1 (2A and 2B) and R2 (3A and 3B). Scale bar: 100 µm. Given the heterogeneity in the size of particles present in the samples two sizes were represented (A large particles and B smaller particles). Approximate sizes of the particles: 1A, 1.3 mm; 1B, 600 µm; 2A, 1.5 mm; and 2B, 800 µm. The black and dashed arrows indicate intact cells and cells with ruptured cell walls, respectively. The proteins and starch granules are designated by the blue and red arrows, respectively.



**Figure 2.** Particle size distribution of the pea ingredients (A) and the two diets (B). Values are presented as means of triplicate.

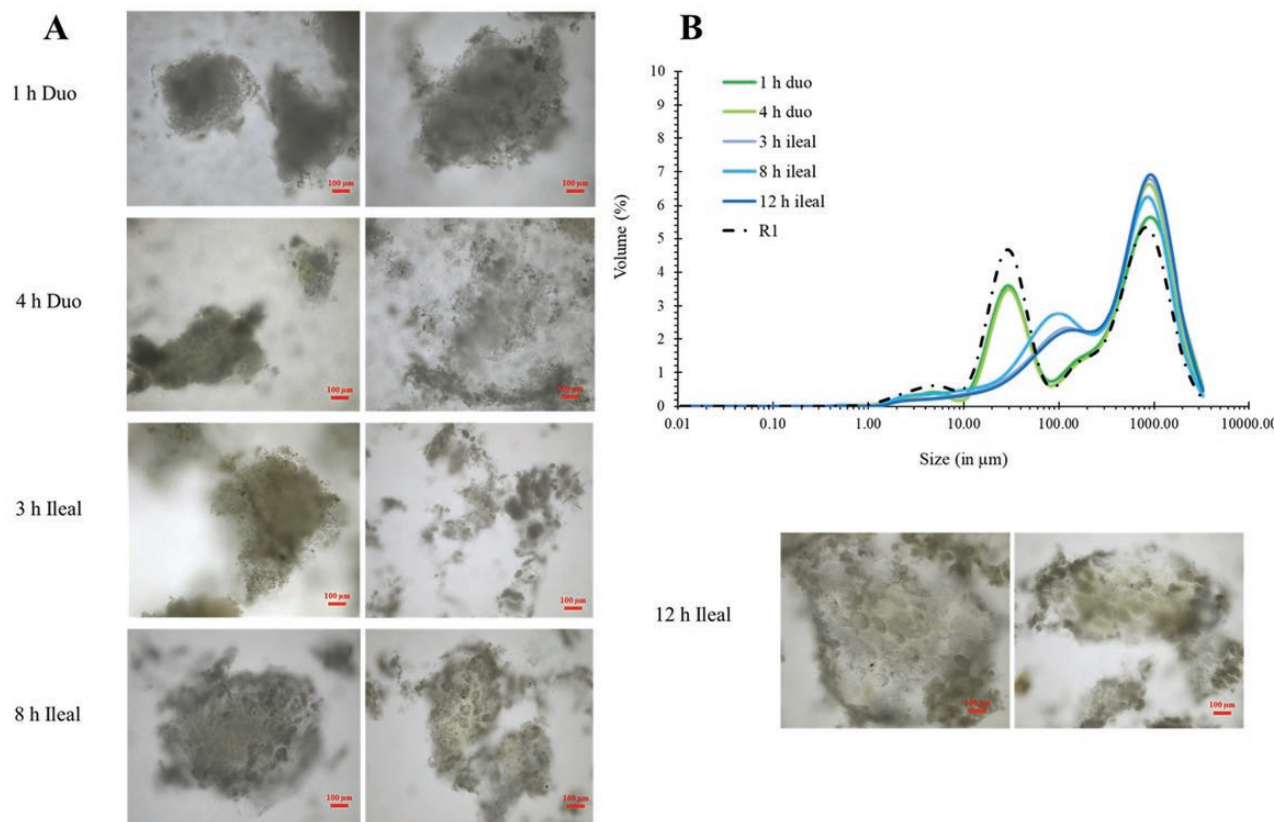
(3 h Duo) and the intestinal phase the digesta collected at the ileal cannula (6 h Ileal). Indeed, for R1 two peaks were obtained, at 30 µm and around 1100 µm; the *in vitro* and *in vivo* curves overlapping completely for the smallest particles (Supplementary Figure S1A). Some variations were noticeable for the particles with larger size and the samples obtained at slaughter. However, only the intensity changed not the pattern. Regarding R2, all curves corresponded well particularly for the gastric phase (Supplementary Figure S1B).

### In vitro and in vivo protein bioaccessibility and digestibility

The percentage points of protein released (solubilized proteins) from the flour and R1 increased between the gas-

tric and intestinal phases (Table 3). Despite being totally bioaccessible (not encapsulated by cell wall), the proteins from R2 were not soluble, thus an important proportion of them precipitated, particularly in the simulated gastric compartment certainly due to the acidic pH. However, their hydrolysis was greater than for R1 (and the flour), and this regardless of the phase. Therefore, at the end of the *in vitro* digestion, approximately 13% of the protein remained unhydrolyzed for R2, and 30% for the flour and R1. The differences between R1 and R2, from the *in vitro* and *in vivo* data, are of the same order of magnitude (~19% difference), however the data obtained after slaughtering were not significant.

Measurement of free amino groups followed the same trend than the results obtained with the Dumas method



**Figure 3.** Typical micrograph images of the particles recovered in the digesta after ingestion of R1 (A) and the corresponding particle size distribution (B). For each time point, two typical images are shown. Digesta were collected at the duodenal cannula (duo) after 1 and 4 h of ingestion; and at the ileal cannula (ileal) after 3, 8, and 12 h of ingestion of R1. The particle size distribution values are presented as means of triplicate.

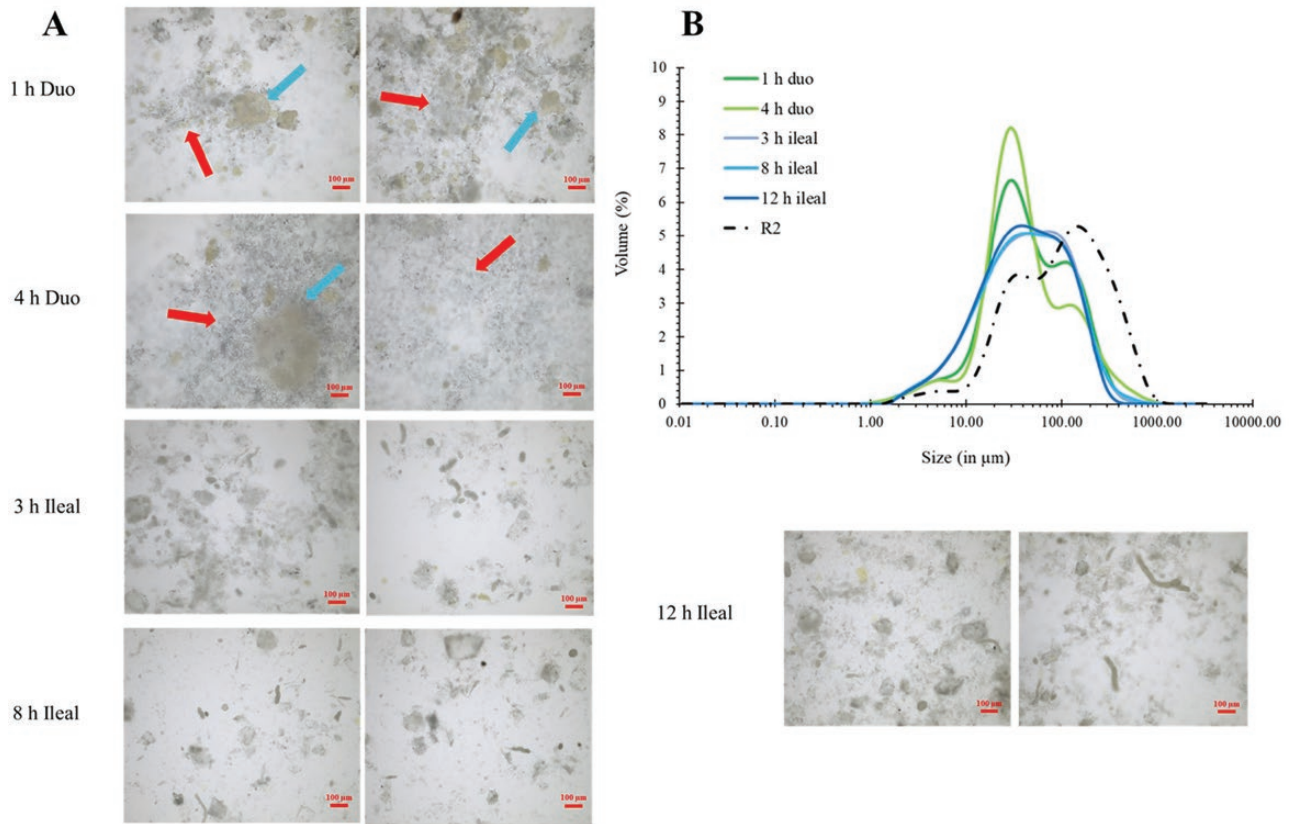
(Figure 8A). Only a small amount of proteolysis products was present in the samples incubated without the enzymes (between 1.1 and 2.3 mM). As the digestion progressed, more products (amino acids or peptides) were generated, about 12 and 37 mM for the gastric and intestinal phases, respectively (flour, R1 and R2 combined). At the end of the intestinal digestion, the quantity of amino groups produced was higher for R2 compared with the flour and R1 ( $P < 0.05$ ), with about 41 mM for the former and 35 mM for the latter.

The conversion of proteins to free amino acids and peptides was also followed using SDS-PAGE (Figure 8B). This technique complemented the quantitative analyses reported above as it gave an account of the size of the proteolysis products. However, the protein patterns appeared to be slightly different between the protein released from the pea flour (R1) and the extracted proteins (R2). After the gastric phase, some proteins were still clearly visible, especially for the flour and R1 (bands in lane D in Figure 8B). Some peptides formed by hydrolysis could be identified: around 70–80 kDa (R2 only), and around 17–10 kDa (both R1 and R2). The acidic environment affected the solubility of the proteins since the bands corresponding to the proteins constitutive of R2 were more intense for the gastric than the intestinal phase (bands in lane B). This could have led to the formation of peptides resistant to pepsin activity (bands in lane D). On the other hand, most of the proteins seem to have been hydrolyzed during the intestinal phase, which is consistent with the *in vitro* protein analyses. Indeed, only the proteins coming from the enzyme and bile preparations are

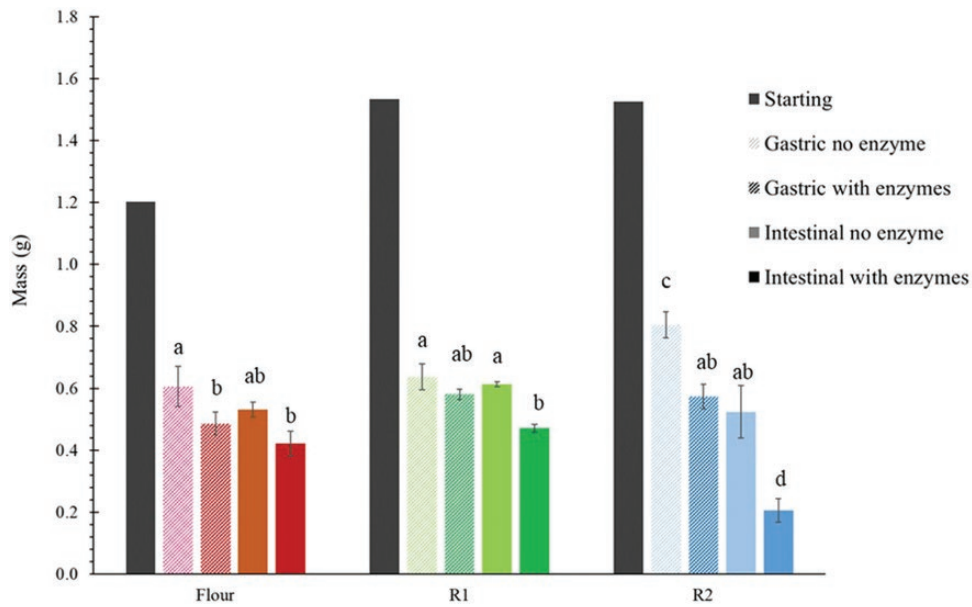
distinguishable (*Control* lane). Finally, the TCA precipitation and the centrifugation steps ensured the removal of nonhydrolyzed proteins, including the enzymes (*TCA* lanes). Therefore, the values obtained from the OPA assay corresponded to the products of proteolysis, the TCA precipitation eliminating proteins and large peptides present in the samples (Rieder et al., 2021).

## Discussion

We have used a combination of biochemical and biophysical analyses to gain insight into the mechanisms dictating the degradation of pea ingredients, with various degree of destructure, during *in vivo* and *in vitro* gastrointestinal digestions. This destructure can be first defined as the level of cell walls integrity, i.e., proportion of intact and ruptured cell walls. The disassembly of pea ingredients differed greatly between the two flours: for R1, large particles (i.e., approximately 1 mm), full of nutrients, were recovered in the distal sections of gastrointestinal tract whereas for R2 only cell wall fragments (i.e., approximately 55 µm), nonhydrolysable by endogenous enzymes, were retrieved in the ileal digesta. The particles from R2 that escaped digestion in the upper gut corresponded to fractured cells given that their size was below the average size of an individual pea cells, which equals to about 150 µm (Edwards et al., 2020; Junejo et al., 2021). The dietary fiber that remained after intestinal digestion, i.e., the cell wall components and resistant starch, could then be fermented by the microbiota present in the colon.

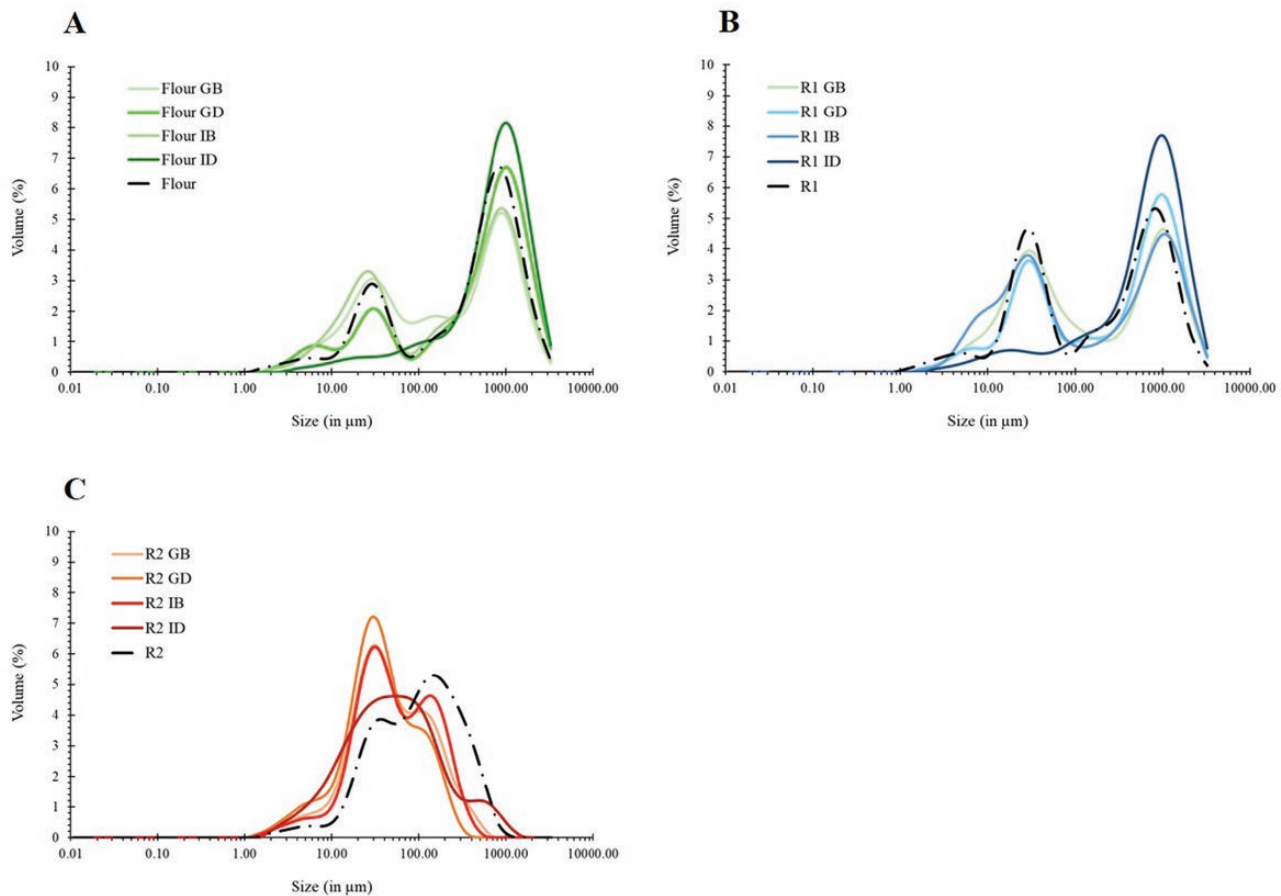


**Figure 4.** Typical micrograph images of the particles recovered in the digesta after ingestion of R2 (A) and the corresponding particle size distribution (B). Digesta were collected at the duodenal cannula (duo) after 1 and 4 h of ingestion, and at the ileal cannula (ileal) after 3, 8, and 12 h of ingestion of R1. For each time point, two typical images are shown. The undigested proteins and starch granules are indicated by the blue and red arrows, respectively. The particle size distribution values are presented as means of triplicate.



**Figure 5.** Mass of dried material, in g, at baseline (starting) and recovered after incubation in digestive fluids (gastric and intestinal phases), without and without enzymes. Different letters indicate significant differences as determined by Kruskal–Wallis test followed by Dunn’s post hoc test ( $P < 0.05$ ).



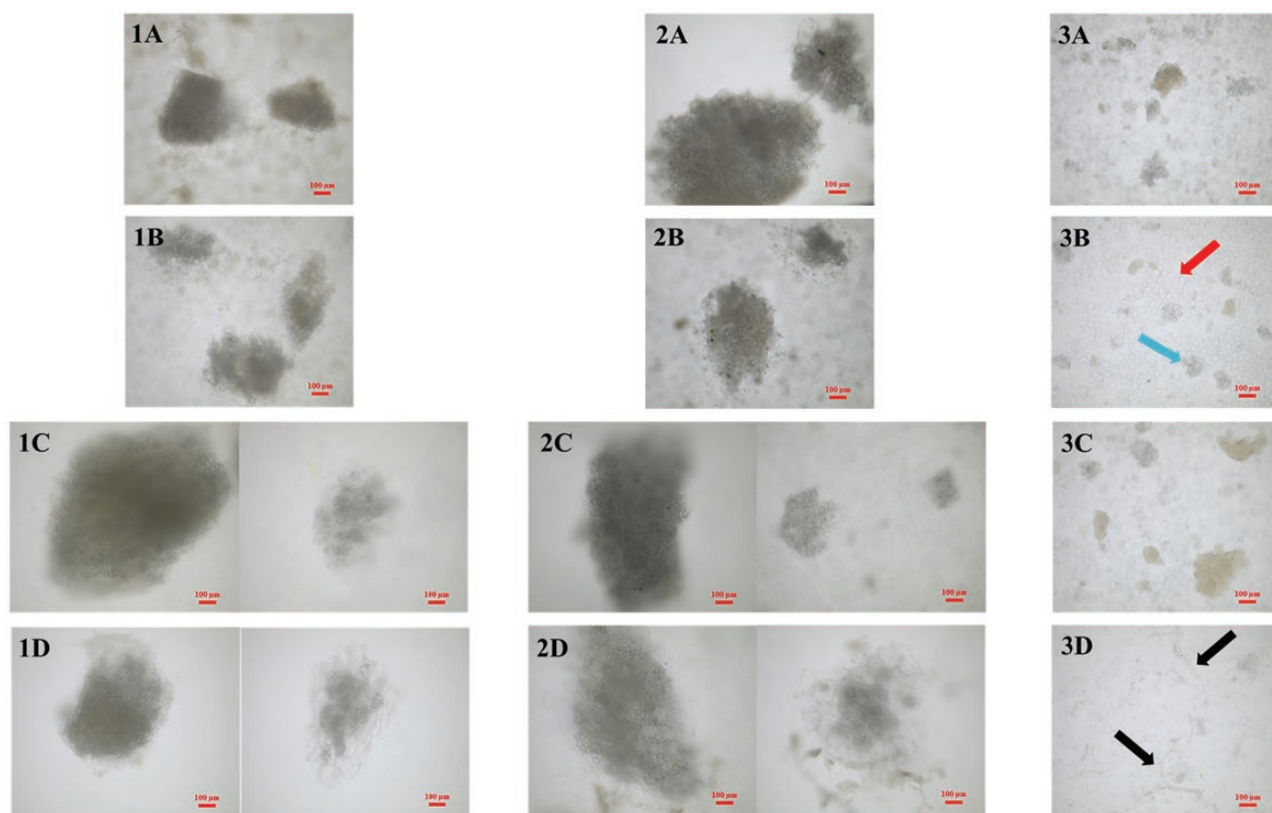


**Figure 6.** Particle size distribution of the digesta recovered after simulated gastrointestinal incubation for the flour, R1 and R2. GB, gastric phase without enzyme; GD, gastric phase with enzymes; IB, intestinal phase without enzyme; and ID, intestinal phase with enzymes.

It is known that soluble and insoluble dietary fibers affect the transit rate of digesta through the gastrointestinal tract, which in turn influences macronutrient digestion (Ratanapaul et al., 2019). However, it is less clear how the organization and structure of the cell wall within particles (form under which dietary fibers are delivered) impact nutrient digestibility. In the current work, we investigated the effects of cell wall integrity on the bioaccessibility (in this study the term is used to describe the release of protein from the feed matrix and does not include hydrolysis) and digestibility of proteins using an *in vivo* and *in vitro* approach. Differences were observed in the release of proteins and their subsequent hydrolysis between the regular and reconstituted pea flours. We assumed that the physical disruption of the cell wall during the pea milling was not sufficient to ensure a complete protein digestion. The pea tissue integrity appeared to have been conserved for some of the particles even though some swelling may have occurred. This behavior along with the protein aggregation may have hindered the penetration of the digestive agents (e.g., enzymes and bile salts) through the cell wall and thereby hindered protein hydrolysis. It was demonstrated that when the proteins were not enclosed within the cell, as it was the case in R2, the proteins were more rapidly broken down. This is consistent with a previous *in vitro* study performed on isolated cell from pea with the Infogest protocol that showed a reduced protein hydrolysis when the cell walls were intact (Junejo et al., 2021). In a study performed on pigs, it was also showed that pea protein isolate had higher nitrogen apparent ileal

digestibility than pea flour, 80.2% and 88.2%, respectively (Woyengo et al., 2015). Another team reported an apparent ileal digestibility of 75.9% for pea flour. Therefore, the digestibility values obtained here are lower than the one found in the literature (64% and 84% for R1 and R2, respectively); variety of the pea used and growing conditions could influence digestibility (Graham and Aman, 1987; Fan and Sauer, 1999). Furthermore, no pea fiber was added to the pea protein diet in the study by Woyengo et al. (2015), thus the potential interaction between those compounds and the effect on the animal physiology (e.g., fluctuation in transit time and/or endogenous losses) were not directly assessed. Previous works also reported that the transformation of the pea seed, i.e., via thermal processing, impacted protein digestibility (Owusu-Asiedu et al., 2002; Park et al., 2010). Generally, information about the behavior of those pea ingredients, particularly the cell wall components, in the different compartments of the pig digestive tract were missing in those trials.

The size and the conformation of the proteins have also an impact on their hydrolysis (Maeda et al., 2022). As previously described, certain plant proteins, particularly pea protein, can be resistant to hydrolysis (Le Gall et al., 2007; Mackie and Macierzanka, 2010; Jiménez-Munoz et al., 2021). The profile of the pea proteins obtained in this work agrees with published results showing both globulins (i.e., convicilin, vicilin, and legumin), and albumins (Creveiu-Gabriel, 1999; Cui et al., 2020; Jiménez-Munoz et al., 2021). The resistant proteins or polypeptides identified are likely to correspond to the



**Figure 7.** Light microscopy images of the flour (1), R1 (2) and R2 (3), after simulated gastric (A and B) and intestinal (C and D) incubations, without (A and C) and with (B and D) enzymes. For the flour and R1, two images, displaying large and small particles recovered at the end of the intestinal phase, are shown. Scale bar: 100  $\mu\text{m}$ . Note the presence of undigested proteins (blue arrow), starch granules (red arrow) and cell wall fragment (black arrows).

**Table 3.** Protein bioaccessibility and digestibility values, as mean percentage points  $\pm$  standard deviation, obtained in vitro and in vivo for R1 and R2<sup>1</sup>

	R1	R2	Difference between R1 and R2(%)	P value
In vitro digestion, %				
Gastric bioaccessibility	62.0 $\pm$ 2.4	16.4 $\pm$ 7.3	45.6	0.020
Gastric hydrolysis	64.7 $\pm$ 1.0	81.4 $\pm$ 0.3	16.7	0.104
Intestinal bioaccessibility	67.2 $\pm$ 1.1	52.1 $\pm$ 7.9	15.2	0.263
Intestinal hydrolysis	69.1 $\pm$ 0.9	87.2 $\pm$ 2.4	18.0	0.016
In vivo digestion, %				
Ileal cannula (n = 6)	63.7 $\pm$ 8.7	84.1 $\pm$ 4.7	20.4	0.002
Ileal - slaughter (n = 3)	47.4 $\pm$ 15.4	65.8 $\pm$ 14.1	18.4	0.245

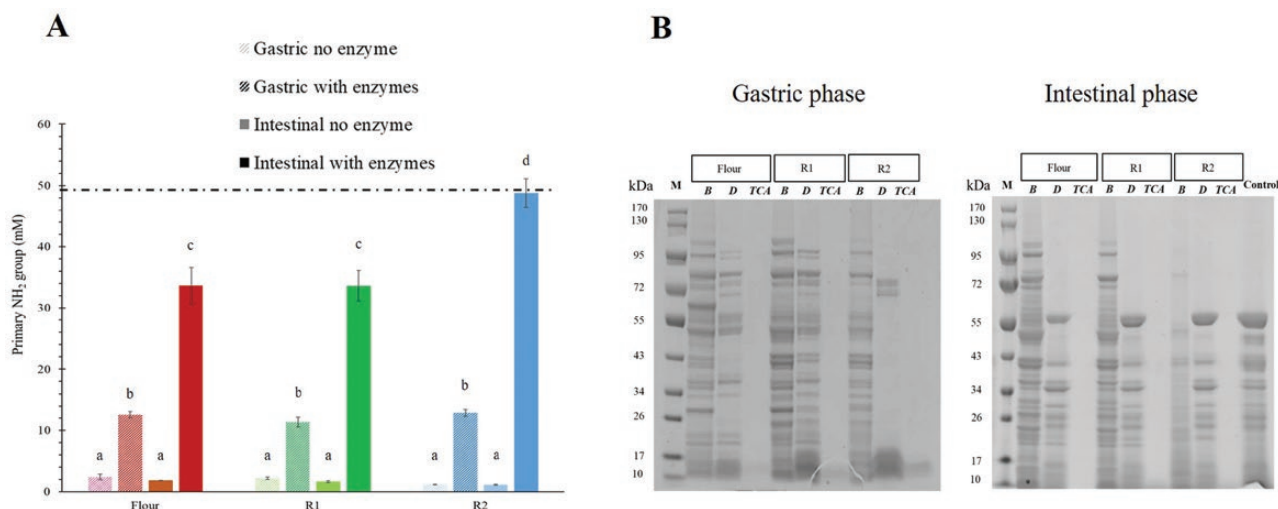
<sup>1</sup> R1 = “regular” pea flour with mostly intact cell walls; R2 = reconstituted pea flour with broken cell walls.

subunits of convicilin (approximately 70 kDa) for the largest, and the  $\beta$ -subunit of legumine and albumin PA1 (approximately 20 kDa) for the smallest (Creveu-Gabriel, 1999). However, the sub-units of viciline (50 kDa) or albumine PA2 (26 kDa) were not found. The solubility of the protein can also affect their digestibility since the proteases have to be in

the same phase than their substrates to hydrolyze them. It is known that a fraction of the pea proteins, called glutelin, is hydrophobic and tends to precipitate (Creveu-Gabriel, 1999; Jiménez-Munoz et al., 2021). The average size of the protein aggregate obtained here, i.e., 128  $\mu\text{m}$ , was similar to the one reported in the literature, i.e., 138  $\mu\text{m}$  (Jiménez-Munoz et al., 2021). As stated by the authors, these aggregates as well as a low solubility can slow down protein hydrolysis.

Feed processing, and the resulting effects on feed structure, has an impact on the rate and extent of protein hydrolysis which in turn affects the site of amino acids and small peptides release and the rate at which they are absorbed (Fouhse et al., 2017; Rojas and Stein, 2017; Zentek and Goodarzi Boroojeni, 2020). Processing could lead to a deconstruction of the plant tissue, thereby changing the general physicochemical properties of the cell wall (e.g., solubilization of polysaccharides, reduction in their molecular weight, swelling and/or rupture of the cell wall) and those of the proteins (e.g., complexation with other compounds, aggregation and solubilization).

The biological relevance of the Infogest static protocol to assess the protein quality of pea has been already investigated (Ariens et al., 2021). However, few studies have looked at the digestibility of protein in complex food matrices, and scarcely by combining in vivo and in vitro experiments (Fan and Sauer, 1999; Woyengo et al., 2015; Egger et al., 2017; Zhou et al., 2018). Moreover, the raw materials as well as the digesta taken during these studies were not always finely characterized. Another strength of this work is that the pea ingredients were included on their own in the diets administered to the



**Figure 8.** Amount of primary amino groups generated during the in vitro hydrolysis of the pea ingredients (A). Different letters indicate significant differences as determined by Kruskal–Wallis test followed by Dunn’s post hoc test ( $P < 0.05$ ). The dashed line corresponds to the maximum amount of primary NH<sub>2</sub> group that would be obtained for a complete hydrolysis (determined from the hydrolysis of pea protein alone). Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) of supernatant of incubated pea ingredients during the simulated gastric and the intestinal phases (B). M, markers; B, incubation without enzyme; D, incubation with enzymes; TCA, incubation with enzymes followed by precipitation with TCA (trichloroacetic acid); and Control, incubation medium with enzymes (pepsin and pancreatin).

pigs, thereby pea was the only source of protein and dietary fiber. This permitted to avoid any interactions with other feed components and to precisely examine the potential encapsulation effect of pea cell walls. Furthermore, this design facilitated the comparison of the digesta collected in the animals to the in vitro samples.

Small disparities between the in vitro and in vivo experiments were observed in the PSD from the samples coming from the gastric phase. This could be caused by the difference in the site of collection (duodenum for in vivo rather than stomach for in vitro) and, therefore, differences in response traits that may have impacted the digestion of (e.g., pH, electrolyte composition and enzymes activity). Also, some particles may have remained in the pigs’ stomach for extended period of time compared to the simulated digestion due to their size. Indeed, large (size >1000–2000  $\mu\text{m}$ ) and hard particles contained in digesta delay gastric emptying as they cannot pass through the pylorus, which is the so-called “sieving effect” (Kong and Singh, 2008). This process is not simulated in static in vitro models, which may explain some of the discrepancies observed in the PSD. This can nevertheless be accounted for by choosing a specific digestion time for each digestion phase, bearing in mind that solely a snapshot of the dynamic aspects of the digestion can be reproduced in vitro due to the complexity of the processes involved.

Overall, the differences in protein digestibility between the two diets (around 19%) were in the same order of magnitude for in vitro and in vivo, although the differences were not significant for the digesta collected after slaughter (a larger sample size would have been needed).

## Conclusions

In this study, we demonstrated that the data obtained in vivo and in vitro were consistent, and that the digestibility of protein depended on the particles size and whether or not they are surrounded by cell walls. Our results provide compelling evidence for an encapsulation mechanism in hindering protein

digestion of pea. Thus, composition alone is not sufficient to predict the digestibility of protein in the pig gastrointestinal tract, additional information about the degree of cell wall integrity is necessary. Although our study had limitations, it improved our understanding of protein release and hydrolysis in pea, and the role play by the feed structure, particularly the cell wall integrity. Further study could be done using semi-dynamic or even fully dynamic models of digestion adjusted to the pig physiology.

## Acknowledgments

We thank Etienne Labussière, Gwenola Le Roy and Philippe Ganier for their assistance with the animal experiment, dietary fibre analysis and the acquisition of the microscopy images. This work was funded by INRAE (PHASE division), France. The animal trial was performed at the INRAE UE 3P experimental facility (INRAE, Saint-Gilles, France).

## Conflict of Interest Statement

The authors declare no real or perceived conflicts of interest.

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