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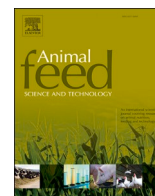
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Cell wall of feeds and their impact on protein digestibility: An in vitro method applied for pig nutrition

Myriam M.L. Grundy^{*}, Julie Tang, Jaap van Milgen, David Renaudeau

PEGASE, INRAE, Institut Agro, 35590 Saint Gilles, France

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ABSTRACT

Plant-derived by-products of the food, drink, and fuel industry are low-cost ingredients, that are routinely used to formulate feeds for livestock, especially for pigs. Often, those ingredients are rich in dietary fibres (DF), which is known to have a negative influence on the digestive use of nutrients in pigs. The effect of DF on nutrient bioaccessibility (i.e., the release of nutrients from the plant tissue) and digestion (i.e., transformation of the plant tissue during digestion and the hydrolysis of nutrients by digestive enzymes) depends on their amount, but more importantly, on their nature and organisation within the cell wall. The aim of this work was to determine how DF from different sources affected protein bioaccessibility and proteolysis. We hypothesised that protein released from the feed matrix and hydrolysis in pig feed depend mainly on the nature and amount of cell wall polysaccharides (i.e., dietary fibre). To test this, we conducted an in vitro study where nine feed ingredients commonly used in pig feeding (i.e., soybean meal, barley, wheat, two varieties of peas, fava bean, chickpea, maize and sugar beet pulp) were digested using a simulated gastrointestinal digestion model. Cell wall integrity and proteolysis were assessed using biochemical, biophysical, and microscopic techniques. Protein bioaccessibility differed among the ingredients ($P < 0.01$), with sugar beet pulp and maize having the lowest fraction of protein released from the plant tissue (about 34 %) and chickpea the greatest (93 %). Protein hydrolysis values also varied greatly among the ingredients ($P < 0.01$) but did not always follow the same trend as that of bioaccessibility. For instance, soybean meal had the highest protein hydrolysis with 96 % and sugar beet pulp the lowest with 50 %. The particle size distribution and microscopy images provide an explanation for these differences, related to the physical barrier, swelling, and rupture of the cell wall. This study illustrated that the cell wall structure is specific for each ingredient, with consequences on bioaccessibility and digestibility that cannot be characterised by the composition in DF alone. In feed formulation, this concept ought to be accounted for to achieve optimal use and uptake of nutrients by the animal.

Abbreviations: AA, amino acids; ADFom, acid detergent fibre expressed exclusive of residual ash; aNDFom, neutral detergent fibre assayed with a heat stable amylase and expressed exclusive of residual ash; B, barley; CP, chickpea; DF, dietary fibres; DM, dry matter; F, fava bean; IDF, insoluble dietary fibre; Lignin sa, Lignin determined by solubilisation of cellulose with sulphuric acid; M, maize; P, pea Ascension variety; PA, pea Assas variety; PSD, particle size distribution; SBM, soybean meal; SBP, sugar beet pulp; SDS-PAGE, Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis; SDF, soluble dietary fibre; W, wheat.

^{*} Corresponding author.

E-mail address: myriam.grundy@inrae.fr (M.M.L. Grundy).

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1. Introduction

A large range of fibre-rich feedstuffs are routinely added to pig diets, usually based on local availability of low-cost ingredients that are often by-products from the food, drink, and fuel industry. Dietary fibres (DF) are found primarily in the cell wall of plants. According to the current definition, DF are carbohydrate polymers that cannot be hydrolysed by mammalian digestive enzymes (McCleary, 2019; Augustin et al., 2020). They include cellulose, hemicellulose, and pectin constitutive of the cell wall, but also resistant starch, non-digestible oligosaccharides (3–10 sugars), and lignin (i.e., a polymer of propyl phenol units). There exists a wide diversity in cell walls, in term of composition and organisation of the polysaccharides within the cell wall (Popper et al., 2011). Therefore, not all sources of DF have similar physicochemical properties due to the nature and spatial distribution of these polysaccharides and associated molecules (e.g., phenolic compounds and proteins); their effects on digestion processes and gut physiology will therefore also vary (Souffrant, 2001; Williams et al., 2019; Li et al., 2021).

Dietary fibres can influence nutrient bioaccessibility (i.e., the release of nutrients from the food matrix that are potentially available for digestion) and digestion (i.e., transformation of the plant tissue during digestion such as swelling or size reduction of particles, and the hydrolysis of nutrients by digestive enzymes) in the upper gut through different mechanisms (Bach Knudsen, 2001; Dégen et al., 2007; Grundy et al., 2016). First, intact cell walls can encapsulate proteins and prevent hydrolysis by proteases. Water-soluble DF have the ability to increase the viscosity of gastric and intestinal contents, thereby hindering the contact between the enzymes and their substrates. Certain polysaccharides can also interact directly with the macronutrients, agents of digestion (e.g., enzymes and bile salts), or both. Finally, they can interact with the mucus layer creating a barrier to absorption of the products of digestion. Other compounds present in the cell wall matrix, associated with DF such as polyphenols, can also impact nutrient metabolism (Saura-Calixto, 2011). As a result, the presence of DF in pig diets can increase endogenous secretions (e.g., enzymes, mucin and digestive fluids as a whole) and nitrogen losses (Souffrant, 2001).

Understanding the role played by DF during digestion is critical for pig nutrition. This knowledge permits to better use different sources of fibre-rich plant-based ingredients in feed formulation and apply specific processing techniques (e.g., grinding, pelleting or addition of enzymes). By doing so, it is possible to manipulate the food matrix and improve nutrient bioaccessibility and digestibility (either decrease or increase it) and thereby meet swine nutritional requirement for each life stage (sow vs. piglet vs. growing pig) (Jarrett and Ashworth, 2018; Li et al., 2021). Dietary fibre is necessary to maintain normal physiological gut function, however high DF content can result in low nutrient digestibility and energy utilisation (Noblet and Le Goff, 2001). In vitro models of digestion are an interesting complementary tool to in vivo studies because they permit to gain insights into the mechanisms involved during digestion of plant-based foods and elucidate the physiological responses observed in vivo. In particular, it is possible to follow the degradation of the food matrix as digestion progresses while studying a wide range of ingredients in well-controlled and standardised conditions.

During the digestion of proteins from plant tissues, different phenomena occur (Grundy et al., 2016; Capuano et al., 2018). First, the proteins have to come into contact with the proteases for the hydrolysis to take place. This can be achieved if i) the proteins “leach out” of the plant cell to reach the aqueous environment where the enzymes are present, or ii) the enzymes penetrate inside the cell by diffusion through the cell wall. In the latter case, the products of proteolysis then have to “leave the cell” to be absorbed by the enterocytes. The proteins released from the plant cell (here called bioaccessibility) are not necessary all fully hydrolysed to absorbable nutrients as some of them may not be soluble or they can interact with other compounds (e.g., binding with antinutrients such as phytate or DF, adsorption to the lipid droplet interface) preventing access of the enzymes to their substrate (Grundy et al., 2016; Parada and Santos, 2016; Popova and Mihaylova, 2019). When the proteolysis happens, it may also not be total and some large peptides, not hydrolysable by the brush-border peptidases, may remain.

The aim of this work was to study how DF, from different sources, had an impact on protein digestibility. More precisely, how the composition, nature, and organisation of DF (cell walls) within plant tissues altered the bioaccessibility and the subsequent digestibility of proteins. To achieve this, we conducted an in vitro study where nine commonly used pig feed ingredients were digested using a simulated, three phases, oro-gastrointestinal static digestion model. Cell wall integrity and protein behaviour during digestion were assessed using biochemical, biophysical and microscopic techniques.

2. Materials and methods

2.1. Materials

Soybean (SBM), barley (B), wheat (W), Assas and Ascension varieties of forage peas (PA and P, respectively), fava bean (F), chickpea (CP), maize grains (M), and dehydrated beet pulp (SBP) were ground using an industrial hammer mill (Stolz, Pontivy, France) 40 HP, capacity 3 T/H, fitted with a 2.5 mm grid (grinding batch of about two tons).

Pepsin from porcine gastric mucosa (#P6887, 2145 U/mg of solid), porcine bile extract (#B8631), 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). Sodium chloride (99.8 %), calcium chloride (99 %), potassium chloride (99 %), potassium dihydrogen phosphate (99 %), sodium bicarbonate (99.5 %), magnesium chloride hexahydrate (99 %), and ammonium carbonate (99.5 %) were purchased from Sigma (Saint Quentin Fallavier, France).

2.2. Composition of the material studied

Protein content was measured based on the Dumas method (LECO, FP828 Carbon/Nitrogen/Protein Determinator, Villepinte, France, AOAC 968.06) using a nitrogen conversion factor of 5.4 for B, W, PA, P, CP, and F; 5.5 for SBM and SBP; and 5.6 for M (Mariotti et al., 2008). Moisture (oven-dried at 104 °C to a constant weight, method 935.29; AOAC, 2016) and ash (method 942.05; AOAC, 2016) contents were also determined.

Neutral detergent fibre (aNDFom), acid detergent fibre (ADFom), and acid detergent lignin (lignin sa), contents were determined using fibre bags (Model F57, Ankom Technology, Macedon, NY, USA) and a fibre analyser (ANKOM DELTA Automated Fibre Analyzer, Ankom Technology, Macedon, NY, US) adapted from the Van Soest method (Van Soest et al., 1991). The samples were also analysed for their composition in soluble (SDF) and insoluble (IDF) DF using the method 991.43; AOAC (2016) (Megazyme kit assay, Megazyme, Product Code: K-TDFR). Total dietary fibre (TDF) was then calculated by adding SDF to IDF. Each set of samples were analysed in triplicate.

2.3. Particle size measurement

The particle size distribution (PSD) of the nine samples (flours) was 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 1.600 for the flours, and the absorbance value was 0.001. The PSD, as average volume percentage, is presented as the means of at least three replicates per sample.

2.4. Water retention capacity

Water retention capacity (WRC), defined as the amount of water retained by the material after centrifugation (Jacobs et al., 2015), was estimated following a procedure adapted from Robertson et al. (2000). Briefly, samples were hydrated in distilled water at room temperature (1:25 w/v). After 24 h, the samples were centrifuged (3000 g for 20 min) and the supernatant carefully removed. Water retention capacity was calculated from the mass of water retained divided by the mass of the initial sample. Analyses were performed in triplicate.

2.5. Simulated *in vitro* digestion

The model of human digestion simulating pig digestion was adapted from a standardised method (Brodkorb et al., 2019). Briefly, after 2 min of incubation at 39 °C at pH 7 to simulate the oral phase, the samples were subjected to gastric (120 min at 39 °C, pH 3; pepsin added) and duodenal (120 min at 39 °C, pH 7; bile salts and pancreatin added) digestions. The composition of the simulated fluids used for each digestion phase can be found elsewhere (Brodkorb et al., 2019). The amount of sample used was adjusted based on protein content (to maintain a constant substrate/peptases ratio) and corresponded to 0.130 g of protein on a dry weight basis. The enzymatic reactions were stopped by increasing the pH to 9 with 5 N NaOH at the end of the intestinal phase. Each digestion was performed in triplicate with (digested sample) and without (blank sample) enzymes. The samples were then analysed as indicated in Section 2.6. Protein not remaining in the feed matrix (pellet) after incubation with the enzymes (see Section 2.6.1. for protein measurements et Section 2.8. for the calculations) were considered to have undergone some digestion and are further referred to as “digested protein”.

2.6. Protein and proteolysis products assessment

2.6.1. Dumas method

After the duodenal phase, the samples (blank - bioaccessible proteins, and digested - digested proteins) were centrifuged (2500 g for 10 min at 4 °C), 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 overnight at 80 °C before being analysed with the LECO combustion analyser as described in Section 2.2. The term bioaccessibility is defined here as the quantity of proteins released from the food matrix and potentially available for digestion (proteolysis) and absorption.

2.6.2. SDS-PAGE

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed on the blank and digested samples using NuPAGE Bis-Tris gels (4–12 % Bis-Tris, Thermo Fisher Scientific, Illkirch-Graffenstaden, France, #NP0322Box). For each sample, 10 µL of reducing agent (DL-dithiothreitol, DTT; stock concentration of 77.5 mg/mL in H₂O) and 25 µL NUPAGE LDS sample buffer (4x, Thermo Fisher Scientific, Illkirch-Graffenstaden, France, #NP0007) were added to 65 µL of supernatant. Then, 15 µL of each sample were loaded into the gel, and after migration (70 V for 30 min and 100 V for 1 h) the gel was stained with Coomassie Blue. PageRuler Prestained Protein (Thermo Fisher Scientific, Illkirch-Graffenstaden, France, #26616) was used as a molecular weight marker.

2.7. Microscopy

Samples for microscopy were collected before (control) and after simulated digestion, mounted on microscopy slides, and then

visualised with an apotome microscope and the Zen software (Apotome™, Zeiss, France). Images were captured using 10 × and 20 × objective lenses.

2.8. Calculation and statistical analysis

Protein bioaccessibility (incubated samples without enzymes) and digestibility (incubated samples with enzymes), expressed in %, were determined using the following equations:

$$\text{Protein bioaccessibility or digestibility} = \left(\frac{m_{\text{Total original proteins}} - m_{\text{Recovered proteins}}}{m_{\text{Total original proteins}}} \right) \times 100 \quad (1)$$

where $m_{\text{Recovered proteins}}$ is the mass in g of protein recovered after digestion and $m_{\text{Total original proteins}}$ is the mass in g of protein originally present in the ingredient.

The mass of material degraded after incubation without (dry mass, DM, bioaccessibility) and with enzyme (DM digestibility) were calculated as below:

$$\text{DM bioaccessibility or digestibility} = \left(\frac{m_{\text{Total original sample}} - m_{\text{Recovered sample}}}{m_{\text{Total original sample}}} \right) \times 100 \quad (2)$$

The data were analysed using R studio (version 4.1.2). For all tests, the significance level was set at $P < 0.05$ (2-tailed) and the data were expressed either as means of technical replicates \pm standard deviation. In this study, an experimental unit corresponded to one tube (one incubation either with or without enzyme). The differences in composition, particle size, WRC, protein bioaccessibility and digestibility between the nine ingredients were analysed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test or Kruskal-Wallis test followed by Dunn's post hoc test as a non-parametric method. The latter analysis was used when the residuals of the ANOVA model did not follow a normal law or was heteroscedastic; therefore, for the variables: particle size, protein bioaccessibility and digestibility. Pearson correlation analyses were conducted to study the relationships between characterisation parameters and protein bioaccessibility/digestibility.

3. Results

3.1. Characterisation of the ingredients

Table 1 shows the chemical composition of the ingredients. The values obtained for aNDFom ranged from 9.5 % to 42.2 %, for M and SBP, respectively; for ADFom from 2.0 % to 19.8 %, for M and SBP, respectively; and for lignin sa from 0.1 % to 1.5 %, for P and SBP, respectively. These data indicate that SBP was the ingredient containing the most DF (aNDFom = 42.2 %). Total dietary fibre measurements confirmed those findings but provided more detail about the solubility in water of these fibres. As an example, the SDF value of SBP was higher than that of M (7.7 % and 1.3 % respectively).

Most ingredients contained particles with a wide range of sizes as shown by the multimodal PSD (Fig. 1). However, SBM and SBP have a fairly monodispersed PSD with a main peak at 806 and 1040 μm , respectively. Overall, the average particle size ranged from 97 for F, to 764 μm for SBP (see Table S1).

The only statistically significant difference in WRC detected among the nine ingredients was for SBP ($P < 0.05$), 9.3 compared with about 3.9 mL/g for the other ingredients (Fig. 2). Thus, the ingredients having particles of the largest sizes and the greatest DF content ($P < 0.001$) had the highest WRC.

Table 1
Chemical composition of the studied ingredients (% on a dry matter basis)^a.

	Ingredients ^b								
	SBM	B	W	PA	P	F	CP	M	SBP
DM (%)	88.3	88.4	88.3	85.1	85.2	88.6	88.2	86.5	88.6
Crude protein (%)	42.0	10.2	10.8	23.7	18.5	21.7	14.4	7.7	11.5
aNDFom (%)	18.0	18.3	15.1	17.2	13.6	17.8	16.4	9.5	42.2
ADFom (%)	5.0	5.7	3.1	6.7	6.3	10.1	4.1	2.0	19.8
ADL ^c (%)	0.3	1.0	1.0	0.7	0.1	1.0	0.7	0.2	1.5
TDF ^d (%)	20.0	21.0	15.9	33.2	33.4	38.9	19.7	16.9	47.6
SDF (%)	2.7	2.7	3.3	3.0	3.5	3.0	3.1	1.3	7.7
IDF (%)	17.3	18.3	12.6	30.2	29.9	35.9	16.6	15.6	39.9
Ash (%)	5.8	2.8	1.6	3.1	2.5	3.8	3.8	1.2	7.2

^a Values are mean of triplicate.

^b SBM, Soybean meal; B, Barley; W, Wheat, PA, Pea Assas variety; P, Pea Ascension variety; F, Fava bean; CP, Chickpea; M, Maize; SBP, Sugar beet pulp.

^c ADL, lignin (sa).

^d TDF, Total dietary fibre estimated from IDF, insoluble dietary fibre, and SDF, soluble dietary fibre.

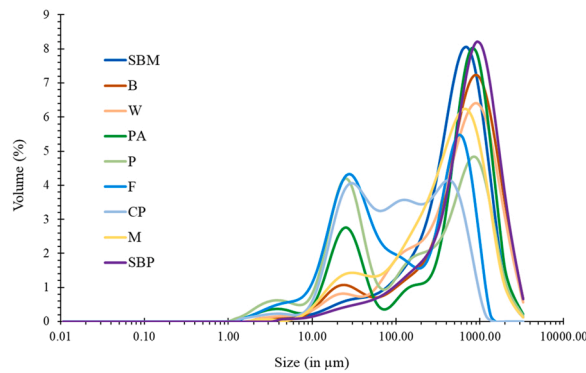


Fig. 1. Particle size distribution of the ingredients studied at baseline. Values are presented as means of triplicate. SBM, soybean meal; B, barley; W, wheat; PA, Assas pea; P, Ascension pea; F, fava bean; CP, chickpea; M, maize; and SBP, sugar beet pulp meal.

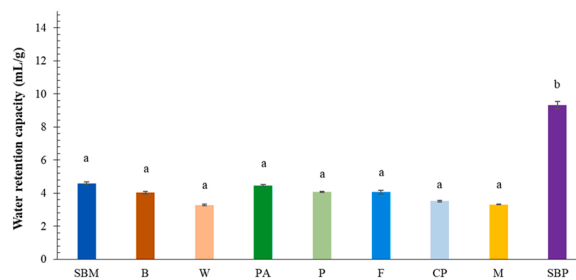


Fig. 2. Water retention capacity of the ingredients studied. Different letters indicate significant differences as determined by one-way ANOVA and Tukey’s post-hoc test ($P < 0.01$). SBM, soybean meal; B, barley; W, wheat; PA, Assas pea; P, Ascension pea; F, fava bean; CP, chickpea; M, maize; and SBP, sugar beet pulp.

3.2. Protein bioaccessibility

Fig. 3 shows a great disparity in the protein bioaccessible among the nine ingredients. Thus, SBM, M, and SBP had low bioaccessibility (down to about 33 % for M; $P < 0.01$) whereas CP had 93 % of its proteins released into the simulated digestive fluids. SDS-PAGE gels also showed variability in the quantity of protein present in the supernatant which gives an account of their solubility (Fig. 3B). Therefore, Pa, P and F had the greatest amount of soluble proteins (based on the presence and intensity of the bands).

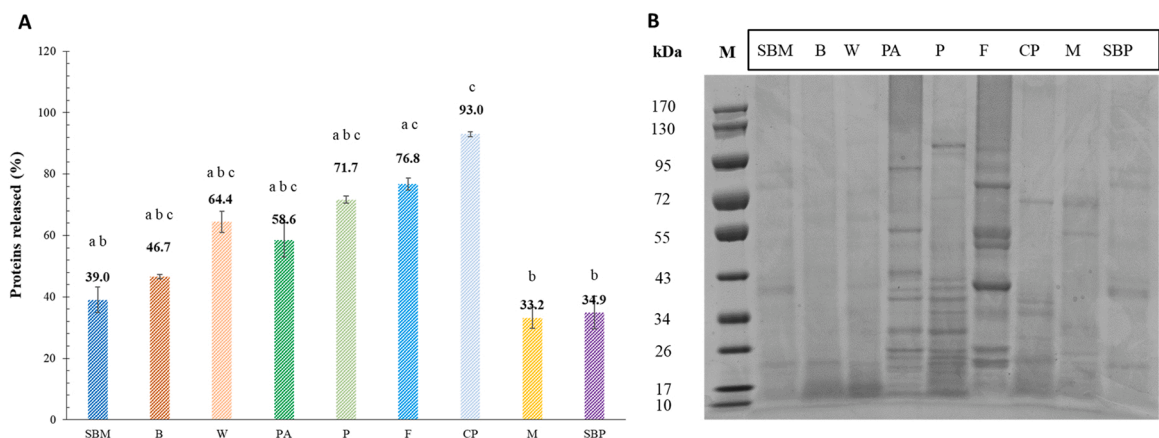


Fig. 3. Proteins bioaccessible from the food matrices after incubation in simulated digestive fluids (measured from blanks). Protein content from the recovered particles (pellet) measured with the Dumas method (A); SDS-PAGE gel of the supernatant (B). Different letters indicate differences as determined by Kruskal-Wallis test followed by Dunn’s post hoc test ($P < 0.01$). SBM, soybean meal; B, barley; W, wheat; PA, Assas pea; P, Ascension pea; F, fava bean; CP, chickpea; M, maize; and SBP, sugar beet pulp.

3.3. Digestion of the ingredients and relationship with chemical composition and structure of the matrix

The quantity of protein hydrolysed, presented in percentage, is depicted in Fig. 4. The SBM and CP were digested to a greater extent than the other ingredients (approximately 96 % and 94 %, respectively), with SBP having the lowest degree of protein digested (50 %, $P < 0.01$). The extent of protein digestion was negatively correlated to DF content (aNDFom, $r = -0.59$; TDF, $r = -0.73$; $P < 0.001$, see correlation matrix Fig. S1) but more importantly to the mean particle size ($r = -0.68$, $P < 0.001$).

Similar to the protein bioaccessibility and digestibility, there were differences between the ingredients in the amount of material degraded during incubation ($P < 0.01$, Fig. 5A). For the two extremes (CP and SBP), we observed little change in the quantity of material degraded without (bioaccessible) and with (digested) enzymes, 24.2 % for SBP and 81.9 % for CP. On the other hand, SBM and M displayed an important increase in the mass of material being digested. Those trends are consistent with the protein digestibility data indicating that the macronutrients of the ingredients (protein but also starch and lipid) would follow the same digestibility pattern (no selectivity in the nutrients that are hydrolysed).

Fig. 5B shows that the smallest particles have been degraded during the digestion. Indeed, we notice a shift of the curve towards the right indicating that the largest particles remained, some of them emptied or partially emptied of nutrients (see Figs. 1 and 6). Furthermore, some swelling occurred during digestion, particularly for W, PA, P, and SBP (Dv90, the particle diameter below which 90 % of the material is contained, increased, $P < 0.05$).

The micrographs in Fig. 6 give information about the structure of the plant tissue before (blank, Fig. 6A and B) and after (digested, Fig. 6C and D) digestion; of particular interest is the integrity of the cell wall and the aspect of the food matrix. As reported with the PSD data, these microscopic images exhibited a great apparent variability in the overall size and appearance of the particles. In the blank samples, the plant tissues appear full of nutrients with presumably intact cell walls. Some of the digested samples (1: SBM, 6: F, 7: CP and 8: M) were depleted of their content whereas some still contained nutrients and even looked swollen (2: B, 3: PA, 4: P and 9: SBP). Milling of CP led to a flour composed predominantly of broken cells (Fig. 6.7) as demonstrated by its median particle size ($\sim 203 \mu\text{m}$ that is about the size of an individual cell as measured by Edwards et al., 2020). Only emptied cells and cell fragments were visible after simulated digestion (Fig. 6.7C and D). Interestingly, the cell wall of SBM (Fig. 6.1C and D) and M (Fig. 6.8C and D) appeared to be porous to digestive agents. For those ingredients, the remaining, emptied cells resulted in structure looking as “ghost particles”.

4. Discussion

4.1. Characterisation of the ingredients

For certain ingredients, a large discrepancy existed between the aNDFom and the TDF values (i.e., PA, P, F, and CP) possibly due to the dissolution of some cell wall polysaccharides (pectin and water-soluble non-starch polysaccharides) in the detergent used to obtain the aNDFom (Van Soest et al., 1991; Bach Knudsen, 2001). Therefore, using both methods of analysis is preferable for ingredients rich in certain soluble polysaccharides such as hemicellulose and pectin.

Despite using the same milling process, important differences were observed among the PSD showing that the grains behave differently under mechanical pressure. The impact of processing techniques such as particle size reduction has been widely studied, and it has been found to improve in vivo nutrient digestibility and pig performance (Lancheros et al., 2020; Lyu et al., 2020; Jo et al., 2021). Indeed, smaller particles result in more ruptured cell walls compared to large particles and thus the majority of the nutrients are freely available for digestion by enzymes present in the upper gut (stomach and small intestine, Grundy et al., 2016). However, feeds that are made of particles that are too small could lead to adverse health effects such as gastric ulcers in sows (Vukmirović et al., 2017). Therefore, the optimum average particle size has been established to be between ~ 400 and $1000 \mu\text{m}$ depending on the ingredient (Healy et al., 1994; Vukmirović et al., 2017; Lancheros et al., 2020; Lyu et al., 2020). Apart from F and CP, all ingredients studied here

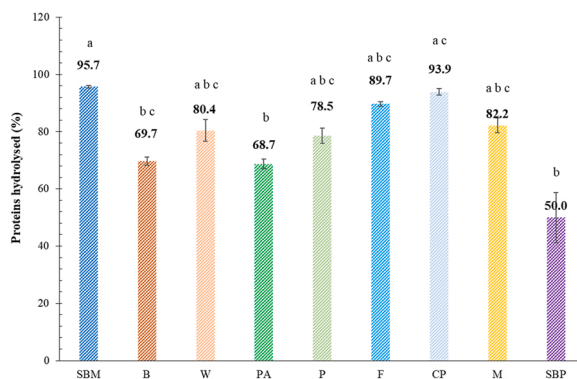


Fig. 4. In vitro protein hydrolysis of the ingredients studied. Different letters indicate differences as determined by Kruskal-Wallis test followed by Dunn's post hoc test ($P < 0.01$). SBM, soybean meal; B, barley; W, wheat; PA, Assas pea; P, Ascension pea; F, fava bean; CP, chickpea; M, maize; and SBP, sugar beet pulp.

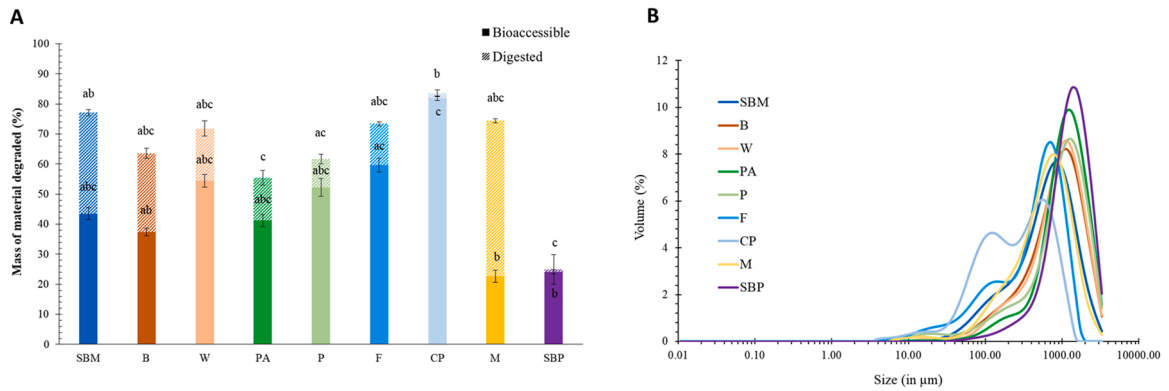


Fig. 5. A) Proportion, in %, of DM of material degraded after incubation in simulated digestive fluids, without (bioaccessible) and with (digested) enzymes. Different letters indicate differences as determined by Kruskal-Wallis test followed by Dunn's post hoc test ($P < 0.01$). B) Particle size distribution of the digestas for the nine ingredients. Values are presented as means of triplicate. SBM, soybean meal; B, barley; W, wheat; PA, Assas pea; P, Ascension pea; F, fava bean; CP, chickpea; M, maize; and SBP, sugar beet pulp.

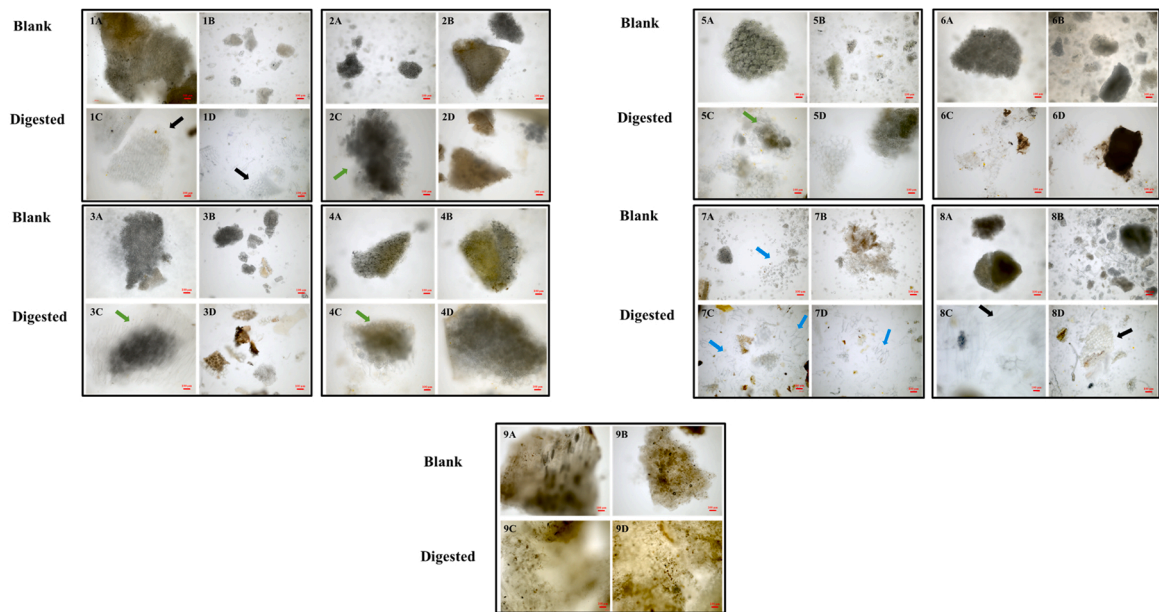


Fig. 6. Light microscopy images of the ingredients at baseline (A and B) and after digestion (C and D). Scale bar: 100 μm . Black arrows: "ghost particles"; green arrows: particles full of nutrients, sometimes swollen; blue arrows: cells with ruptured cell walls. 1: soybean meal; 2: barley; 3: wheat; 4: pea Assas variety; 5: pea Ascension variety; 6: fava bean; 7: chickpea; 8: maize; and 9: sugar beet pulp.

were within that size range.

Water retention capacity analysis is one easy way of estimating the hydration properties of a DF source and provides some insight about its fate in the gastrointestinal tract, an aqueous environment where the food matrix can swell and consequently impacts gut motility and digesta consistency (Robertson et al., 2000; Bach Knudsen et al., 2012). The WRC data showed that SBP had a contrasting behaviour when in presence of water compared with the other ingredients. Sugar beet pulp contained water-soluble polysaccharides, polysaccharides that swell in presence of water but remained entrapped within the food matrix (Figs. 6.9 and S2).

4.2. Effect of different sources of DF on protein bioaccessibility and digestibility

Protein analysis of the blank samples provided information about the porosity of the cell wall, the solubility of the present proteins, and whether or not the proteins could diffuse freely from the food matrix (potentially available for proteolysis), while the results on digestibility gave an account of the proteins that were hydrolysed by the proteases. Previous studies have shown that DF tended to impact negatively nutrient digestibility and absorption by pigs, however, the way this is achieved still remain unclear, hence some contradictory outcomes (Noblet and Le Goff, 2001; Lindberg, 2014; Agyekum and Nyachoti, 2017; Li et al., 2021). Based on their

nature, amount and distribution within the cell wall, their behaviour in the gastrointestinal tract (e.g., solubility, and entrapment of nutrient) and their subsequent effect on nutrient uptake will differ.

Consistent with the present results, other in vitro experiments found substantial differences in protein hydrolysis extent and kinetics between diverse sources of DF (Boisen and Fernandez, 1995; Jezierny et al., 2010; Salazar-Villanea et al., 2016b; Chen et al., 2019). Overall, the end point values varied between studies which is not surprising given the variability in the digestion conditions used (e.g., enzyme quantities, incubation time, and pH). Also, information about the physicochemical characteristics of the DF sources, particularly particle size, were missing which make comparison with the present study challenging. Nonetheless, recently some researchers have included some information about the particle size and hydration behaviour of the ingredients and feeds they investigated (Zhou et al., 2018; Lyu et al., 2021).

As mentioned above, the particle size distribution can give an account of the number of intact cells. When the size of particles

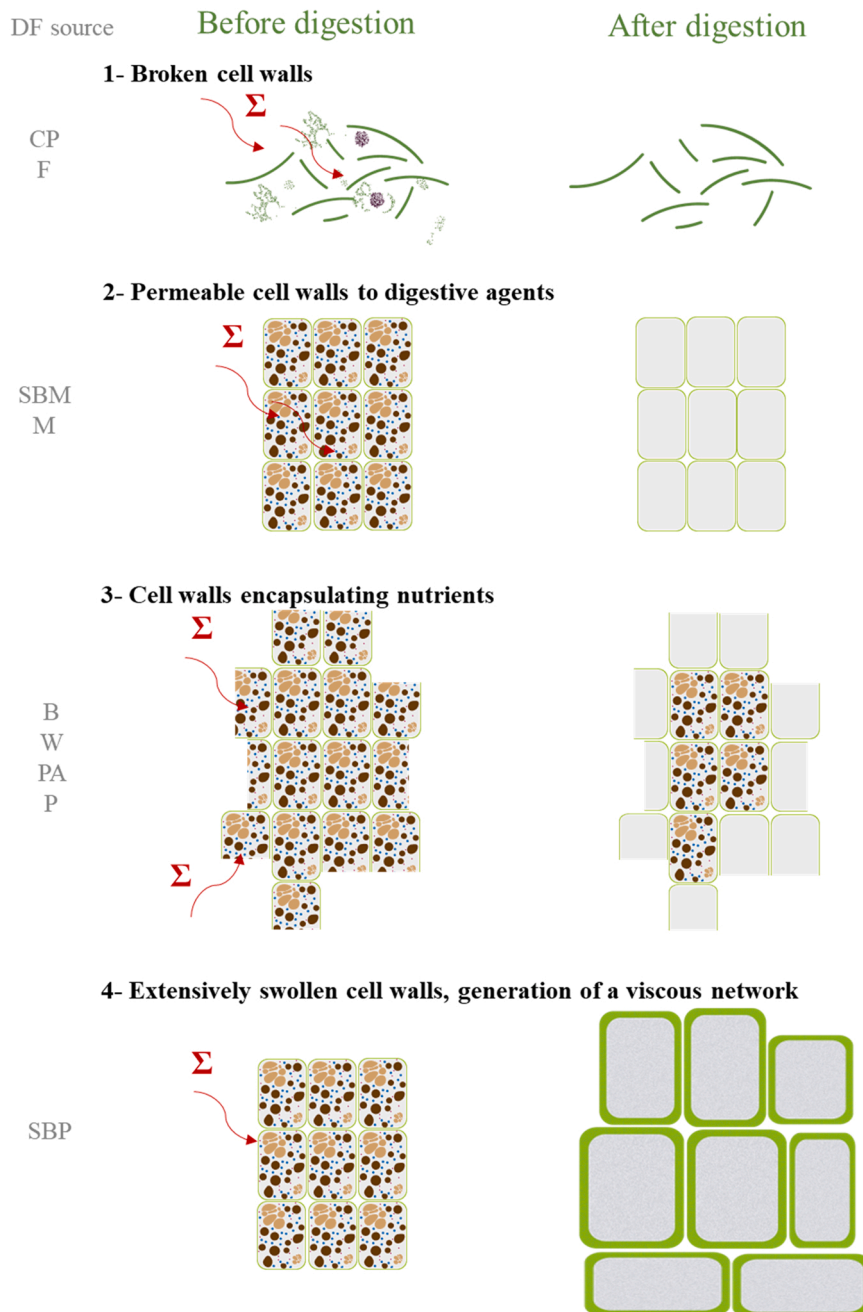


Fig. 7. Schematic representation of cell wall behaviours during gastrointestinal digestion.

increased, protein hydrolysis decreased since large particles have a greater number of intact cells and thereby nutrients encapsulated within the food matrix (Grundy et al., 2016; Lyu et al., 2021). However, given that the amount of proteins bioaccessible from the SBM and M particles was low but their digestion were rather high, other mechanisms are likely to influence this process (see Fig. 6).

Moreover, this *in vitro* analysis showed that despite having relatively high protein bioaccessibility, protein hydrolysis of peas (sample P) did not increase in presence of enzymes. Protein bioaccessibility differed from protein solubility for some of the ingredients (W and CP) as illustrated in Fig. 3B (the intensity of the bands was not matching the bioaccessibility values). It also showed the variability in protein sizes, and presumably types, among the ingredients. All those parameters together with the cell wall will have consequences on protein hydrolysis. When looking at the products of proteolysis via SDS-PAGE (Fig. S3), some peptides resistant (~ 20 kDa) to digestion could be identified as previously reported (Le Gall et al., 2007). The structure of the protein can influence their hydrolysis, the storage proteins present in pea being mainly of the globular type, namely legumin, vicilin, and convicilin (Fukushima, 1991; Salazar-Villanea et al., 2016a). This structure can be altered during processing, for example grinding and pelleting, which will generate proteins with another conformation that could be only partially hydrolysed. Furthermore, legumes such as pea are known to contain numerous antinutrients that can inhibit or prevent proteases activity, including phytate, trypsin inhibitors, and tannins (Khattab and Arntfield, 2009; Popova and Mihaylova, 2019). Therefore, even though the cell wall plays a major role in controlling protein digestion, other mechanisms are involved.

Little attention has been paid to the fate of the cell wall during the digestion process in the upper gut of pig, which is critical to evaluate digestion kinetics and thus the site of nutrient hydrolysis (stomach vs. small intestine), as well as understanding fluctuation in transit time, and endogenous protein secretion and losses. All those factors can affect metabolism (e.g., postprandial plasma profile of amino acids and peptides) and the efficiency of nutrient utilisation (Li et al., 2021). The effect on the physiological responses (e.g., blood profile) differs depending on the nature of the cell wall polysaccharides and whether the cell walls are intact or not. For instance, solubilised polysaccharides could directly interact with the mucosa and therefore lead to greater secretions of endogenous proteins (mucins) (Morel et al., 2005). However, those processes are complex and still need to be unveiled (Souffrant, 2001). Accumulating evidence from *in vitro* experiments conducted on separated cells from various plant origins demonstrated the physical barrier effect (reduced or limited porosity) of cell wall; enzymes were thus able to diffuse inside the cells and hydrolyse their substrates (protein, starch and lipid) (Bhattarai et al., 2017; Li et al., 2019; Junejo et al., 2021; Pälchen et al., 2021). However, contrary to many plant cells, soybean appears to be permeable to proteases notably when it underwent some form of processing (Zahir et al., 2020).

4.3. Underlying mechanisms governing protein digestibility in the nine studied ingredients

By means of biochemical, biophysical, and microscopic techniques the characterisation of the ingredients before and after digestion, the DF composition, their organisation within the plant tissue, and the level of degradation of the ingredients (e.g., degree of intact cell walls, swelling, porosity, and encapsulation of nutrients) were conducted. This, together with the *in vitro* simulated digestion method, permitted to uncover some of the mechanisms influencing protein hydrolysis. Based on the results described above, four main scenarios could be identified (Fig. 7):

- *Very high bioaccessibility and hydrolysis*, as observed for CP and F, where the majority of the cell walls were rupture and the proteins readily available for digestion.
- *Low bioaccessibility and high hydrolysis*, which was the case for SBM and M, where proteins could not diffuse easily out of the cell however proteases and other enzymes could penetrate the cell wall as illustrated by the “ghost particles” recovered after digestion (Fig. 6.1 and 6.8).
- *Medium bioaccessibility and hydrolysis*, as for instance for B and PA, where an important quantity of nutrients remained enclosed within the core of the particle. This phenomenon was sometimes coupled with swelling of the cell wall (e.g., for B).
- *Low bioaccessibility and hydrolysis*, as observed for SBP, where the DFs extensively swell during digestion but still prevent either the protein to leach out of the food matrix or the enzymes to penetrate the cell.

Therefore, cell wall can hinder protein hydrolysis by preventing or limiting the diffusion of digestive enzymes into plant cells (e.g., B, PA, and P). However, other processes can also lead to a reduced hydrolysis such as swelling of the feed matrix (SBP), presence of antinutrients, and protein aggregation (PA and P) or the proteins structure that prevents their solubilisation (B and W).

4.4. Evaluation of the *in vitro* model of digestion

The model used in this work was adapted to pig (incubation temperature) from the Infogest standard protocol (Brodkorb et al., 2019). The method has been extensively used for investigating the digestion of foods aimed at humans, however a good consistency between *in vitro* and animal (pig) study data has been recorded (Egger et al., 2017; Grundy et al., 2020; Sousa et al., 2020). Similarly, the results obtained in this study are in agreement with nitrogen digestibility values from nutritional tables, bearing in mind that the latter are for faecal digestion (Fig. S4; INRA et al., 2020).

Further research could be made to better estimate the size of the solubilized peptides during digestion, e.g., with size exclusion chromatography to give a better estimate of protein hydrolysis (Rieder et al., 2021). The quantity of enzymes used could also be reduced in order to detect accurately the products of proteolysis using biochemical techniques such as SDS-PAGE and *o*-phthalaldehyde (OPA) spectrophotometric assay. Semi-dynamic models aiming at simulating pig digestion processes should be developed to simulate some of the dynamic events occurring during digestion (e.g., enzyme and acid secretion, and gastric emptying) similarly to

the one existing for humans (Mulet-Cabero et al., 2020). The inclusion of mechanical processing during the oral phase could also improve the model but to achieve this there is a need to better understand the processing occurring in the mouth of pig (mastication and resulting particle size reduction).

At the present time, we are unable to reproduce the complexity of the phenomena occurring in the digestive tract of pigs. However, *in vitro* assays are useful tools to characterise feeds or ingredients digestibility without the complexity of the response of the animal. Indeed, contrary to *in vivo* experiments, it is possible to follow, for example, the bioaccessibility of nutrients and other components of the feeds by incubating the ingredients without any enzymes. Moreover, the kinetics of these processes (e.g., fast or slow proteolysis) can be determined, which could be useful for understanding physiological responses (e.g., hormone secretions) taking place *in vivo*. In experiments performed in pigs, protein digestibility is determined empirically based on the difference between dietary protein intake and unabsorbed protein generally collected at the terminal ileum. With the method from the present study, protein digestibility can be estimated from the amount of protein remaining in the feed matrix after digestion minus the original protein content of the ingredient and the proteins contained in the pepsin, pancreatin and bile preparations (removal via filtration). It is also possible to have an overview of the content of the aqueous phase – components that would get absorbed (e.g., products of enzyme activity) or delivered to the distal part of the gastrointestinal tract (e.g., DF solubilised, cell wall fragments, and polyphenols). To fully understand the mechanisms involved and the “real” impact of DF on protein digestibility in pigs, both *in vitro* and *in vivo* should be conducted on the same ingredients or feeds. Unfortunately, it was not possible to achieve this in the current work, notably, because of the large number, but limited supply available, of the ingredients investigated. Moreover, experiments should be replicated on different ingredient batches to obtain biological replicates and confirm that the observations made in this study are consistent across different sources of ingredients.

5. Conclusions

In the present work, insight into the roles played by cell walls from various DF sources was gained, which provided some explanations regarding some of the divergences in protein digestibility observed *in vivo*. This study highlighted the diversity in cell wall behaviour during digestion and the importance of characterising DF sources; composition alone being not sufficient to predict digestibility. When formulating feed for pigs, it is therefore important to consider the ingredients and the physicochemical properties of their cell wall to achieve the desired outcome (optimal protein digestion, dietary fibre health impact such as diverse microbiota and bulk effect). Further studies should investigate the combined effect of ingredients and the processes applied to them before being consumed by the animals (e.g., pelleting, or addition of water).

CRedit authorship contribution statement

Myriam M.L. Grundy: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Funding acquisition, Supervision, Project administration. **Julie Tang:** Investigation, Data curation, Formal analysis. **Jaap van Milgen:** Conceptualization, Writing – review & editing, Funding acquisition, Methodology, Project administration. **David Renaudeau:** Conceptualization, Project administration, Writing – review & editing, Methodology, Resources, Funding acquisition.

Conflict of Interest

‘Declarations of interest: none’.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.anifeedsci.2022.115467](https://doi.org/10.1016/j.anifeedsci.2022.115467).

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