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In vitro digestibility and solubility of phosphorus of three plant-based meat analogues

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Abstract

Interest in plant-based meat analogues has increased and can be expected to be applied to pet foods, which necessitates the understanding of the nutrient supply in those foods. Our primary aim was to advance our understanding of the digestive properties of sterilized plant-based meat analogues. The impact of the preparatory processing steps on the solubility of meat analogues was studied. Meat analogues were made by mixing water, salt, and wheat gluten with soy protein isolate, pea protein isolate, or faba bean concentrate. Mixed materials were processed into model meat analogues using shear cell technology. Products were canned in water or gravy and sterilized. An animal-based canned pet food was made as a reference. Products sampled at the processing steps (mixing, shearing, sterilization) were digested in vitro. Samples of digestate were taken at the gastric phase (0 and 120 min) and small intestinal phase (120, 200, 280, and 360 min) for analysis of protein hydrolysis. The extent digestion of nitrogen and dry matter was determined at the end of incubation. Total phosphorus, soluble phosphorus after acid treatment, and after acid and enzymatic treatment were determined. The degree of hydrolysis after gastric digestion was low but increased immediately in the small intestinal phase; products based on pea had the highest values (56%). Nitrogen digestibility was above 90% for all materials at each processing step, indicating that bioactive compounds were absent or inactivated in the protein isolates and concentrate. Phytate seemed to play a minor role in meat analogues, but phosphorus solubility was influenced by processing. Shearing decreased soluble phosphorus, but this effect was partly reversed by sterilization. Nutrient digestibility as well as phosphorus solubility in plant-based products was higher than or comparable with the reference pet food. These findings show that the digestive properties of the tested plant-based meat analogues do not limit the supply of amino acids and phosphorus.

KEYWORDS

in vitro digestibility, pet food technology, phosphorus, plant-based, vegan

1 | INTRODUCTION

The market for plant-based meat analogues for human consumption is increasing (Santo et al., 2020), which also fuels the interest in meat analogues among pet food manufacturers. These meat analogues are developed to resemble the properties of meat with respect to taste and texture, and to a lesser extent, nutritional value. Most meat analogues are made via extrusion, but other methods are under development. One of those processes is based on shear cell technology (Dekkers et al., 2018), which can be used to create the desired fibrous (meat-like) structures. Dry ingredients are mixed with water to reach 50%–70% moisture, after which the mixture is sheared at 90°C–140°C for about 30 min (Grabowska et al., 2016). The sheared product can be cut into pieces, canned with a sauce or gravy, and sterilized as in conventional wet pet foods (Wehrmaker et al., 2021). The ingredients can vary and often include a combination of wheat gluten with proteins from soybeans, peas, fava beans, and even sunflower (Cornet et al., 2021; Jia et al., 2022; Schreuders et al., 2019). However, recipes with wheat gluten and soy protein isolate, pea protein isolate, or faba bean concentrate resulted in the most consistent fibrous products in the shear cell to date. In terms of environmental impact, meat analogue-based pet foods would differ from conventional wet pet foods as both the footprints of the ingredients and the energy consumption for making meat-based chunks differ. For human food applications, it is often claimed that plant-based meat analogues can be produced more efficiently than meat (e.g., Smetana et al., 2015), but for pet food applications, similar studies are still required.

Pet foods are often the only food that pets consume and, therefore, the foods need to be complete and balanced. However, lower digestibility compared with animal-based pet food would potentially compromise the supply of nutrients, even if the product would be completely balanced, which explains concerns with plant-based pet food (Brown, 2009). The animal products used to supply most of the proteins do not have cell walls that render nutrients unavailable and do not contain bioactive substances that potentially lower the bioavailability of nutrients. Although whole, unrefined, raw plant-derived protein sources can be less digestible than animal products (Ogawa et al., 2018), plant protein isolates and plant protein concentrates are well digested in pigs (Pedersen et al., 2016) and in vitro (Ayala-Rodríguez et al., 2022; Laguna et al., 2017; Qiu et al., 2013).

Apart from the choice of the protein source, the bioavailability of amino acids can also be affected by a multitude of physical and chemical changes caused by shear, moisture, and heat applied in processing (Salazar-Villanea et al., 2016; Svihus & Zimonja, 2011). Although the protein digestibility of meat analogues has been studied in vitro (Duque-Estrada et al., 2019; Xie et al., 2022), the impacts of the specific and multiple processing steps in creating these products are still unknown.

Phytic acid is a bioactive substance of particular interest in the development of plant-based meat analogues in pet foods. Phytic acid and its salt derivative (phytate) are the primary storage form of phosphorus (P) in plants and the animal can be provided with P if it can be released. P can be released through phytate hydrolysis by enzymes before consumption, by dietary phytase, or through endogenous phosphatase and phytase from the intestinal epithelium or microbiota (Rodehutscord et al., 2016). Although phytic acid is considered to be fairly heat stable (Alonso, Grant, et al., 2000; Yoshida et al., 1980), a better understanding of the impact of processing, including sterilization in an aqueous solution, on P bioavailability for meat analogues is important to formulate foods that supply sufficient but not excess P, which potentially causes adverse health effects on organs such as the kidneys (Dobenecker et al., 2021) and pollutes the environment (Smith & Schindler, 2009). Apart from its role in P bioavailability, phytic acid may also reduce protein digestibility (Carnovale et al., 1988).

Given the potential application of meat analogues in sterilized pet foods, our primary aim was to advance our understanding of the nutritional properties of canned plant-based meat analogues in terms of in vitro digestibility and P solubility. Our hypotheses were that the nutrient quality of plant-based meat analogues is sufficiently high to make them a suitable alternative to animal products in traditional animal-based pet food and that the processing steps increase P solubility. Here, we use a digestion protocol developed for dogs, but it can be expected that results hold for cats as well, because protein digestion efficiencies are similar between cats and dogs (Clauss et al., 2010), despite subtle differences in digestive physiology between dogs and cats (see e.g., NRC, 2006).

2 | METHODS

2.1 | Production of meat analogues and pet food

In this study, fibrous products were made as a model for meat analogues. These model products are therefore referred to as meat analogues in this paper. The meat analogues evaluated were based on wheat gluten, either mixed with soy protein isolate (MaSoy), pea protein isolate (MaPea), or faba bean concentrate (MaFaba) (Table 1). For each meat analogue, three batches of ingredients were made independently to be mixed, sheared (120°C, 30 min) in a small conical shear cell (Manski et al., 2007), and cut into chunks measuring 5 × 10 × 10 mm. A complete animal-based pet food was produced in chunks in a single batch as a reference, as described previously (Wehrmaker et al., 2021). Each batch of meat analogue and the batch of pet food chunks were canned with water or a gravy (water with salt and carrageenan), sealed, and sterilized (126.6°C, 21 min, 1.36 bar) in a Steritech steam air autoclave (European Container Processing & Systems S.A). One can from a batch of each sterilized product was randomly selected and analyzed for composition.

2.2 | In vitro digestion

The following samples were taken for in vitro digestion experiments: one from each of the three batches of sterilized canned meat

TABLE 1 Ingredient composition (%) of three types of meat analogues and the reference pet food.

Ingredients	MaSoy	MaPea	MaFaba	Pet food
Soy protein isolate	15.0			
Pea protein isolate		17.5		
Faba bean concentrate			22.5	
Wheat gluten	15.0	17.5	22.5	
NaCl	0.5	0.5	0.5	1.0
Water	69.5	64.5	54.4	7.8
Poultry				43.3
Meat by-products (liver, spleen)				22.2
Meat				15.6
Animal plasma				3.3
Animal by-product meal				2.7
Whole grain wheat flour				1.6
Vitamins, minerals, taurine				1.6
Air-dried animal blood cells				1.1
Sodium tripolyphosphate				0.7
Canola oil				0.02

Abbreviations: MaFaba, meat analogue based on wheat gluten and faba bean concentrate; MaPea, meat analogue based on wheat gluten and pea protein isolate; MaSoy, meat analogue based on wheat gluten and soy protein isolate.

analogues and a random selection of three cans from one batch of animal-based reference pet food. Samples from the three batches of mixed and sheared products before canning were also analyzed to assess the impact of shearing on dry matter (DM) and nitrogen (N) digestibility. All samples were homogenized by milling (Ika A11 basic Staufen) while being cooled with liquid N. The homogenized samples were then digested in vitro using a modified method of Hervera et al. (2007) and Hervera et al. (2009), simulating gastric and intestinal digestive conditions in dogs. Samples (0.02–0.05 g N) were incubated in pH-stat equipment (877 Titrino plus Titrator [Metrohm Ion Analysis, Metrohm]). Pre-heated phosphate buffer solution (25 mL, 0.1 M, pH 6.0) and HCl solution (10 mL, 0.2 M) were added to the vessel containing the sample. The pH was adjusted to 2.0 with 1 M HCl solution and a fresh pepsin solution (1 mL, 25 g/L, porcine pepsin 2000 FIP U/g; Merck 7190) was added. The samples were incubated at 39°C under constant magnetic stirring (300 rpm). The reaction mixture was monitored for pH and maintained at pH 2 for 2 h by adding 1 M HCl if needed. Fresh pancreatin solution was prepared by mixing 2 g of pancreatin (Porcine pancreas 8*USP; SigmaP-7545) in 40 mL phosphate buffer (0.2 M, pH 6.8) under continuous magnetic stirring for 15 min. After incubating for 2 h, 9 mL of phosphate buffer (0.2 M, pH 6.8) and 5 mL of 0.6 M NaOH were added to the solution; the pH was adjusted to 6.8 with 1 M NaOH solution, after which 1 mL of the supernatant of the pancreatin solution was added. pH was monitored and adjusted with 1 M NaOH if necessary. The hydrolysis was continued for 4 h under the same conditions. Samples of the digestate (1 mL) were taken at 0 min, 120 min (end of the gastric phase), 120 min (start of the intestinal phase), 200 min, 280 min, and 360 min. The samples were immediately flash frozen at −80°C and stored at −20°C until further analyses. At the end of the intestinal phase, digestates were collected, cooled on ice for 10 min, and then centrifuged (30 min, 20,000 × *g*, 4°C). The pellets (insoluble fraction) were removed from the supernatant (soluble fraction) by decanting, freeze-dried, and ground in a porcelain mortar and pestle before chemical analyses.

2.3 | Chemical analyses

Homogenized canned products were analyzed for moisture, N, crude fat, crude fibre, crude ash, and total P. Moisture content was analyzed after drying the sample at 103°C to a constant weight (Reg (EC) 152/2009, III, A). Nitrogen content was analyzed using the Kjeldahl method (Reg (EC) 152/2009, III, C) and crude fat content according to Weibull-Stoldt (Reg (EC) 152/2009, III, H, Procedure B). Crude fibre was analyzed using a procedure with sulphuric acid/potassium lye (Reg (EC) 152/2009, III, I). Crude ash was determined by ashing at 500°C (Reg (EC) 152/2009, III, M). Total P was analyzed using inductively coupled plasma optical emission spectroscopy (ICP-OES; DIN EN 15510 2017-10) as a reference value for the analysis of acid- and enzyme-released P. The analyses were performed by an accredited laboratory (Intertek Food Service GmbH).

The amount of P released after acid treatment and P released after acid plus enzyme treatment (i.e., phytase and alkaline phosphatase) was determined using a Megazyme assay (K-PHYT 05/17; Megazyme) as described by McKie and McCleary (2016). The sample weight was 1.0 g for mixed ingredients, MaFaba sheared, MaFaba sterilized, one sterilized reference sample in water, and all sterilized reference samples in gravy. All the other samples had absorbance below 0.1; therefore, a modified sample extraction procedure with samples of 2.5 g was used for further analyses. The difference in sample weight was accounted for when calculating the P concentrations. Samples were extracted with 20 mL HCl (0.66 M) overnight at ambient temperature. Phosphorus after acid treatment and P after acid plus enzyme treatment were determined by measuring the absorbance (655 nm) in a spectrophotometer (DR3900; Hach). Three samples per product were analyzed once at each processing step.

To evaluate the progress of in vitro protein digestion over time, samples from digestates were analyzed for free amino groups by optical parametric amplifier (OPA) spectroscopy (DR3900; Hach) assay (340 nm) with an L-serine (ReagentPlus; Sigma-Aldrich) standard curve and for the amount of cleaved peptide bonds by bicinchoninic acid (BCA) assay (562 nm) according to the microplate reader protocol of Thermo Scientific (Multiskan FC; Thermo Fisher). Samples were hydrolyzed in 12 M HCl for 24 h at 95°C to determine the total number of peptide bonds in the meat analogues and pet food (h_{tot}). Free amino groups were measured on hydrolysates by OPA spectroscopy assay (340 nm). To evaluate in vitro digestibility at

the end of incubation, mixed ingredients, sheared, and homogenized canned products were analyzed for DM by drying to a constant weight in an oven overnight at 105°C and for N using the Dumas method (Rapid N Exceed; Elementar Analysensysteme) (Dumas, 1831); undigested residues were also analyzed for N using the Dumas method.

2.4 | Calculations and statistical analyses

The content of organic matter in sterilized products was calculated as DM content minus crude ash content, and crude protein (CP) content was calculated as the N content × 6.25. Organic matter, CP, crude fat, and crude fibre contents are expressed on a DM basis.

The mean from a standard curve from 0.00 to 7.50 μg P was used to calculate P released after acid treatment and P released after acid plus enzyme treatment, according to McKie and McCleary (2016).

$$\text{Phosphorus (g/100 g)} = \frac{\text{mean} \times V \times F}{10{,}000 \times w \times v} \times \Delta A \text{ phosphorus},$$

where mean is the average value of P standards (μg/Δ*A* phosphorus), *V* is the original sample volume (mL), *F* is the dilution factor, Δ*A* is the change in absorbance of the sample, 10,000 converts μg/g to g/100 g, *w* is the weight of original sample material (g) and *v* is the sample volume (used in the calorimetric determination step). The protein concentration (g/L) was calculated from a calibration curve bovine serum albumin (0–1 g/L) after subtraction of blanks. The degree of hydrolysis (DH) was defined as the degree of cleaved peptide bonds (*h*) and was calculated according to Nielsen et al. (2001):

$$h = \frac{\text{ser} - \text{NH}_2 - \beta}{\alpha},$$

where ser-NH_2 is serine equivalents (in mequiv/g protein) according to a standard curve corrected for dilutions and blanks divided by the amount of protein in the sample, β is $-NH_2$ before digestion, and α is mequiv/g protein (assumed to be 1).

$$\text{DH} = \frac{h}{h_{\text{tot}}} \times 100\%,$$

where h_{tot} is the total number of peptide bonds as determined by acid hydrolysis.

In vitro DM and N digestibility values were calculated as follows:

$$\text{Digestibility} = \frac{(W_{\text{before}} - W_{\text{after}})}{W_{\text{before}}} \times 100\%,$$

where W_{before} is the weight (g) of sample in DM or N present before incubation, and W_{after} is the weight (g) of residue in DM or N after incubation.

Data were analyzed using a mixed-effects model in PROC MIXED in SAS (version 9.4; SAS Institute). Data from pet foods were only used as a reference and not included in the statistical analyses. To understand whether meat analogues differed in in vitro DM and N digestibility and whether the solution in which these were sterilized mattered, a model was used that included the fixed effects of the type of meat analogue (MaFaba, MaPea, and MaSoy), medium (water and gravy), the interaction between type and medium (T × M) and the random effect of the batch. To understand whether shearing had an impact on the digestibility of meat analogues, we used a second model that included the fixed effects of meat analogue type (MaFaba, MaPea, and MaSoy), shearing (before and after), the interaction between type and shearing, and the random effect of the batch. As sample weigh-ins for sheared products and sterilized products were deemed to differ too much, no statistical analysis was performed to evaluate whether the final step in the process (i.e., sterilization) had an impact on digestibility. To better understand whether meat analogues differed in soluble P after HCl treatment and after HCl plus enzyme treatment and whether the solution in which these were sterilized affected these parameters, a model was used that included the fixed effects of meat analogue type (MaFaba, MaPea, and MaSoy), medium (water and gravy), the interaction between type and medium (T × M) and the random effect of batch. Furthermore, we calculated whether the processing steps led to differing P solubility irrespective of the type of medium used. The statistical model included meat analogue type (MaFaba, MaPea, and MaSoy), processing applied (mixing, shearing, and sterilizing), and the interaction between type and processing (T × P). The assumed normality of the error term (residuals) was examined using the Shapiro–Wilks test and confirmed for all statistical analyses except for P released after HCl and enzyme treatment as affected by meat analogue type and processing. These data were analyzed after an inverse transformation. In the case of significance, post hoc comparisons of least square means were performed. Differences were considered statistically significant at $p < 0.05$.

3 | RESULTS

3.1 | Chemical composition of sterilized meat analogues and pet food

The DM content of meat analogues sterilized in water and gravy ranged between 13.1% and 19.5%; the DM content was lowest for MaSoy, intermediate for MaPea, and highest for MaFaba (Table 2). The CP content ranged from 72.8% to 87.8% of DM and was lowest for MaFaba, intermediate for MaPea, and highest for MaSoy. Crude fat content of the meat analogues was in general low and highest for MaPea in gravy (7.6% DM). Crude fibre content was below 1% of DM in meat analogues.

3.2 | In vitro protein digestion kinetics

The DH, as measured by free amino groups, increased in all samples (9%–26%; Figure 1) from min 120 of the gastric phase to the same

TABLE 2 Chemical composition (% of dry matter) of three types of meat analogues and the reference pet food sterilized in water or gravy.

	Sterilized in water				Sterilized in gravy			
	MaSoy	MaPea	MaFaba	Pet food	MaSoy	MaPea	MaFaba	Pet food
Dry matter	13.8	15.6	18.4	17.4	13.1	15.7	19.5	18
Organic matter	95.7	94.9	95.1	88.5	99.2	94.9	94.9	88.3
Crude protein	86.2	81.4	72.8	55.7	87.8	80.9	72.8	55.0
Crude fat	2.9	7.1	4.3	27.6	5.3	7.6	5.1	26.7
Crude fibre	<dl	<dl	<dl	0.6	<dl	<dl	<dl	0.6
Total phosphorus	0.4	0.4	0.5	1.1	0.5	0.4	0.5	1.1

Abbreviations: dl, detection level = 0.1% on an as-is basis; MaFaba, meat analogue based on wheat gluten and faba bean concentrate; MaPea, meat analogue based on wheat gluten and pea protein isolate; MaSoy, meat analogue based on wheat gluten and soy protein isolate.

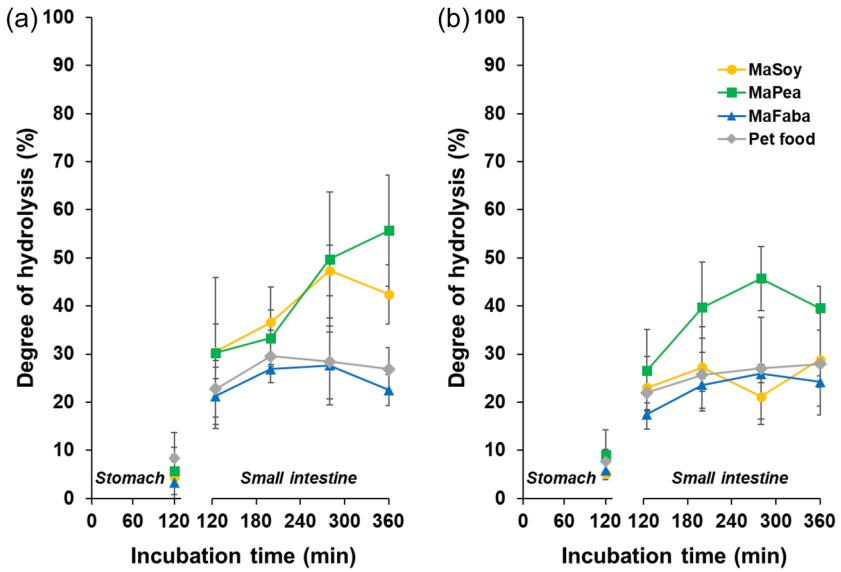

FIGURE 1 Degree of hydrolysis of sterilized meat analogues (Ma) based on wheat gluten with soy protein isolate (MaSoy), pea protein isolate (MaPea), faba bean protein concentrate (MaFaba), and conventional pet food as reference measured during *in vitro* digestion at 120 min (end of the gastric phase and start of the small intestinal phase), 200, 280, and 360 min. (a) Sterilized in water; (b) sterilized in gravy. Lines are added to guide the eye. Error bars represent the standard deviation. [Color figure can be viewed at wileyonlinelibrary.com]

time point but under intestinal digestion conditions (min 120 of the intestinal phase). After that, the DH increased further until min 280 in all samples except for MaSoy sterilized in gravy, which showed a decrease at min 280. From min 280 to min 360, the DH increased in MaSoy sterilized in gravy and MaPea sterilized in water, whereas in all other samples, the DH decreased slightly again at the last time point (min 360). The DH at min 360 reached from 23% in MaFaba sterilized in water to up to 56% in MaPea sterilized in water, averaging 34% for sterilized products.

The protein concentration, as measured by the total number of peptide bonds (BCA assay), decreased in all samples during the course of gastric and intestinal digestion (Figure 2). At 120 min of the gastric phase, a decrease in peptide bonds could be observed for MaSoy, MaPea, and pet food sterilized in water, whereas they increased for MaPea sterilized in water, MaSoy, MaPea, MaFaba, and pet food sterilized in gravy. From then on, peptide bonds steadily decreased except for pet food sterilized in gravy, in which an increase from 200 to 280 min was observed. In general, a plateau seemed to be reached after 200 min.

3.3 | Extent of in vitro dry matter and nitrogen digestibility

The extent of in vitro DM digestibility of the sterilized meat analogues ranged from 77.3% (MaPea in gravy) to 81.9% (MaSoy in gravy), whereas N digestibility ranged from 91.6% (MaPea in gravy) to 94.2% (MaSoy in water) (Table 3). The pet food, used as a reference product, had in vitro DM digestibility values of 56.6% and 57.6% and N digestibility values of 91.8% and 91.7% when sterilized in water and in gravy respectively. The type of medium (water or gravy) did not affect the in vitro digestibility (DM, N) of the meat analogues ($p > 0.05$ for T × M and for medium). The composition of the meat analogues did not influence in vitro digestibility values ($p > 0.05$ for type).

In vitro DM digestibility of mixed ingredients was lowest for MaFaba (72.8%) and highest for MaSoy (83.2%). After shearing, MaFaba had the lowest digestibility (71.7%) and MaPea the highest (81.4%) (Table 4). For N, the digestibility values were similar for the ingredient mixtures and for sheared meat analogues. Shearing had a

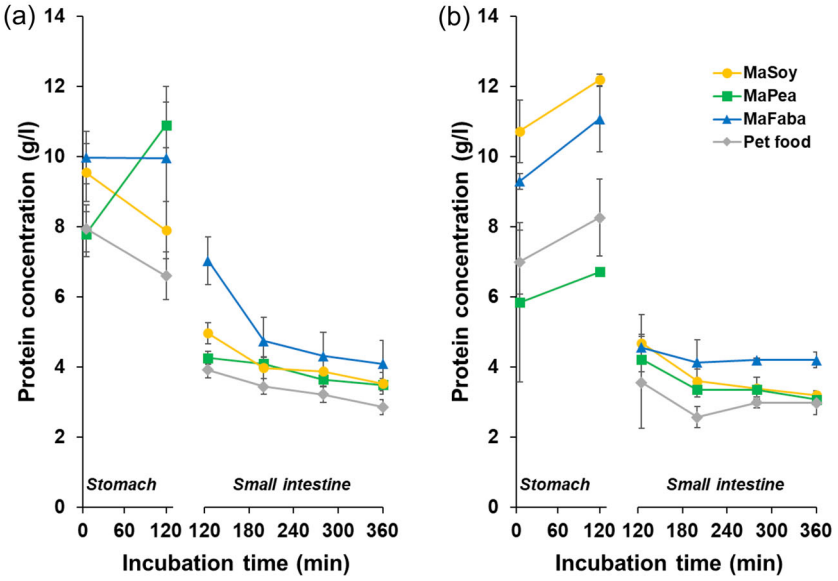

FIGURE 2 Protein concentration as measured by peptide bonds of sterilized meat analogues (Ma) based on wheat gluten with soy protein isolate (MaSoy), pea protein isolate (MaPea), faba bean protein concentrate (MaFaba) and conventional pet food as reference measured during in vitro digestion at 0 min and 120 min (end of the gastric phase and start of the small intestinal phase), 200, 280, and 360 min. (a) Sterilized in water; (b) sterilized in gravy. Lines are added to guide the eye. Error bars represent the standard deviation. [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 3 In vitro dry matter and nitrogen digestibility (%) of three types of meat analogues sterilized in water or gravy.

	Sterilized in water			Sterilized in gravy				*p*-Value		
	MaFaba	MaPea	MaSoy	MaFaba	MaPea	MaSoy	Pooled SE	Type	Medium	T × M
Dry matter	78.0	77.9	81.9	79.7	77.3	81.7	1.8	0.066	0.745	0.763
Nitrogen	92.3	92.2	94.2	92.5	91.6	93.0	0.9	0.201	0.507	0.741

Abbreviations: MaFaba, meat analogue based on wheat gluten and faba bean concentrate; MaPea, meat analogue based on wheat gluten and pea protein isolate; MaSoy, meat analogue based on wheat gluten and soy protein isolate; SE, standard error; T × M, interaction between meat analogue type and medium (water or gravy) in which it was sterilized.

TABLE 4 In vitro dry matter and nitrogen digestibility (%) for three types of meat analogues before (mixed) and after shearing (sheared).

	Mixed			Sheared				*p*-Value		
	MaFaba	MaPea	MaSoy	MaFaba	MaPea	MaSoy	Pooled SE	Type	Shear	T × S
Dry matter	72.8	80.1	83.2	71.7	81.4	79.8	1.4	<0.001	0.362	0.275
Nitrogen	96.0	94.0	96.1	93.0	94.8	94.8	0.8	0.396	0.125	0.119

Abbreviations: MaFaba, meat analogue based on wheat gluten and faba bean concentrate; MaPea, meat analogue based on wheat gluten and pea protein isolate; MaSoy, meat analogue based on wheat gluten and soy protein isolate; SE, standard error; T × S, interaction between meat analogue type and effect of shearing.

consistent effect on the digestibility (DM, N) of the meat analogues ($p > 0.05$ for T × S and for shear). Meat analogues only differed in in vitro DM digestibility ($p < 0.001$ for type); MaFaba had lower digestibility than MaSoy ($p = 0.004$) and MaPea ($p = 0.002$) at this stage of the process (i.e., before sterilization).

3.4 | Phosphorus solubility

Total P (in g/100 g DM) as determined by ICP-OES was 0.44 ± 0.02 in MaSoy, 0.48 ± 0.03 in MaPea, 0.53 ± 0.04 in MaFaba, and 1.10 ± 0.01 in the pet food (Figure 3). For mixtures, P released after acid (HCl) treatment and after acid plus enzyme treatment were in the range of total P and similar for the different meat analogue recipes. Shearing decreased P released after acid treatment to 0.08 g/100 g DM for MaSoy and MaPea and to 0.17 g/100 g DM for MaFaba ($p < 0.0001$). After sterilization, values of P after acid treatment were increased, ranging from 0.17 and 0.18 g/100 g DM (MaSoy and MaPea respectively) to 0.35 g/100 g DM for MaFaba sterilized in water and 0.31 g/100 g DM for MaFaba sterilized in gravy ($p < 0.0001$). Compared with the mixtures, shearing decreased the amount of P released after acid plus enzyme treatment to 0.29 g/100 g DM in MaSoy ($p < 0.0001$), to 0.31 g/100 g DM in MaPea ($p < 0.0001$), and to 0.49 g/100 g DM in MaFaba ($p = 0.3648$). Values measured after sterilization were slightly higher than values found after shearing for all meat analogues: MaSoy, $p = 0.0380$; MaPea, $p = 0.0077$; MaFaba, $p = 0.0122$. The sterilized product appeared to have higher levels than the mixed product only for MaFaba, although this did not reach

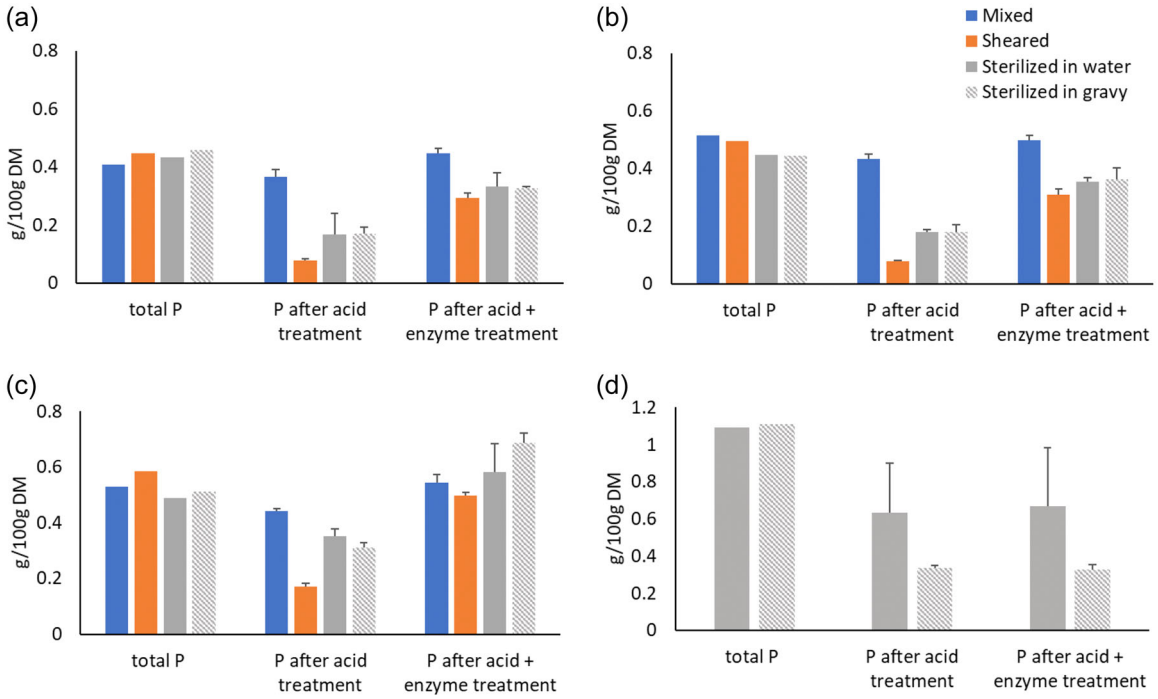

FIGURE 3 Total phosphorus content and phosphorus solubility of mixed, sheared, and sterilized meat analogues (Ma) based on wheat gluten with (a) soy protein isolate (MaSoy), (b) pea protein isolate (MaPea), (c) faba bean protein concentrate (MaFaba), and (d) conventional pet food as reference. Solubility was measured as phosphorus released after acid (HCl) treatment and after acid plus enzyme (phytase, alkaline phosphatase) treatment. Error bars represent the standard deviation. DM, dry matter. [Color figure can be viewed at wileyonlinelibrary.com]

statistical significance ($p = 0.1120$). For pet food, the P released after acid treatment was considerably lower than the total P content; the pet food sterilized in water showed a higher release of P than pet food sterilized in gravy. Furthermore, treating the pet food with enzymes did not change the release of P.

4 | DISCUSSION

The results confirm the hypothesis that the products were highly digestible in vitro. Furthermore, shearing did not affect the in vitro DM and N digestibility of the products. Shearing led to decreased P solubility, which was not expected. However, this decrease was partly reversed by further processing in a medium (sterilization).

4.1 | Method of measuring the extent of DM digestion

The in vitro DM digestibility values of the canned pet foods were up to 34.8% lower than the average reported by van Zelst et al. (2015) (91.4% ± 2.6%). Two reasons could account for this: the quality of the ingredients used and the method used to collect the undigested residue after incubation. In vitro DM digestibility of fresh meats and dry meat meals was found to vary from 89% to 92% and from 52% to 72% respectively (Montegiove et al., 2021). However, the pet food used in the present study contained only small amounts of dry animal by-product meal, and N digestibility was in the expected high range. The method of separation of DM at the end of incubation was likely the reason for the relatively low digestibility values. In the present study, the digested fraction was separated from the undigested residue by centrifugation. Low DM digestibility could have been caused by the fat that remained in the pellet, which was not separated from the undigested fraction by washing the residue on a filter as performed in other studies (Hervera et al., 2009; van Zelst et al., 2015). However, the fat content of meat analogues was low because the ingredients are low in fat and/or the fat had been removed, whereas the ingredients of the animal-based pet food are naturally high in fat and the recipe also contained oil. We, therefore, hypothesize that the difference in fat contents and the approach to separate the digested from the undigested fraction in this in vitro method underlies the relatively low DM digestibility value of the reference pet food.

4.2 | In vitro digestibility

The DH after the gastric phase of incubation ranged from 3.4% (MaFaba sterilized in gravy) to 9.1% (MaPea sterilized in gravy). These outcomes are in accordance with the in vitro study of Ren et al. (2018) in which different sterilized preparations of soy protein isolate and soy protein isolate with black soybean seed extract had a DH of <10% at the 60 min gastric phase. The DH of pet food after the gastric phase was comparable with the DH of meat analogues.

Cooked plant-based meat analogues based on textured soy protein concentrate reached approx. 7% DH after 60 min of in vitro gastric digestion and 15.8% DH after 120 min (Zhou et al., 2021). Similarly, Rivera del Rio et al. (2020) measured gastric in vitro digestion of unheated and heated soy and pea protein isolate (90°C and 120°C for 30 min respectively) and showed that the DH after 120 min of gastric digestion increased from about 6%–16%. They observed that pepsin-resistant peptides were present in the digesta. Similarly, sheared soy protein concentrate, soy protein isolate, and soy protein isolate with pectin (at 100°C, 120°C, and 140°C for 30 min respectively) were digested in vitro for up to 180 min under gastric conditions and DH values between 3% and 15% were found. The DH was influenced by the processing applied, the raw materials used, and the particle size of ground meat analogues (Duque-Estrada et al., 2019). Tian et al. (2019) showed that gastric DH of soy protein isolate initially increased when heated up to 85°C for 15 min, but then a decrease was observed. The same was true for heating time; DH increased up to 30 min at 85°C but decreased at 60 min.

The DH in the small intestinal phase in the present study for meat analogues as well as pet food was lower than reported earlier for heated (5 min, 90°C) pea protein isolate (up to approx. 70%) and pea protein-rich flour (up to approx. 85%), but similar to pea flour (up to approx. 60%) (Rivera Del Rio et al., 2022). Cooked, but not sterilized, meat analogue based on textured soy protein concentrate was reported to have a DH of 54% (Zhou et al., 2021). Ren et al. (2018) found that the DH of sterilized (121°C, 15 min) soy protein isolate at the end of 120 min intestinal in vitro digestion was about 37%, similar to the present results. The somewhat lower DH values obtained in the present study might relate to the complexity of the samples, which also contained gluten and were more extensively processed than the protein sources evaluated in the studies in the aforementioned literature.

When comparing gastric and small intestinal digestion, the DH increases immediately in the small intestinal phase of in vitro incubation. This is also apparent and illustrated by the protein concentration (Figure 2), which remains stable in the gastric phase but decreases quickly at the first intestinal measurement. This increase was observed in plant-based meat analogues as well as in pet food. This is likely due to increased protein solubility and thus accessibility to digestive enzymes after the change in pH from the gastric to intestinal phase at pH 6.8. A similar effect was observed for soybean meal, rapeseed meal, whey powder, dried porcine plasma protein, mealworm, and black soldier fly larvae when the pH was changed from 3.5 to 6.8 (Chen et al., 2019). However, this effect was not reported for wheat gluten, probably due to the fact that the solubility of wheat gluten is less dependent on pH. The fast increase in DH followed by a plateau reached after 60 min of intestinal digestion was also observed in commercial textured soy protein concentrates (Zhou et al., 2021). The fast increase in DH might be caused not only by changes in pH but also by the specificity of digestive enzymes applied. Pepsin mostly cleaves peptide bonds of hydrophobic and aromatic amino acids, whereas pancreatin mostly cleaves bonds of basic amino acids and aromatic amino acids (Gong et al., 2022). Furthermore, it can be assumed that intact proteins were hydrolyzed with the fast zipper-type mechanism following Linderstrøm-Lang's theory (Linderstrøm-Lang, 1952) at the beginning of the intestinal phase when aggregated proteins became soluble (Abrahamse et al., 2022). For all samples, including pet food, the DH plateaus reached during in vitro digestion shows that the full extent of protein digestion was reached.

The extent of in vitro N digestibility was high for all sterilized meat analogues and the reference pet food (above 90%), which is in line with values for pet foods and with digestibility values of products with similar plant proteins. The findings for these pet foods agree with the average ± standard deviation values of 95.9% ± 2.1% reported for 20 processed and unprocessed canned pet foods using a comparable in vitro digestion procedure (van Zelst et al., 2015). Notably, MaFaba had lower DM digestibility values than other meat analogues before sterilization, which was not the case after sterilization. Digestibility data for meat analogues evaluated with similar methods are scarce. The N digestibility for MaFaba and MaPea was comparable with values of 89% and 91% for infant formula consisting of 50% milk protein and either 50% faba bean concentrate or pea protein concentrate, using an *in vitro* dynamic system (Le Roux et al., 2020). Although the apparent faecal digestibility measurement overestimates N digestibility at the ileal level (Hendriks et al., 2012), the apparent faecal N digestibility of extruded foods with 30% dehulled faba beans was 90.0% (Corsato Alvarenga et al., 2020). Finally, to further advance our understanding of the nutritional value of meat analogues, it is of interest to look beyond N digestibility and focus on (in vitro) amino acid bio-availability in relation to amino acid requirements in dogs and cats in future studies (see also Wehrmaker et al., 2022). These next steps in research and development on plant-based pet foods also include advancements in dog- and cat-specific formulations to have complete (e.g., including taurine) and balanced nutrient profiles (e.g., amino acids), as well as quantifying environmental impact for comparison with conventional pet foods.

We could not observe an effect of shearing on DM or N digestibility of MaSoy, MaPea, or MaFaba, which differs from other findings reported in the literature. When raw and extruded faba beans and peas were digested *in vitro* by trypsin and chymotrypsin, protein digestibility after extrusion increased by more than 20% to 87.4% and 83.0% respectively (Alonso, Aguirre, et al., 2000). This was attributed to a reduction of trypsin and chymotrypsin inhibitors. Trypsin inhibitors are mainly present in the cotyledons of soybeans, peas, and faba beans (Avilés-Gaxiola et al., 2018); however, trypsin inhibitor activity in faba bean hulls was two-fold that in the cotyledon in some reports (Savage & Morrison, 2003). Wheat gluten only contains α-amylase inhibitor (Gélinas & Gagnon, 2018). Reduction of trypsin inhibitor activity could be demonstrated in faba bean isolate produced using a wet process (Vogelsang-O'Dwyer et al., 2020). Trypsin inhibitors are probably removed during protein fractionation due to the high or low pH applied during the protein extraction process (Avilés-Gaxiola et al., 2018). As expected, the raw materials used for meat analogues (protein isolates or concentrates) were

extracted using a method that removes trypsin inhibitors (Anderson & Wolf, 1995). Zentek and Goodarzi Boroojeni (2020) also concluded that increases in protein digestibility after hydrothermal processing can be attributed to reductions in bioactive compounds of plant ingredients. The high digestibility values can also be explained in part by the wheat gluten content of the recipes because wheat gluten already has an apparent faecal N digestibility up to 98% in dogs (Kendall & Holme, 1982) and up to 99% in Australian silver perch (Allan et al., 2000). This effect was especially pronounced in MaFaba; 57% of the protein was from wheat gluten in MaFaba, 49% in MaPea, and 48% in MaSoy. However, meat analogues were highly digestible independently of the protein source mixed with gluten.

4.3 | Phosphorus solubility

Plant-based materials contain considerable amounts of P in the form of phytic acid, which hinders the availability of P to the animal. The fractionation method used to make the ingredients (i.e., concentrates, isolates) and additional processing to create the final products can influence the solubility of P (Amat et al., 2022). A good understanding of P solubility is required when formulating complete pet foods to achieve appropriate P levels and an appropriate Ca to P ratio (Luo & Xie, 2013; Naczk et al., 1986). In addition, phytic acid, as well as other phosphates, chelate with minerals such as Cu, Ca, Fe, Mn, and Zn (Humer et al., 2015), making them less available to the animal. Hence, not only P but also other minerals might be less available in phytic acid-rich pet foods. The method applied in this study to determine P solubility in defined conditions after processing of meat analogues with different plant ingredients aimed to determine maximal P solubility. The method first exposes the products to an acid environment and then to the digestive enzymes alkaline phosphatase and phytase (Dersjant-Li et al., 2015). Most P from phytic acid is believed to be released in the acidic environment of the stomach through dietary phytases (Seynaeve et al., 2000). The addition of phytase in the method is of physiological and practical relevance. Phytase is produced by intestinal microbiota in vivo (Seynaeve et al., 2000), and if phytate levels in plant-based pet foods decrease the nutritional value too much, the addition of phytase to plant-based pet foods may be an option in the future.

The mixed ingredients had similar P levels after acid treatment to total P levels measured by ICP-OES. The addition of enzymes hardly increased P levels. This suggests that most P can be liberated/solubilized by acid and only a fraction is linked to phytate (or in the form of phosphates linked to insoluble compounds). Phytase produced by intestinal microbes has been reported to contribute to the utilization of dietary phytate in chickens (Józefiak et al., 2016). Similar utilization can also be expected in canines (Pereira et al., 2020), suggesting that the application of phytase would not be necessary for adequate utilization of P for meat analogues. Shearing of the mixed products decreased soluble P, which was most pronounced when P was released by the acid treatment (i.e., without enzymes). Different underlying mechanisms for the decrease in soluble P can play a role, e.g., complexation of phytic acid with proteins, complexation of phytic acid with minerals, or complexation of P with protein and/or minerals, especially Ca. This was most likely also the case here. Complexation of phytic acid with protein is dependent on pH (Cheryan & Rackis, 1980). The pH of protein in sterilized products after milling ranged from 6.42 ± 0.07 (MaFaba in water) to 7.05 ± 0.05 (pet food in gravy) (Table S1). Phytic acid-protein complexes can be formed at pH 5–7 (Cheryan & Rackis, 1980); however, it is not clear whether the pH at the shearing step was in a range that could have promoted phytic acid–protein complexation. Thus, the experimental results show a change in P solubility, but so far, the outcomes do not allow a conclusive explanation; the exact mechanisms need to be explored further.

Sterilization then increased the levels of soluble P again, and in MaFaba, the reduction in soluble P was even completely reversed after sterilization. Enhanced mineral HCl extractability after pressure cooking (15 min, 121°C) is reported for pigeon pea and could be caused by the release of mineral-P complexes (Duhan et al., 2004). The differences in P solubility might also be related to changes in product solubility, which is key for the efficacy of phytase (Schlemmer et al., 2009). Softening of the sheared product after sterilization due to additional water uptake has been observed (Wehrmaker et al., 2021), and it is hypothesized that phytate–protein complexes can become more accessible for enzymes through processing (Selle et al., 2012). Thus, this could also have been the case here, explaining part of the increased release of P after sterilization.

A study on dogs showed a higher bioavailability of P when Zn proteinate was used but did not show differences in P and Zn availability after feeding a high phytate diet without and with phytase for 5 weeks, suggesting that adaptation of phytate digestion may be taking place (Pereira et al., 2020). Whether this is indeed the case in canines and whether it is relevant in releasing P and possibly minerals bound during processing remains to be elucidated. In general, P can be absorbed in the small and large intestine in dogs (Schünemann et al., 1989). Further research should focus on the adaptiveness and responsiveness of canine intestinal microbiota to P provided in plant-based diets and subsequent P absorption. P levels in isolates and wheat gluten were generally low, especially compared with the P requirements and losses that might occur. However, P levels were comparable with or higher than in the reference pet food, which again was comparable with on average 53% P solubility for wet pet foods after in vitro gastric and small intestinal digestion reported previously (Soutar et al., 2021). That study examined P solubility in vitro with the application of taurine-conjugated bile salts after water extraction for 30 min, excluding P buffers to circumvent the addition of P during the in vitro digestion. This suggests that only minor supplementation is necessary to make a complete and balanced plant-based meat analogue pet food.

4.4 | Effect of sterilization medium

No effect of the sterilization medium, i.e., water or gravy, was observed for P solubility, DM digestibility, or N digestibility. Although

sterilization in water or gravy could yield differences in the physical properties of the meat analogues (Wehrmaker et al., 2021), we did not observe an impact on the nutritional properties investigated in the present study. Possibly, dilution with liquids in the in vitro system decreases the impact of gelling agents on digestion as well as on P solubility. However, gelling agents have been found to affect nutrient digestion. However, in vivo, Karr-Lilienthal et al. (2002) observed higher ileal amino acid digestibility when a canned dog food contained gelling agents (0.2% guar gum and 0.5% carrageenan, 0.2% locust bean gum and 0.5% carrageenan) compared with the food without gelling agents. This was attributed to the increased transit time due to increased viscosity, which is not accounted for in a static in vitro digestion system as used here. Clearly, the role of gelling agents in plant-based dog foods as opposed to animal-based dog foods should also be studied in vivo.

5 | CONCLUSIONS

The in vitro N digestibility of the three sterilized canned plant-based model products for meat analogues was high and in line with pet food digestibility. The rate of in vitro protein digestion was low in the gastric phase and increased quickly in the intestinal phase but remained low. Phytate seemed to play a minor role in the P solubility of the plant-based products and solubility was high for the sterilized products. However, P solubility was influenced by processing, implying that processing steps should be designed carefully to ensure adequate P supply. When considering protein digestibility and P solubility, the plant-based products performed similar to the sterilized canned dog food, facilitating the implementation of meat analogues in plant-based dog foods.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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