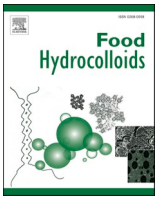
Food Hydrocolloids 150 (2024) 109732

Contents lists available at ScienceDirect

Food Hydrocolloids

journal homepage: www.elsevier.com/locate/foodhyd



Physico-chemical properties of pea fibre and pea protein blends and the implications for *in vitro* batch fermentation using human inoculum

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ARTICLE INFO

Keywords:
Yellow pea
Pea hull fibre
Pea protein
Rheology
Microstructure
In vitro human colonic fermentation

ABSTRACT

The incorporation of fibre into pea protein matrices influences their microstructure, yet our understanding of their gut fermentability remains unexplored. In this study, dietary fibres and protein from yellow pea were investigated for their physico-chemical properties and impact on *in vitro* colonic fermentation using human inoculum. Pea fibre and pea protein blends were studied at different pH and after thermal treatment at 95 °C for 30 min with oscillatory rheology, static light scattering and confocal laser scanning microscopy. The effect on *in vitro* colonic fermentation was evaluated measuring gas production, ammonia, and short chain fatty acid (SCFA) production. Rheology indicated that during thermal treatment a firmer gel is formed close to the protein isoelectric point with a structure characterised by aggregation, but less particle swelling compared to other pH. Addition of fibre led to higher storage modulus (G'), with the fibre dominating the rheological properties. Fermentation of samples containing protein led to higher levels of ammonia and SCFA compared to only fibres. Blends produced higher amounts of valerate, *i*-valerate and caproate, and lower amounts of ammonia. Reduced fermentation of proteins in the presence of fibres was also reflected in a more intact microstructure of the protein particles in the digesta. Although thermal treatment of blends caused particle swelling and induced gelation, only small differences could be discerned in the *in vitro* colonic fermentation outcomes. Our results highlight that potentially harmful fermentation products from protein, such as ammonia, were reduced in the presence of pea hull fibre.

1. Introduction

Intake of plant-based protein and dietary fibre is important for a healthy diet. Yellow pea (*Pisum sativum* L.) is a legume rich in both protein and dietary fibre and is of interest for its nutritional value, low allergenicity and possibility to grow in temperate climates (Boukid, Rosell, & Castellari, 2021; Roy, Boye, & Simpson, 2010). Field pea consist of 20–30 % protein, split into two fractions; globulins and albumins (Gueguen & Barbot, 1988). Globulins are salt soluble and consist of legumin, vicilin and convicilin, while albumins are smaller water-soluble proteins (Karaca, Low, & Nickerson, 2011). The pea protein fraction has properties beneficial to a wide variety of food systems, such as stabilisers in emulsions (Burger & Zhang, 2019), foams (Cui et al., 2020; Kornet, Yang, Venema, van der Linden, & Sagis, 2021) and texturisers (Kornet, Penris, et al., 2021; Moreno et al., 2020). Other large fractions of field pea are starch (~60 %) and fibre (Stone, Karalash, Tyler, Warkentin, & Nickerson, 2015; Tiwari & Singh, 2012). The fibre fraction is circa 15–25 % of the total pea and is mainly present in the pea hull (Dalgetty & Baik, 2003).

Addition of polysaccharides to pea proteins is known to affect its functionality and textural features. However, the magnitude and type of change is specific to the added polysaccharide. Schreuders and co-workers showed that the addition of pectin or cellulose to pea protein changed the mechanical properties of the systems (Schreuders et al., 2022). Higher concentration of pectin added to the protein resulted in increased elasticity, while a higher amount of cellulose barely affected

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https://doi.org/10.1016/j.foodhyd.2024.109732
Received 13 October 2023; Received in revised form 18 December 2023; Accepted 2 January 2024
Available online 3 January 2024

the results. The difference in mechanical properties stems from protein-polysaccharide interactions, as no pectin gelation was induced. Furthermore, mixing plant protein and fibre leads to changes in mechanical and textural properties (Johansson et al., 2021) however, the effect on the digestibility and fermentability of these food systems remains unclear. Addition of pectin to pea protein dispersions was demonstrated to increase the dispersibility of the system at lower pH (Lan, Chen, & Rao, 2018). The pea protein particles and pectin could form soluble complexes, enhancing the colloidal stability.

Dietary fibres (to which several polysaccharides belong) are indigestible by humans and their degradation occurs during microbial fermentation in the colon (Buttriss & Stokes, 2008; Titgemeyer, Bourquin, Fahey, & Garleb, 1991). Proteins are digested to a large part before reaching the colon, but the proteins that bypass digestion and reaches the large intestine, are fermented by the gut microbiota leading to the production of metabolites with potential health detrimental effects (Gilani, Cockell, & Sepehr, 2019). The percentage of ingested protein that bypass digestion and reach the colon depends on the type and amount of proteins consumed, but is generally around 10 % (Scott, Gratz, Sheridan, Flint, & Duncan, 2013). Plant proteins can be encapsulated in cell walls, which is the case for legumes such as pea. Depending on the structure of the cell wall (intact or broken), the bioaccessibility of the protein in the digestive tract differs. Intact cell walls can maintain their cellular structure until they reach the large intestine, where the cell wall will be fermented and thus exposing protein and other macromolecules to the microbiota (Bhattarai et al., 2021).

Compared to dietary fibre fermentation, protein fermentation results in a diverse range of metabolites, with the main products being SCFA, branched chain fatty acids (BCFA) and nitrogenous compounds such as ammonia (Cummings & Macfarlane, 1991). Ammonia is known to affect the intestinal tissue and can be correlated to tumour growth in the colon. Other toxic metabolites produced in lesser amounts are phenols, amines and sulphites (Macfarlane & Macfarlane, 1997). Amines are believed to act as precursor to nitrosamines which is a known carcinogen detectable in human excrement, while high concentrations of sulphites are linked to diseases such as inflammatory bowel disease (IBD) (Scott et al., 2013). However, the presence of dietary fibres reduces the production of toxic metabolites by promoting microbial proliferation and thereby increasing the need for peptides in bacterial proteosynthesis (Bernalier-Donadille, 2010; Smith & Macfarlane, 1996).

The objective of this study is to investigate the influence of pea fibres on metabolites generated from pea protein colonic fermentation, particularly in the context of potential impacts on the large intestine. The research specifically focuses on discerning the effects of non-thermally and thermally treated pea protein, pea fibres, and their blends on total gas production, ammonia production, and short-chain fatty acid (SCFA) generation during *in vitro* colonic fermentation. Prior to the fermentation process, an exhaustive characterisation of microstructure and rheological properties for both non-thermally and thermally treated samples was conducted to distinguish the individual contributions of microstructure, rheological properties, and composition to *in vitro* colonic fermentation. The findings are expected to provide valuable insights into the complex interplay of pea fibre and protein components in the context of colonic fermentation, contributing to our understanding of dietary impacts on gut health.

2. Materials and methods

2.1. Materials

The yellow pea protein isolate (*Pisum Sativum*) used was Pisane® C9 (Cosucra Groupe Warcoing S.A., Belgium) with 88% protein content on dry basis. The yellow pea hull fibre used was Vestkorn Fibradan® F20X (Vestkorn Milling A/S, Denmark) with 89.5% total dietary fibres on dry basis. The composition of the pea fibres has been reported by Karlsson et al., briefly, pea fibre is mainly composed of cellulose (~60 %), hemicelluloses (~25 %) and pectins (~15 %) (Karlsson et al., 2023). Starch analysis was performed for both the pea hull fibre and pea protein samples, and showed that pea hull fibre and pea protein contained <1 % of starch. The pea protein was also subjected to monosaccharide analysis, showing that <2 % of the pea protein was carbohydrates. In addition, the samples contain <10 % of moisture, as given by the suppliers. Therefore, we believe that none of these impurities would have a significant impact on the results. All chemicals used were obtained from Merck (Darmstadt, Germany) if not otherwise stated and were of analytical grade.

2.2. Sample preparation

Dispersions containing 15 % (wt %) yellow pea protein isolate (PPI) and yellow pea hull fibre (PF) was prepared. The procedure involved adding 51.17 g of the sample to 300 mL of deionized water, to obtain a total protein or fibre concentration of 15 wt %. The blend was then homogenized using a Silverson at 7000 rpm for 2 min (L5M-A, Chesham, UK) and an Ultra-Turrax at 16 000 rpm for 1 min (T18 digital, IKA Works GmbH & Co. KG, Staufen, Germany) to reduce particle size and improve the overall dispersibility of the samples. Six samples were prepared for the rheological and microstructure measurements and six samples for the *in vitro* colonic fermentation experiments (Table 1).

2.3. Analysis of protein and fibre

2.3.1. Particle size

Mastersizer 2000 (Malvern Instruments Ltd, Malvern, UK) equipped with a 2000 Hydro-SM accessory was used to measure the particle size distribution (PSD). A 100 mL volume of deionized water was added to the accessory. A small amount (approximately 0.5 mL) of each sample was pipetted into the water. The PSD analysis was performed using a refractive index of 1.47, an absorption of 0.01, and an obscuration range of 5–10%. Since the particles in the dispersions had an irregular shape, the model for irregular particles was selected. The instrument software (Mastersizer 2000; version 6.01) calculated the PSD based on the intensity profile of the scattered light. The surface area-based $D_{[3,2]}$ diameters were obtained for every sample, with equation (1).

$$D_{[3,2]} = \sum_i n_i d_i^3 \Big/ \sum_i n_i d_i^2 \qquad \text{eq. 1}$$

2.3.2. Zeta potential

The zeta potential of protein and fibre were measured using a

Table 1
Composition of prepared aqueous dispersions used for rheology and microstructural studies or *in vitro* colonic fermentation and microstructural studies. Solid content was kept constant at 15 wt %. Fibre T, F50T and protein T have been thermally treated at 95 °C for 30 min.

Sample	Ratio Fibre: Protein	Fibre concentration (wt %)	Protein concentration (wt %)
Rheology and microstructure			
Fibre	100:0	15	0
F80	80:20	12	3
F60	60:40	9	6
F40	40:60	6	9
F20	20:80	3	12
Protein	0:100	0	15
in vitro colonic fermentation			
Fibre	100:0	15	0
Fibre T	100:0	15	0
F50	50:50	7.5	7.5
F50T	50:50	7.5	7.5
Protein	0:100	0	15
Protein T	0:100	0	15

Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK) within the pH range of 3–10. A 0.1 mg/mL dispersion of pre-homogenized protein or fibre were prepared in 0.1 M NaCl aqueous solution, and pH was adjusted using 1 M HCl or 1 M NaOH. The samples were sonicated before measurement. The cuvette used for the measurement was a folded capillary zeta cell (Malvern Instruments Ltd, Worcestershire, UK). All samples were done in triplicate with 1–100 subruns.

2.4. Rheological effects of thermal treatment

The rheological measurements were conducted using a DHR-3 rheometer (TA Instruments, New Castle, USA) equipped with a parallel plate geometry of 40 mm diameter, with a solvent trap. The gap between the plates was set to 1 mm. Temperature control was achieved using a Peltier plate, and evaporation was controlled using a custom-built cover from TA Instruments. Constant strain amplitude (γ = 0.5%) and frequency (f = 6.28 rad/s) were used during the thermal treatment (samples held at 95 °C for 30 min). Strain sweeps were performed to determine the linear viscoelastic region (LVR) and a strain of 0.5 % was chosen within the LVR to perform frequency sweeps. Predispersed and homogenized samples of protein were set to different pH values (2, 4.5 and 7) and tested. Fibre, F80, F60, F40, F20 were only ran at pH 7. Before the measurements, the samples were stirred and covered with aluminium foil at 20 °C for 24 h. The samples were then added to the rheometer at 20 °C and heated at a rate of 5 °C/min until T = 95 °C. The temperature was held at 95 °C for 30 min before cooling to 20 °C at a rate of 1 °C/min. Subsequently, a frequency sweep ranging from 0.01 rad/s to 100 rad/s was performed at 20 °C with a strain amplitude of 0.5 %.

2.5. Microstructural characterisation

2.5.1. Confocal laser scanning microscopy (CLSM)

Gels of pea protein and pea protein-pea fibre blends were prepared for confocal laser scanning microscopy (CLSM). Dispersions of 15 wt % protein prepared as mentioned above were set to pH values of 2, 4.5 and 7 with 1 M HCl or 1 M NaOH and mixed at 20 °C for 1 h 15 wt % of protein and fibre blends was set to pH 7. The dispersion (3 mL) was added to a stainless-steel cylinder with a diameter of 16 mm and sealed with a rubber stopper with a hole to prevent pressure build up. The metal cylinder was placed in a water bath at 95 °C for 30 min and thereafter cooled to 20 °C overnight.

Texas red at 0.2 % (wt %) in water (Titus, Haugland, Sharrow, & Segal, 1982) and Direct Red 23 at 0.2 % (wt %) in water (Ursache, Andersen, Marhavý, & Geldner, 2018) were used to stain the protein and fibre respectively. The sample was placed in a metal cup attached to a glass slide and the stain was added. The stain was let to diffuse through the sample for 20 min before the micrographs were taken. Micrographs were taken using a Leica TCS SP5 (Leica Microsystems GmbH, Heidelberg, Germany) equipped with a HXC PL APO lambda blue 20 × 0.70 IMM UV objective. Wavelengths of the excitation laser used for Texas Red and Direct Red 23 were 594 nm and 543 nm respectively. Emission wavelengths were 610–649 nm for Texas Red and 549–565 nm for Direct Red 23. At least five micrographs were taken of each sample.

2.5.2. Light microscopy (LM)

Samples collected before and after *in vitro* colonic fermentation were cut into 7 μm thick sections in a Leica CM3050S cryostat. Sections were applied to microscopy slides. Proteins were stained with Light Green at 0.2 % (wt %) in 0.3% (wt %) acetic acid in water, before examined with an Olympus BX53 light microscope (Olympus Life Science, Tokyo, Japan) with a 10x objective. Micrographs were captured with a CMos SC50 camera (Olympus Life Science) and processed with the Olympus software cellSense Entry version 2.3.

2.6. In vitro colonic fermentation

2.6.1. Preparation of medium and substrate

A total volume of 1500 mL was made by combining basal solution, phosphate buffer, bicarbonate, and vitamins. The medium contained all the necessary essential nutrients such as nitrogen, vitamins, and minerals but lacked a carbon source (formulation shown in Supplementary Table S1). This medium was then divided into seven 500 mL Schott bottles by adding 89 mL to each bottle. The bottles were sterilized by autoclaving at 121 °C for 20 min. To eliminate oxygen, the medium was bubbled with nitrogen for 45 min, and 1 mL of reducing agent was added to create anaerobic conditions.

There were 6 substrates subjected to *in vitro* fermentation, where two substrates of the same compositions were prepared; one with thermal treatment and one non-thermally treated. The three different compositions were protein, fibre and equal proportions of fibre and protein (F50). For the thermally treated substrates, a 3 mL solution of 15 wt % protein and/or fibre was prepared and mixed at 20 °C for 1 h. The mixture was then added to a stainless-steel cylinder and heated in a water bath at 95 °C for 30 min. Afterward, the substrates were cooled overnight at room temperature. The non-thermally treated substrates were stirred at 20 °C for 1 h. Substrates were sterilized in a laminar airflow (LAF) bench using UV-light for 30 min. Varying amount of substrate was weighed into 50 mL falcon tubes under sterile conditions for the samples. 1.5 g was weighed for the protein and fibre, and 3 g was weighed for the protein and fibre blend. Thermally treated samples are denoted fibre T, F50T and protein T. Each substrate was prepared and tested in triplicate.

2.6.2. Inoculum preparation

The inoculum preparation was conducted in a laminar airflow (LAF) bench to maintain sterile conditions. Faeces were obtained from three human volunteers who had unrestricted diets and no history of gastrointestinal diseases. Each experiment was performed in triplicate, using a separate donor for each run. The faecal samples were diluted to a concentration of 20 wt % by mixing them with a sterile 50 mM phosphate buffer solution. The mixture was homogenized to ensure uniformity and then filtered through a sieve bag to remove any solid particles. It is important to note that the inoculum preparation took place within 2 h of the faecal collection to maintain the viability of the microorganisms. Experiments were conducted following ethical guidelines approved by the Swedish Ethical Review Authority (2022-01696-01).

2.6.3. Batch in vitro fermentation

To each substrate 10 mL faecal inoculum was added and mixed on a vortex. The substrate and faecal inoculum mixture were transferred to the bottles containing the anaerobic medium using a syringe, adding up to a total volume of 100 mL. The bottles were kept at 37 °C for 24 h with stirring and sampling occurred at 0, 8 and 24 h. A time limit of 24 h was selected to reduce build-up of metabolites, as the batch *in vitro* colonic fermentation set up lack influx and efflux of substrate and fermentation products. Collected samples were centrifuged at 4 °C and 18 000 rpm to stop the fermentation process and separate the pellet and supernatant. The pH was measured in the supernatant, and the samples were stored at 80 °C until further use. Total gas produced was measured over time in each bottle using the Gas Endeavor system (BPC Instruments, Sweden). The Gas Endeavor system allows to measure low gas volume and flow that is produced during the *in vitro* colonic fermentation. Moreover, a blank containing only the medium and faecal inoculum was prepared.

2.7. Total ammonia

Total ammonia content of the fermented samples at time points 0, 8 and 24 h was determined using an ammonia assay kit from Megazyme Ltd (Bray, Ireland). Briefly, 200 μL of water per well was added to a 96-well plate. 30 μL of buffer with 2-oxoglutarate at pH 8, 20 μL of NADPH

and 10 μL of samples (or standard) was thereafter added. Absorbance was measured at 340 nm, and then 2 μL GIDH was added to induce a reaction. The 96-well plate was shaken for 5 min and then absorbance was measured at 340 nm again. ΔA was calculated as the difference between the two measurements. The final ammonia concentration was calculated according to eq. (2).

$$c_{sample} = \frac{\Delta A_{sample}}{\Delta A_{standard}} * c_{standard} * F \quad \text{eq.2}$$

Where c_{sample} is the sample concentration in g/L, ΔA_{sample} is the difference in absorbance before and after reaction for the sample, $\Delta A_{standard}$ is the difference in absorbance before and after reaction for the standard, $c_{standard}$ is the concentration of the standard in g/L and F is the dilution factor. All samples were measured in duplicate.

2.8. Short chain fatty acid analysis

To validate the functionality of the *in vitro* colonic fermentation, the concentrations of acetate, propionate, and butyrate were monitored at time intervals 0, 8, and 24 h. The analysis of SCFA was carried out using UHPLC-qToF-MS following the 3-nitrophenylhydrazine (3-NPH) derivatization method, as previously done by (Dei Cas et al., 2020). In short, a 50 μL portion of the fermentation extract was mixed with 90 μL of cold methanol containing internal standards (IS) at a concentration of 10 μg/mL each, including acetic acid-d4, butyric acid-d8, and propionic acid-d2. The mixture was subjected to 5 min of ultrasonication, followed by centrifugation at 10 000 g for 5 min at 4 °C. Subsequently, a 100 μL aliquot was transferred to an LC vial. For the derivatization step, the sample was combined with 50 mM 3-NPH (50 μL), 50 mM N-ethyl-carbodiimide (EDC, 50 μL), and 7% pyridine (50 μL). After a 1-h incubation, the derivatization reaction was halted by adding 0.2% formic acid, and the sample was subjected to UHPLC-qToF-MS analysis.

The UHPLC system was equipped with an Acquity BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters) using a mobile phase consisting of 0.1% formic acid in water and acetonitrile. The elution gradient (0.4 mL/min) commenced at 10% acetonitrile (0–2 min), followed by a linear increase to 100% acetonitrile (2–4 min), maintaining 100% acetonitrile (4–6 min), and concluding with re-equilibration at 10% acetonitrile for 4 min. The column temperature was held at 50 °C, while the autosampler temperature was maintained at 10 °C. Mass spectra were acquired in negative ion mode with an m/z range of 70–1500. The parameters for the ESI source were set with collision energy 0 V, capillary voltage 3.6 kV and nozzle voltage 1500 V. N2 pressure at the nebulizer, the flow rate and temperature were set at 21 psi, 10 L/min, and 379 °C, respectively. The injection volume was 5 μL and data acquisition and processing were performed using MassHunter Workstation Software (Agilent).

2.9. Statistical analysis

Statistical analyses of particle size, ammonia and SCFA production were evaluated with one-way analysis of variance (ANOVA) in IBM SPSS Statistics with a significance of $p < 0.05$.

3. Results and discussion

3.1. Effect of pH on the zeta potential of pea hull fibre and pea protein

For the pea protein dispersion at a concentration of 2 mg/ml, the zeta potential varied from 20 mV at pH 2 down to −20 mV at pH 10 (Fig. 1). The pH had a large impact on the zeta potential between pH 3 and 6, after which the absolute value levelled out, and remained close to −20 mV. The isoelectric point (pI) of the protein, as determined here by the zeta potential was approximately 4.2, a value that agrees well with literature (Tanger, Engel, & Kulozik, 2020).

The zeta potential of the fibre sample was negative in the pH range tested (Fig. 1). The zeta potential decreased with increasing pH range from −8 mV at pH 3 to −20 at pH 6. The absolute value of the zeta potential of the pea fibre remained similar (between −20 and −22.5) within the pH range of 6–10. This indicates electrostatic repulsion at pH values < 6 (Bhattacharjee, 2016). In addition, the variation of the zeta potential of fibre as a function of pH resembled that of cellulose, albeit a more negative charge at higher pH (Myśliwiec, Chylińska, Szymańska-Chargot, Chibowski, & Zdunek, 2016).

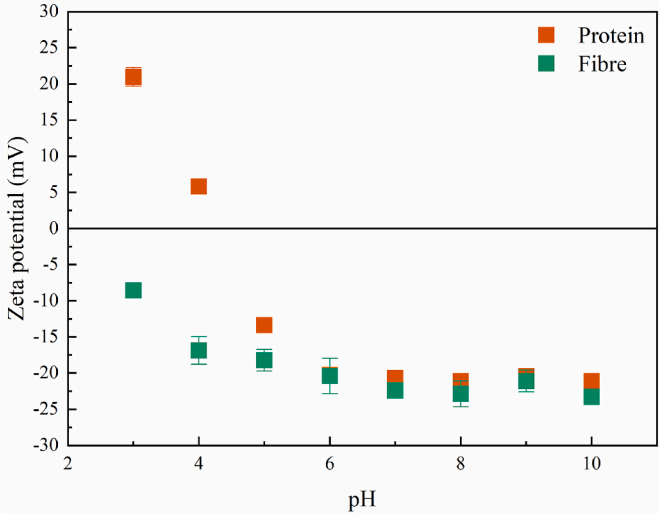

Fig. 1. Zeta potential of fibre and protein dispersions at an ionic strength of 0.1 M adjusted with NaCl and at T = 20 °C.

3.2. Pea protein particle size dependence on pH and temperature

The particle size distribution for the dispersed protein was measured at three pH values: 2, 4.5 and 7 (Fig. 2). At pH 7, one large band was visible at ~40 μm indicating an unimodal sample, with no presence of particles above 100 μm. Both pH 2 and 4.5 displayed a more complex particle size distribution with a shoulder peak below 10 μm and one peak above 100 μm. The size population above 100 μm could be due to aggregation of protein particles, as pH of 4.5 is close to the pI of the pea protein used. At pI, the electrostatic repulsion between the protein

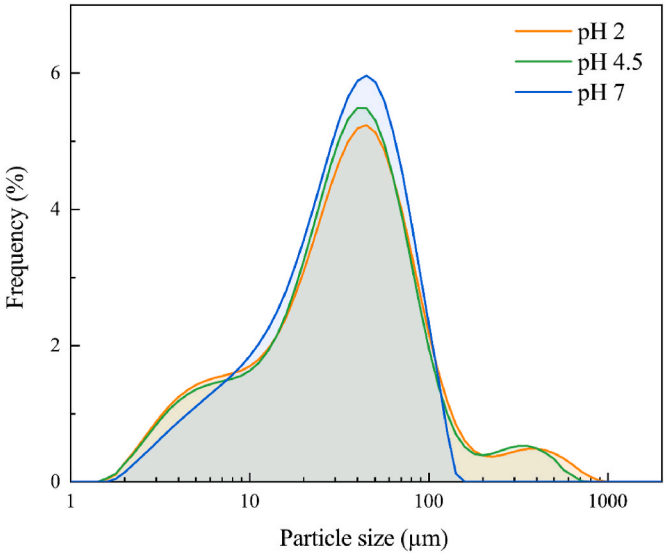

Fig. 2. A. Particle size distribution of pea protein dispersion at pH 2 (orange), 4.5 (green) and 7 (blue).

particles will be at a minimum, thus promoting aggregation in the sample. The increased presence of particles below 10 μm could possibly also be explained by aggregation of smaller protein particles but also by increased density of protein particles close to pI (Moll, Salminen, Seitz, Schmitt, & Weiss, 2022). The increased density might be due to less absorbed water by the protein particles at pH values close to pI, and thereby less swollen particles.

$D_{[3,2]}$ values for the three pH values tested are presented in Table 2. The $D_{[3,2]}$ for pH 7 (19.2 μm) was statistically significantly larger compared to pH 2 and 4.5, with $D_{[3,2]}$ values of 17.3 μm and 17.6 μm respectively. In samples with wide particle size distributions (PSD), $D_{[4,3]}$ will be heavily influenced by large particles while $D_{[3,2]}$ will be more sensitive to smaller particle populations. Therefore, due to the presence of aggregates, $D_{[3,2]}$ was selected for further discussion. The differences in particles size values between pH 7 and the other two pH can then be explained by the occurrence of the small (<10 μm) population and less swelling.

The differences in particle size measured by laser diffraction were also visualised in microscopy images (Fig. 3). The particles size seemed smaller at pH 2 (Fig. 3A) and 4.5 (Fig. 3B) compared to pH 7 (Fig. 3C). This agrees well with the $D_{[3,2]}$ values measured by the laser diffraction (Table 2). Similar trends can be observed after heating with pH 7 (Fig. 3F) displaying larger particle size compared to pH 4.5 (Fig. 3E). Furthermore, after heating we could also observe swelling at pH 2 (Fig. 3D).

3.3. Rheological properties of heated and non-heated pea protein dispersion at different pH values

The rheological properties during thermal treatment (95 °C for 30 min) of pea protein were measured at an initial pH of 2, 4.5 and 7 (Fig. 4). At the end of the thermal cycle, the storage modulus (G′) was higher than the loss modulus (G″) for all pH values measured. The thermal treatment of the protein was defined to occur at the temperature at which G′ increases rapidly but G″ remains similar. Such increase started at ~50 °C independent of pH. The temperature at which gelation started is consistent with previous studies on thermal treatment of pea protein (Kornet, Penris, et al., 2021; Kornet, Shek, et al., 2021). Once 95 °C was reached, and the temperature was held constant for 30 min, G′ plateaued and, only a slow rise in G′ was noticed upon cooling. In absolute values, the highest G′ was obtained at pH 4.5, followed by pH 2 and lastly pH 7. The pH 4.5 is close to the pI for pea protein and the particles are expected to aggregate, resulting in higher G′ and a firmer gel (Johansson et al., 2021). The difference between pH 2 and pH 7 can be explained by a higher tendency to form aggregates at pH 2, as the solution has passed through the pI during the addition of HCl.

During thermal treatment all samples swelled, and the larger particles became more distinct (Fig. 3). The solid-like behaviour stems from increased interactions between the protein particles due to denaturation (formation of aggregates) and decreased affinity to water. However, the primary reason for the gel-like properties is due to the swelling of the particles because of steric hindrance, thereby forming a particulate gel (Berghout, Boom, & van der Goot, 2015). The pea protein suspensions can be considered as concentrated suspensions of weakly interacting soft protein particles. Increasing the concentration, and thus the volume fraction (φ), of soft particles will eventually reach a critical condition where the bulk rheology exhibits elastic properties (Boehm, Warren, Baier, Gidley, & Stokes, 2019). Above the critical packing fraction (φ_c) the system will exhibit solid-like behaviour. In the case of pea protein particles, swelling occurs during thermal treatment causing the particles to occupy more volume resulting in increased elasticity due to steric hindrance.

Table 2
Surface area moment mean ($D_{[3,2]}$) values for protein at pH 2, 4.5 and 7.

Sample	$D_{[3,2]}$ (μm)
Pea protein at pH 2	17.3 ± 0.1
Pea protein at pH 4.5	17.6 ± 0.3
Pea protein pH 7	19.2 ± 0.1

3.4. Rheological properties of heated and non-heated pea hull fibre and pea protein blends

The small differences measured in particle size and microstructure of pea protein indicated that changing the pH would have minimal effect on the pea fibre and pea protein interactions. Therefore, we selected pH 7 as representative pH to carry out the rheological measurements in blends with different protein and fibre ratio. The highest G′ was observed in samples with a large amount of fibre; fibre and F80 (Fig. 5). G′ was lower in samples with predominant protein content, with the lowest observed in F20 and protein. The rheological properties of dispersions of this same pea fibre were previously reported by (Karlsson et al., 2023). Focusing on G′ at T = 20 °C shows that the rheology of the fibre dispersion dominates, where the fibre dispersion is characterised by a paste like behaviour with G′ of 2 kPa. However, even if high fibre content results in larger G′, one observes more blurred sol-gel transition in the mixed systems as compared to protein. The sharp increase in G′ at 50 °C, indicating thermal gelation of protein is not as pronounced as fibre concentration increases. This reveals that the addition of pea fibre to pea protein limits the thermal gelling properties of the protein. A possible explanation could be that the pea protein swells to a lower extent or can no longer form a network when heated. Indeed, there are some indications of less swelling of pea protein particles in the presence of fibre using CLSM imaging (Supplementary Fig. S1).

3.5. In vitro colonic fermentation of heated and non-heated pea hull fibre and pea protein blends

Samples subjected to *in vitro* colonic fermentation were pure fibre, pure protein and a 50:50 blend of fibre and protein. Based on rheology, all these samples were affected by thermal treatment. In addition, thermal treatment is common in food processing therefore, both non-thermally (fibre, protein and F50) and thermally treated (fibre T, protein T and F50T) samples were studied. Colonic batch fermentation produces end-products that affect the pH. Fermentation of carbohydrates and protein produces SCFA which reduces the pH, while protein can also produce alkaline products such as ammonia. The pH varied considerably between the samples, with protein and protein T having the highest pH at 24 h (7.13 and 7.16 respectively), statistically significantly higher than fibre and fibre T (6.05 and 5.84). Furthermore, the blends of fibre and protein in samples F50 and F50T resulted in pH of 6.67 and 6.66 respectively. The pH values for all samples before fermentation did not differ significantly, ranging from pH 7.1 for F50T to pH 7.4 for protein. For pH values at all timepoints, see Supplementary Table S2. In addition, as pH at all timepoints were similar, and close to those observed within the human colon (Yamamura, Inoue, Nishino, & Yamasaki, 2023), the lack of pH control in the batch *in vitro* colonic fermentation experiments is not expected to affect the result. Production of ammonia and branched chain fatty acids (BCFA) from protein fermentation has been shown to be affected by pH, with lower pH (5.5) decreasing the production of these metabolites (Smith & Macfarlane, 1998).

3.5.1. Gas production during in vitro colonic fermentation

Gas is a product formed during colonic fermentation of carbohydrates and proteins. The amount of gas produced gives an indication of how well a substrate is fermented. The blended samples, F50 and F50T, produced the highest amount of gas with 114 mL and 111 mL respectively (Fig. 6). Protein and protein T produced similar amounts of gas compared to the fibre and fibre T, ranging from 66 mL to 75 mL after 24

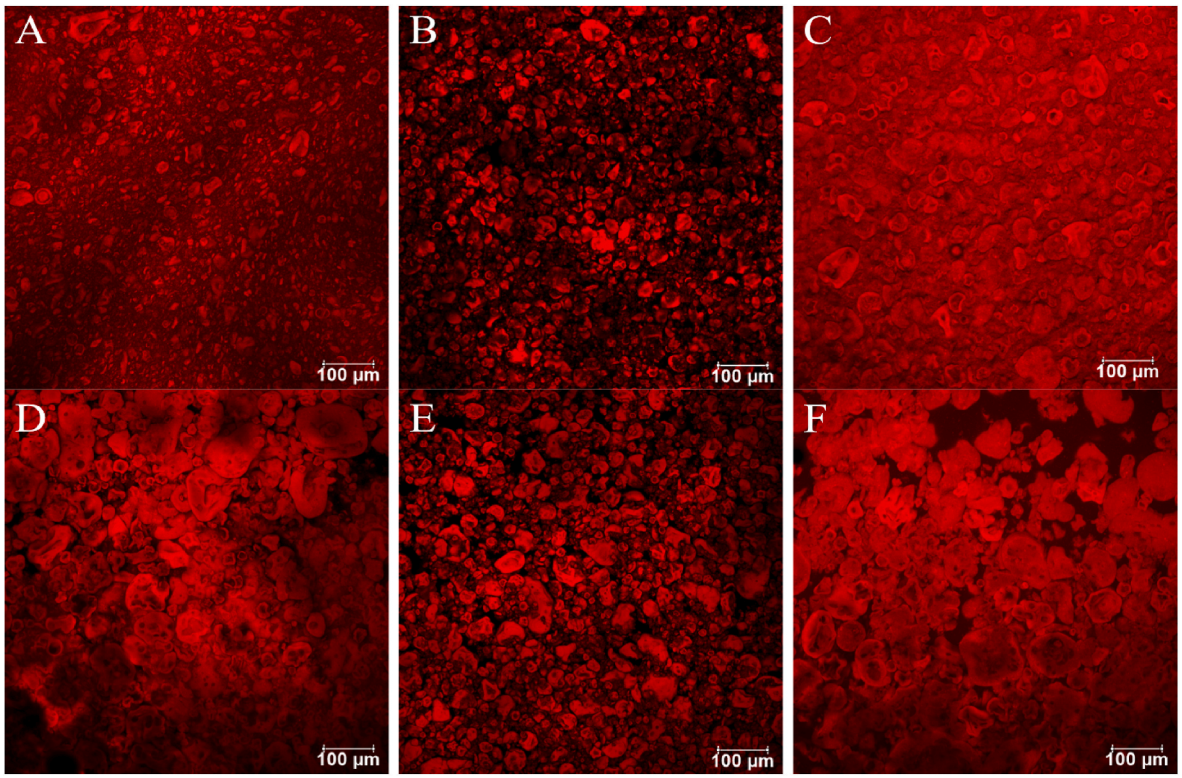

Fig. 3. Confocal laser scanning microscopy (CLSM) micrographs of protein dispersion at 15 wt % for three pH before and after heating. (A) pH 2 before heating, (B) pH 4.5 before heating, (C) pH 7 before heating, (D) pH 2 after heating, (E) pH 4.5 after heating and (F) pH 7 after heating.

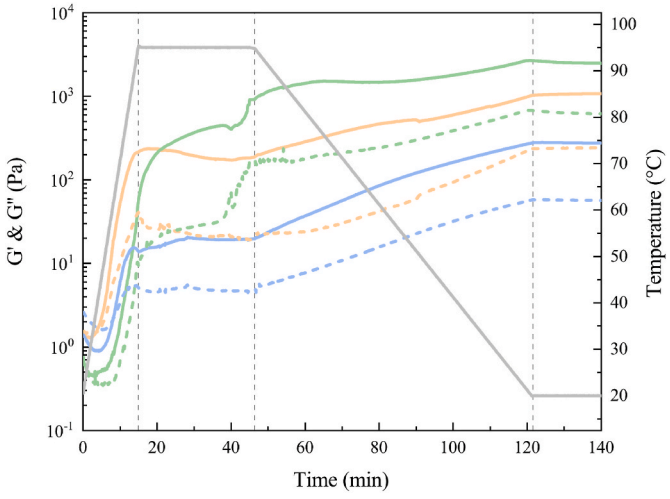

Fig. 4. Evolution of G' (solid) and G'' (dashed) as a function of time and temperature at pH 2 (orange), pH 4.5 (green) and pH 7 (blue) of 15 wt % dispersions. Strain of 0.5 % and frequency of 6.25 rad^{-1} was used. The grey line shows the temperature profile as a function of time.

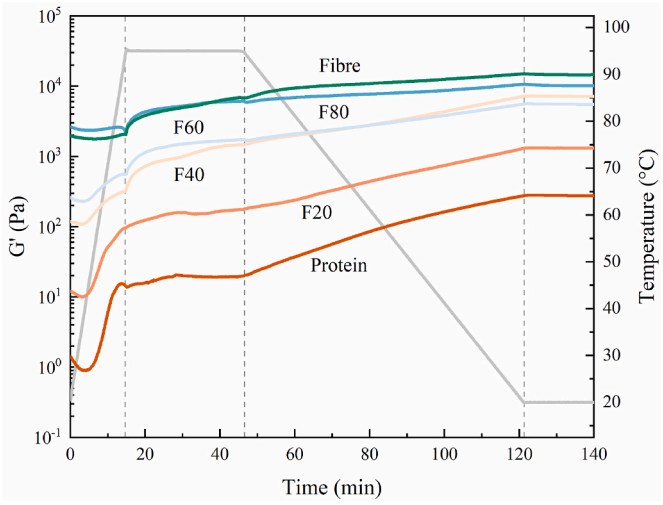

Fig. 5. Evolution of G′ for dispersions with six different ratios of protein and fibre, all having a total dry content of 15 wt %. The thermal cycle consists of heating from 20 to 90 °C, holding at 90 °C and cooling to 20 °C. The samples were fibre, 80 % fibre and 20 % protein (F80), 60 % fibre and 40 % protein (F60), 40 % fibre and 60 % protein (F40), and 20 % fibre and 80 % protein (F20) and protein.

h. The high gas production in F50 and F50T is due to the higher substrate content (3 g/100 mL) in comparison to the pure samples (1.5 g/100 mL). The heat treatment of the samples did not lead to statistically significant differences in gas production.

3.5.2. *Ammonia production during in vitro colonic fermentation*

In general, the total ammonia production during fermentation was higher for samples containing protein after 24 h (Fig. 7). Furthermore, the thermally treated protein sample showed higher levels of ammonia than the unheated sample, suggesting that a structure characterized by larger (less dense) protein particles may affect bacterial growth and decrease the utilization of this metabolite by the microbiota (Fig. 3). Ammonia is produced as an end-product, usually from deamination of amino acids in the colon (Blachier, Mariotti, Huneau, & Tomé, 2007). However, there was no discernible difference in the total ammonia produced during the initial 8 h, as the concentration of ammonia for all samples are between 10 and 20 mmol/L during that time. After 24 h, the protein and F50 produced similar amount of ammonia, 59 mmol/L and

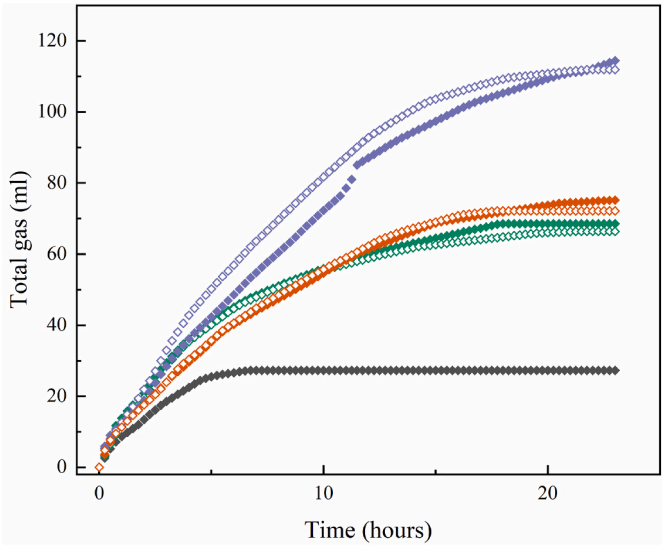

Fig. 6. Average total amount of gas production over 24 h for thermally (open) and non-thermally (solid) treated samples. Each value is an average from three runs. The samples analysed were fibre (green), 50 % fibre and 50 % protein (purple), protein (orange) and, control (black).

49 mmol/L respectively indicating that the presence of fibres during fermentation of the non-thermally treated samples did not significantly affect the production of ammonia originating from the presence of protein. However, when samples were heat-treated prior to fermentation, a statistically significant difference between the protein T sample and the F50T sample was observed (89 mmol/L and 42 mmol/L ammonia production, respectively). This suggests that the presence of fibre during fermentation may stimulate the use of ammonia for microbial growth, at least for the heat-treated samples.

3.5.3. *Short chain fatty acid production during in vitro colonic fermentation*

When dietary fibres and protein reach the large intestine, the main end-products of fermentation are SCFA. The total amount of SCFA produced was the highest in the samples containing protein, with F50T (66.2 mmol/L), F50 (64.0 mmol/L), P (57.5 mmol/L) and PT (47.3 mmol/L) after 24 h (Fig. 8). Although F50 and F50T had twice the amount of substrate compared to the pure samples, it did not produce more SCFA in comparison to protein and protein T. The fibre samples had the lowest SCFA production, with fibre at 17.9 mmol/L and fibre T at 21.9 mmol/L. The same trend was observed after 8 h fermentation (Supplementary Table S3, S4 and S5). SCFA production was statistically significantly higher in any sample containing protein compared to fibre and fibre T. Generally, protein fermentation is correlated to less SCFA production compared to carbohydrates. However, pea hull fibre has inherently low fermentability due to its high cellulose content and crystallinity (Lebet, Arrigoni, & Amadò, 1998). Well-fermentable fibres (e.g., pectin), can produce around 60 mmol/L at a lower substrate concentration (0.4 g/87 mL), which is significantly higher compared to the pea hull fibre (Lu, Flanagan, Mikkelsen, Williams, & Gidley, 2022). The thermal treatment of the samples did not affect the total SCFA production in any of the samples. The large variation within the samples likely stems from inherent differences in the volunteer's microbiota. With diversity in the microbial composition, they will utilise the

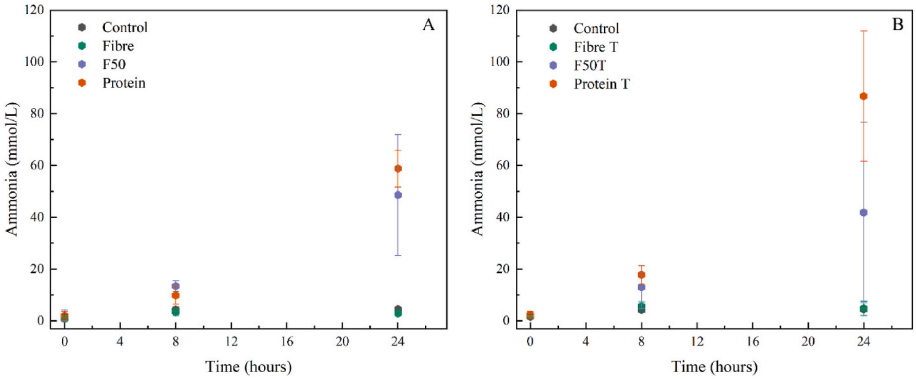

Fig. 7. Total ammonia produced during fermentation for three fibre to protein ratios measured at timepoints 0, 8 and 24 h. (A) Ammonia produced in mmol/L in the non-heated samples. (B) Ammonia produced in mmol/L in the heated samples. At 24 h, protein T produced significantly more ammonia compared to all other samples ($p < 0.05$).

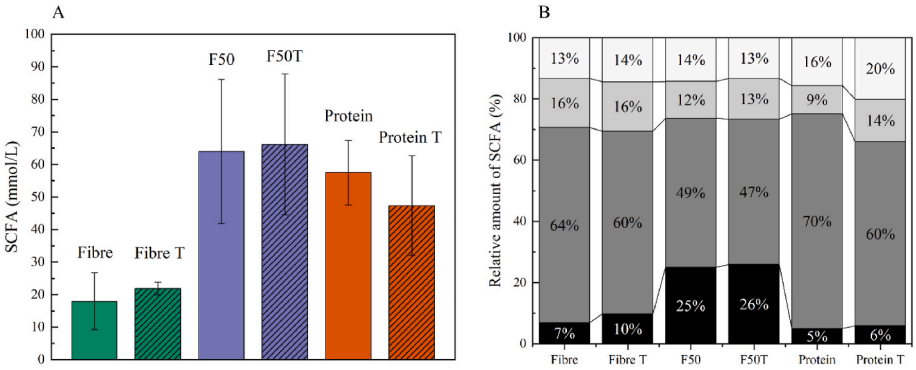

Fig. 8. (A) Total amount of SCFA produced after 24 h of *in vitro* colonic fermentation for fibre (green), F50 (purple) and protein (orange). Non-thermally treated sample are filled, and thermally treated samples are dashed. (B) Relative amount of butyrate (white), propionate (light grey), acetate (dark grey) and other SCFA (valerate, *i*-valerate and caproate) (black) produced at 24 h for the six samples.

substrates differently resulting in varying amounts of end products, e.g., SCFA.

Differences between samples were found in the relative amounts of SCFA produced. The ratio of butyrate produced was statistically significantly higher in protein and protein T (16 % and 20 %) compared to fibre, fibre T, F50 and F50T. Fermentation of specific amino acids produce different end products (SCFA), with glutamate and lysine mainly producing butyrate (Smith & Macfarlane, 1997). Indeed, pea protein contain significant quantities of both glutamate and lysine, which could be one explanation for the larger butyrate fraction (Leterme, Monmart, & Baudart, 1990). Moreover, butyrate measurements include both *n*-butyrate and *i*-butyrate which inflate the relative butyrate production for the protein samples. *n*-butyrate is a SCFA produced during both fibre and protein fermentation, while *i*-butyrate is a branched SCFA that can only be formed from branched-chained amino acids such as valine, present in pea protein (Hespell & Smith, 1983). Butyrate has been correlated to improved host health however, other nitrogenous compounds produced from protein fermentation are known to have harmful effects (Jaskiewicz et al., 1996). The propionate fraction was similar between all samples, but slightly higher in fibre and fibre T (16 % for both samples). The proportion of acetate produced were comparable between fibre, fibre T, protein and protein T, where all samples had between 60 and 70 %. However, F50 and F50T had smaller ratios of acetate (49 % and 47 %) due to the higher production of valerate, *i*-valerate and caproate (25 % and 26 % respectively). The more diverse SCFA profile in the blend of fibre and protein may be explained by the higher availability of nitrogen that stimulate microbial growth and the consequent activation of different enzymes and cross-feeding pathways. Moreover, the average large level of valerate, *i*-valerate and caproate observed in F50 and F50T samples was mainly influenced by one of the donors (donor 1), which produced four to five times more compared to the other two donors (Supplementary Table S3, S4 and S5).

The fermentability of pea fibre and pea protein seems to be dependent on the ratio protein to fibre present in the sample. The total gas production was similar when fibre and protein were fermented separately, but differences could be observed in pH, ammonia and SCFA. Higher pH is known to promote microbiota that produces more nitrogenous compounds such as ammonia, which in turn raises the pH further, whereas low pH and the presence of carbohydrates reduces peptide and amino acid fermentation. However, in the presence of fibre, less ammonia was produced due to carbohydrates being a favourable carbon source or the ammonia being used for bacterial growth, resulting in lower accumulation of ammonia in the batch cultures (CummingsHill, Bone, Branch, & Jenkins, 1979). Nonetheless, results from *in vivo* colonic fermentation and from clinical trials, have shown that carbohydrates reduce the fermentation of protein and amino acids (Ito et al., 1993; Mortensen, Holtug, Bonnén, & Clausen, 1990).

3.5.4. *Microstructural characterisation before and after in vitro colonic fermentation*

In all samples differences could be observed in the microstructure of the protein fraction remaining after *in vitro* fermentation (Fig. 9). In the samples with pure proteins (protein and protein T) only small protein particles could be found after fermentation, whereas in the samples where proteins are mixed with fibres (F50 and F50T) both small and large protein particles were seen. This shows that the fermentation of proteins is less effective when fibres are present, which indicates the preference of the microbiota towards fibres and support findings suggesting that the presence of fibres can mitigate potentially harmful products from protein fermentation (Mortensen et al., 1990). There were no clear differences in the structure as function of thermal treatment, as observed in the micrographs, even if variation could be measured in the total ammonia concentration.

4. Conclusion

We investigated how pH and thermal treatment of pea hull fibre and pea protein blends affect their physico-chemical properties and their *in vitro* fermentation using human inoculum. Changing the pH influenced the particle size, charge and rheology of the pea protein. Smaller particle size and aggregation was observed close to pH 4.2 (pI of pea protein) and resulted in larger G'. Thermal treatment induced heat-set gelation forming a pea protein particle network. With fibre added to the protein dispersion, G′ increased and the rheology was dominated by the fibre.

Differences in the *in vitro* colonic fermentation could be observed between fibre, protein, and the blends. Highest SCFA production was measured in the pea protein and the fibre and protein blend, however, there was variation in which type of fatty acids were formed. The blend produced higher ratios of valerate, *i*-valerate and caproate, while protein formed higher ratios of acetate and butyrate. Lower ammonia production was observed in the blend compared to protein as a result of thermal treatment. Similarly, less protein degradation could be visually in blends after 24 h of *in vitro* colonic fermentation. This can be due to increased proteosynthesis or bacterial preference for carbohydrates. The results indicate that the presence of fibre can mitigate the formation or accumulation of harmful nitrogenous compounds such as ammonia during colonic fermentation. However, more studies are needed to confirm the effect of fibre on protein fermentation, the microstructural impact of protein and fibres on faecal fermentation as well as changes in microbiota composition.

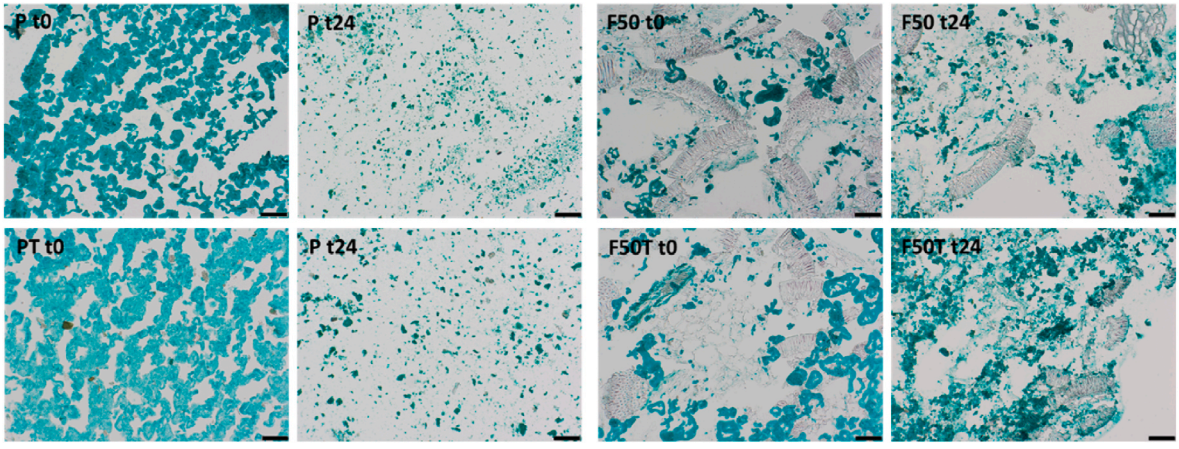

Fig. 9. Microstructure of protein (green) before (t0) and after (t24) *in vitro* colonic fermentation for 24 h. Samples shown are non-thermally protein (P); thermally treated protein (PT); non-thermally 50 % fibre and 50 % protein (F50) and thermally treated 50 % fibre and 50 % protein (F50T). Scale bar 100 μm.

CRediT authorship contribution statement

Jakob Karlsson: Investigation, Validation, Visualization, Writing – original draft. **Patricia Lopez-Sanchez:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing – review & editing. **Tatiana Milena Marques:** Conceptualization, Supervision, Writing – review & editing. **Tuulia Hyötyläinen:** Validation, Writing – review & editing. **Victor Castro-Alves:** Investigation, Validation. **Annika Krona:** Investigation, Supervision. **Anna Ström:** Conceptualization, Funding acquisition, Supervision, Writing – review & editing.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Acknowledgements

The study was performed within the PANSweden project which acknowledge financial support from the Swedish research council, FORMAS grant number 2020-02843. We acknowledge Orkla for providing fibre samples and Lantmännen for providing protein samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2024.109732.

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