THESIS FOR THE DEGREE OF LICENTIATE OF ENGINEERING

Exploring Physico-Chemical Properties and Colonic Fermentation of Pea Hull Fibre and Pea Protein Blends

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ABSTRACT

Yellow pea (*Pisum sativum L.*) is a legume with high protein and dietary fibre content commonly used for human consumption. Yellow pea is consumed as it is but also used as part of food products, in form of protein concentrate, protein isolate, dietary fibre etc. The digestion and colon fermentation of ingested foods are dependent on factors, such as composition and interaction of macromolecules. Knowledge about how more complex mixtures of plant-based nutrients changes due to processing and how it affects colon fermentation are lacking. The aim of this thesis is to study pea hull fibre and pea protein blends and determine the effect of compositional differences and thermal treatment to *in vitro* colonic fermentation.

Rheology, microscopy, and zeta potential measurements were used to study technical function of pea hull fibre and pea protein blends. The fermentation products of the pea hull fibre and protein were studied using *in vitro* colonic fermentation, with determination of pH reduction, gas production and short chain fatty acid formation. Chemical characterisation of the pea fibres was determined using high performance anion exchange chromatography and uronic acid assay. Variables used in the study was ratio of fibre and protein, thermal exposure, and pH.

Pea hull fibre content was dominated by glucose and uronic acid, believed to originate from cellulose and pectin like polysaccharides. Pea hull fibres and pea protein were largely insoluble, and the fibre particles were observed to be larger in size (98.4 μm) than the protein particles (19.2 μm). Thermal treatment (exposure at 95 ˚C for 30 minutes) of fibre and protein blends increased the storage modulus of the single components as well as their blends. The increase in storage modulus is attributed to swelling of particles, and not release of solubilised polysaccharides. The *in vitro* colonic fermentation experiment showed that the products formed from the fibre and protein blends were affected by the ratio of fibre and protein. Treatment with heating only lead to small differences in the fermentation outcome. The results show that fibre and protein composition did affect rheological and *in vitro* fermentation outcome, but also highlights that changes can be observed due to thermal treatment.

Keywords: pea hull fibre, pea protein, rheology, *in vitro* colonic fermentation

List of Publications

The thesis is based on the following appended papers:

I. **Effect of heating of pea fibres on their swelling, rheological properties and *in vitro* colon fermentation**
Jakob Karlsson, Patricia Lopez-Sanchez, Tatiana Milena Marques, Tuulia Hyötyläinen, Victor Castro-Alves, Annika Krona and Anna Ström
Food Hydrocolloids 147 (2024): 109306

II. **Physico-chemical properties of pea fibre and pea protein blends and the implications for in vitro batch fermentation using human inoculum**
Jakob Karlsson, Patricia Lopez-Sanchez, Tatiana Milena Marques, Tuulia Hyötyläinen, Victor Castro-Alves, Annika Krona and Anna Ström
Manuscript

Contribution Report

The author of this thesis has made the following contributions to the publications included:

I. Main author. Responsible for planning of experimental work with co-authors. Performed all experimental work except particle size measurement, short chain fatty acid analysis and light- and confocal laser scanning microscopy.

II. Main author. Responsible for planning of experimental work with co-authors. Performed all experimental work, except particle size measurement, short chain fatty acid analysis and light microscopy.

Contents

1

Introduction

A growing population leads to higher demands on resources, and that includes the agricultural sector [1]. To meet the future needs of the world, food production must grow together with increased sustainability of the agricultural and food system. The food sector accounts today for 34 % of all human generated greenhouse gas emissions (GHG) [2]. A contribution to the GHG is animal handling and animal-based food products [3]. Animals are inefficient producers of food, as they require high amounts of water, land and fossil fuels compared to plant-based alternatives [4]. To reduce the environmental strain, we have to decrease the agricultural footprint, and this can be achieved by scaling down agricultural expansion, reducing waste and shifting our diets from animal derived proteins to plant proteins [5]. In the US, the grain used to feed livestock is equivalent to providing food for eight hundred million people, and in Europe it is possible to grow food to feed the entire European population on plant derived proteins [3, 6].

With a shift towards a more plant-based diet, we need knowledge about the health impact from such diet. A plant-based diet has shown correlations to decreased cardiovascular diseases, lower cancer risk and less occurrence of type 2 diabetes [7-9]. Main sources for plant proteins are legumes (e.g., soybean, pea, and bean) and cereals (e.g., wheat, oat, and rice) [10]. However, proteins from plants have lower protein quality compared to whey and meat, with reduced digestibility and presence of essential amino acids [11]. Legumes lack sulfur-containing amino acids (cysteine and methionine) while cereals have reduced levels of lysine [12, 13]. In addition, the existence of anti-nutritional factors (ANF) in plant-based proteins could further reduce the digestibility [14]. Consumption of a more plant-based diet does, however, increase the intake of complex carbohydrates, both soluble and insoluble [15]. These carbohydrates are non-digestible by humans and are instead metabolized by the microbial communities in the large intestine. That process produces beneficial short chain fatty acids (SCFA), which are favorable for the health of the hosts [16].

Another important aspect when evaluating food nutrition is the processing. Processing covers a multitude of methods that changes the properties and structures of foods. Example of

processing units are; milling, freezing heating and extrusion cooking [17]. There has been an abundance of negative reports connected to food processing, e.g., nutritional degradation and addition of sugar and *trans* fats, but it is also important to evaluate the beneficial outcome. Prolonged shelf-life, improved bioavailability of several compounds and enhanced functional properties are some aspects [18].

This thesis focuses on providing insights in how compositional differences and treatment can affect the rheological properties and *in vitro* colonic fermentation of pea hull fibre and pea protein blends. Treatments of pea hull fibre and pea protein can affect their functional and viscoelastic properties [19, 20]. Furthermore, it is of interest how these properties can affect colonic fermentation, as it can influence health [21]. The objective of this thesis is therefore split into two parts: (*i*) investigating possible interactions between pea hull fibre and pea protein through modification in pH and thermal treatment and (*ii*) explore how composition and thermal treatment can affect *in vitro* colonic fermentation outcome.

Paper I cover the main components in pea hull fibre and how thermal treatment affect the swelling and rheological properties of pea hull fibre dispersions. It also focuses on if any changes in swelling and rheological properties can be correlated to *in vitro* colonic fermentation outcome. In Paper II, interactions between pea hull fibre and pea protein are investigated at different pH and temperature using zeta potential and rheology. The effect of compositional differences and thermal treatment on *in vitro* colonic fermentation is also covered.

2

Background

2.1 Macro composition of food

Before diving into physico-chemical properties and health aspects of food products, it is good to get an understanding of their composition. Food products contains protein, carbohydrates, fats, minerals, and vitamins in various degrees, and they are all essentials for human wellbeing [22]. Protein is used by the human body as a building block for e.g., muscles and enzymes while carbohydrates and fats are used as an energy source. Minerals and vitamins are seen as micronutrients as they are only acquired in small amounts.

Protein and carbohydrates are polymeric molecules being built up by monomers. Protein uses amino acids as building blocks, of which there are 20 in the human body all with different side chain [23]. This means there are a large variety in protein structure and conformation, depending on the presence and order of amino acids. Thereby, there are high variation in protein types, where some are more suitable for human nutrition but also interesting from a techno functional point of view for food technology [24]. Carbohydrates is a broad term covering any oligomeric and polymeric structure using monosaccharides as a building block [25]. Chains consisting of less than 10 monosaccharides are called oligosaccharides, while longer chains are called/referred to as polysaccharides. The polysaccharide chains generally have less variety compared to protein, consisting of only one or a few different types of monosaccharides [26]. The monosaccharide structure is a ring with a varying amount of carbon (C), commonly 5-carbon (pentose) and 6-carbon (hexose) structures, and hydroxyl groups (-OH) bound to different carbons. Figure 2.1 display examples of protein and polysaccharide structures and their building blocks.

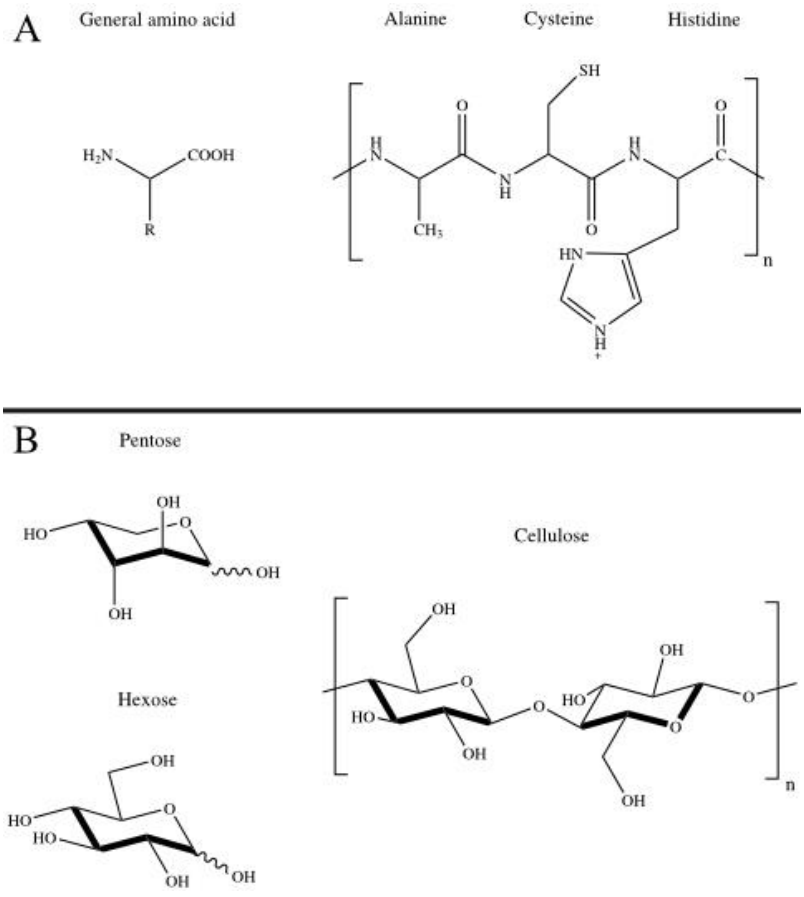

Figure 2.1. Example structure of protein and polysaccharide, and their monomeric units. A. General amino acid structure with amino group (NH_2), carboxylic group (COOH) and a varying side chain (-R). Example of a polypeptide chain (protein) consisting of alanine, cysteine, and histidine. B. Two common monosaccharide structures, pentose, and hexose. Example of the polysaccharide cellulose built up by the repeating monomeric unit glucose.

2.2 Food product texture

Food products comes in different shapes and forms, and to assess e.g., processing and structure, rheological studies can be used. Rheology studies the flow and deformation of matter, where the key concept *stress* plays a major part. Internal stress generally stems from an external force being applied to a body, resulting in a change of the original shape, or so called *deformation* [27]. In food technology, systems are often viscoelastic, meaning they have both viscous (liquid) and elastic (solid) properties [28]. To determine how solid or liquid a system is, oscillatory experiments can be used [29]. By moving an upper wall periodically at a set frequency and amplitude, the system will deform and thereby cause a responding shear stress. This shear stress can be split into two magnitudes, storage modulus (G') and loss modulus (G"). The storage modulus corresponds to the elastic contributions to the system and the loss modulus to the viscous contributions. Then, by comparing G' and G", it is possible to determine if a

system is more solid-like or liquid-like. If G' is larger than G", a viscoelastic solid is formed (Fig. 2.2). One example is the formation of heat set gels as it is a common method for various food applications [30]. Gels are in between solids and liquids, and during formation a transition from liquid (sol) to solid (gel) takes place. When fully formed, the gel is a three-dimensional network with a structural integrity, resulting in the structure being somewhat resistant to flow and deformation. To determine if such a structure has formed, the parameters storage modulus (G') and loss modulus (G") can be used.

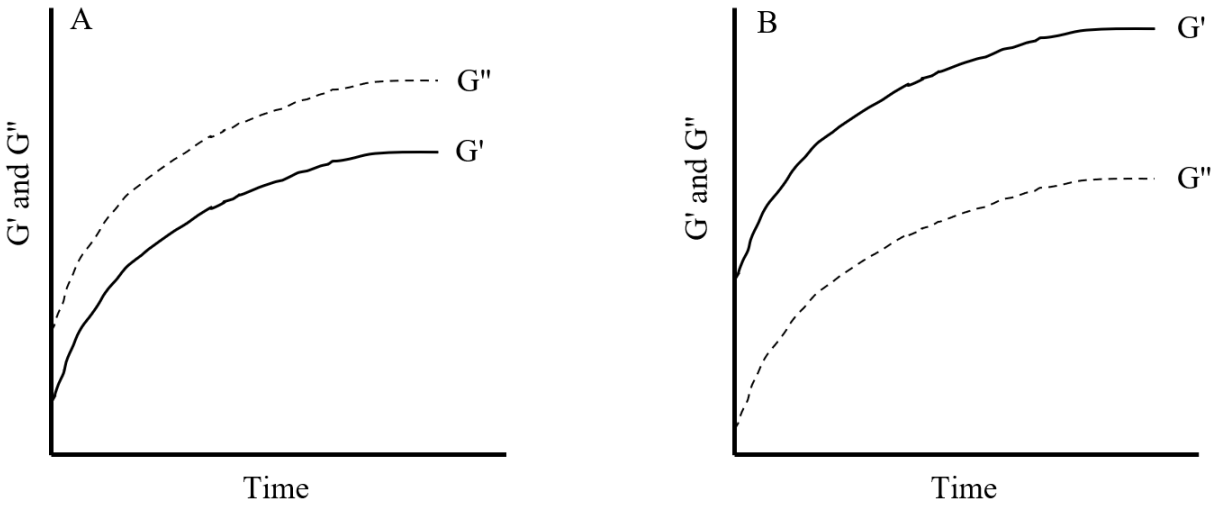

Figure 2.2. Schematic image of two systems. A. G" is larger than G' representing a viscoelastic liquid (system will behave as a liquid). B. G' is larger than G" representing a viscoelastic solid (system will behave as a solid).

Protein and polysaccharides can be used to induce gelation, depending on the desired products, e.g., jams, jellies or yoghurts [30]. Generally, unfolding occurs to expose reactive sites and then aggregation to form larger molecular complexes [31]. An important property that strongly affects the function of protein is the state of denaturation. Native protein in their natural conformation is soluble in water, but when subjected to changes in the environment, e.g., pH and temperature, the protein can become denatured. During denaturation, aggregation of the protein occurs causing the formation of heat set gels. These gels are generally fine stranded gels with large difference between G' and G". Commercial protein products are often denatured, given thermal treatment during extraction. Also these can be used for gel formation, but they have lower solubility, and the gel structure is based on larger particles compared to the fine stranded network obtained from a heated native protein [32]. Using a milder extraction method could yield higher fractions of native proteins, but it is also not as yield efficient as using harsher methodology to obtain denatured proteins [33]. With the extensive research revolving around plant-based proteins, several of available plant proteins complement animal-based proteins in

functional properties in selected food applications [34-36]. One plant-protein that is used greatly in food industry is soy. Due to its technical applications, it is used in a large variety of products requiring emulsification, texturing, water- and oil absorption [37]. Specifically, meat analogues are a field where soy protein excels, as it is possible to form macro- and microstructural fibres that can mimic the texture of meat. Extrusion is often applied to texturize the meat, and solidification can be performed via thermal gelation [38]. Another legume protein used for plant-based food products is pea. Contrary to soy, peas are possible to grow at temperate climates which makes pea interesting for the Nordics [39]. Pea protein has also shown potential to form meat analogues, but is also used in baked goods, meat products and beverages [40]. The formation of heat-set gels from pea protein has shown promise. Formation of gels similar to whey protein has been produced but is dependent on the fraction of pea protein used [32, 33]. Salt concentration and pH also affects the gelling properties of pea protein, with higher salt concentration resulting in higher gelling temperature. The optimal pH for gel formation was found to be around pH 4, close to the isoelectric point (pI) of pea protein [41].

Food products are often a blend between protein, carbohydrates/fibres, and lipid, with this thesis the focus is mainly on protein and fibre blends. Carbohydrates are a common constitute, and depending on the source and type, the carbohydrates have a varying effect on the food properties. Ways to modify rheological properties are e.g., the addition of starch or fibres to protein. This can alter various aspects such as total fibre content, but also functional changes in terms of thickening and water-holding [42]. When producing meat analogues, inclusion of polysaccharides has shown to enhance the formation of a fibrous structure [43, 44]. Soybean fibre, pectin and starch are some of the carbohydrates utilised [45, 46]. A common property between these carbohydrates is their high water dispersibility, which is needed to form a desired fibrous structure [47]. A comparative study between pectin and cellulose added to soy and pea protein isolate showed that addition of pectin enables the formation of fibrous structure, as the blend was exposed to high shear stresses, coupled with increased elasticity after heating [47]. The texture also became mushier after the pectin was added. The cellulose did however not form fibrous structure with soy and pea protein. Adding unrefined fractions of lentil carbohydrates to pea protein has shown to increase the material strength and G' [48]. The carbohydrate fraction mainly contained starch and insoluble cell wall polysaccharides, and the contributing effects were annotated to the starch. Pea hull fibres have several uses in the food sector, mainly to boost fibre content but also to alter functional properties [42]. The addition of pea hull fibres to meat analogues improve the water holding capacity, which in turn can reduce

production cost without affecting the sensory characteristics [49]. However, compared to other legume fibres, for example chickpea and lentil, pea hull fibres have low water holding capacity. The possibility to not drastically change the sensory properties with the addition of pea hull fibres is one of its strengths when one wants to fortify food products with dietary fibres [50, 51].

2.3 Colonic fermentation

To live a healthy life, it is key to ingest a variety of macro- and micronutrients, such as previously mentioned protein, fat, carbohydrate, minerals and vitamins [22]. Countries and their health agencies have set up guidelines for the general population for their dietary intake. The health agencies of the Nordic countries released a report during 2023 with updated dietary guidelines. According to the report, 25 - 40 % of the energy intake should come from fats, 45 - 60 % from carbohydrates and 10 - 20 % from proteins [52]. For proteins that equates to 0.83 g/kg body weight per day. The dietary fibre intake should be at least 25 g/day for females and 35 g/day for males. Dietary fibres are not an energy source for humans, as they are indigestible. Instead dietary fibres are fermented by the microbiota in the large intestine [53]. Dietary fibres are complex polysaccharides (e.g., cellulose or pectin), oligosaccharides, gums and mucilages, stemming naturally from plants such as cereals, fruits and legumes. Even if dietary fibre does not directly contribute to the energy intake of humans, they are valuable as their intake has been correlated to several health benefits [54] body weight control and metabolic function, specifically related to glucose control and insulin sensitivity [55, 56]. Dietary fibre intake has also been associated to reduced risk of cardiovascular diseases and increased colonic health [57].

The health benefits of dietary fibre can be attributed to a variety of properties, both physical and chemical. Soluble fibres can thicken or form gels in the stomach and small intestine, reducing the food transit time and the reduce the uptake of glucose [58]. Insoluble fibres have water-holding capacity, meaning they can absorb harmful molecules and bile acids, thereby reducing the uptake. [59]. Both soluble and insoluble fibres can be fermented by the bacterial community in the colon, producing multiple healthful metabolites, even if soluble fibres are readily fermentable while insoluble are less fermentable [59]. The main products are short chain fatty acids (SCFA), carbon dioxide and hydrogen gas. SCFA are fatty acids with 1 to 6 carbons, with acetate (C2), propionate (C3) and butyrate (C4) being the most commonly produced during colonic fermentation. Longer and branched chain fatty acids (BCFA) (*i*-butyrate, valerate and

i-valerate) are also produced in various amounts depending on the substrate. BCFA are produced from protein fermentation, not from dietary fibres [60]. A large majority of the SCFA produced are absorbed by the colon, where the different types contribute to various metabolic pathways. Butyrate is primarily used as an energy source by the colonic epithelial cells, while acetate and propionate are transported further to the liver [61]. Propionate has been reported to lower the cholesterol synthesis in the liver, and therefore attributed to reduced risk of cardiovascular diseases. Acetate on the other hand is known to be vital in the production of longer SCFA.

It is not only dietary fibres that gets fermented, other carbohydrates, proteins and fats that is not digested will also reach the colon. When digestible carbohydrates, such as starch, reaches the colon and is fermented similar metabolic products to dietary fibres are produced. However, when protein undergo colonic fermentation, vastly different compounds are metabolised. Depending on what type of protein is consumed, circa 10 % of daily ingested protein reaches the colon [62]. Plant-based proteins are generally less fermented compared to animal-based proteins due to being encapsulated inside a cell wall consisting of complex polysaccharides. Therefore, the proteins are not accessible to the gastric and intestine digestive enzymes and is not available until the cell wall has been fermented in the colon. In addition to SCFA acids, branched chain fatty acids (BCFA), sulphites, ammonia and other nitrous compounds are produced during protein fermentation. These nitrous and sulphuric molecules have been correlated to harmful effects, such as increased cancer risk and inflammatory bowel disease (IBD) [62]. With carbohydrates present, the bacterial community will proliferate and thereby use the metabolites produced from protein fermentation for proteosynthesis, for their own growth [63]. Depending on the substrate available for fermentation, different bacterial species will thrive. Bacteria predominantly responsible for protein fermentation are *Bacteroides* and *Clostridium* species [64]. These bacteria are also often more prominent in the distal colon, where the majority of proteins are fermented [65].

The *in vitro* colonic fermentation of pea hull fibres is known to produce SCFA and gas when fermented [66, 67]. The susceptibility to fermentation was low compared to other dietary fibres, e.g., apple fibres. Crystalline nature formed by the high cellulose content in pea hull fibres could be a contributing reason for this. Some studies on more complex pea systems have been performed, but mainly with using pig faecal inoculum. There, pea hull fibre did increase SCFA production and did promote bacterial nitrogen incorporation with protein present [68]. Fermentation of whole pea cells has been performed with both pig and human faecal inoculum,

with varying results observed [69, 70]. Xiong et.al., observed that the type of macronutrient exposed to bacterial fermentation and not structure did determine the outcome [70]. Comparatively, when pig faecal inoculum was used could more complex results be seen. Processing and structure of the cells had similar effect to the macromolecules on the fermentation metabolites produced [69]. This highlights the complexity of fermentation outcome connected to the composition and structural difference of food. In this thesis, a novel way is introduced to investigate how macronutrient and treatment can affect pea fibre and pea protein *in vitro* colonic fermentation.

3

Experimental

3.1 Compositional analysis

3.1.1 Neutral monosaccharide analysis

The determination of the yellow pea fibres monosaccharide composition followed a modified method as described by Sluiter et.al. [71]. Briefly, the fibres were hydrolysed using H_2SO_4 and then further diluted with deionized water. An internal standard of fucose was added. The monosaccharide analysis was run on high-performance anion exchange chromatography coupled with pulsed amperometry detector (HPAEC-PAD) (ICS 3000 Dionex, Sunnyvale, USA). Column used was a CarboPac PA 1 analytical 4 × 250 nm. The solvent used was Milli-Q (MQ) with injections of 60 % 200 mM NaOh and 40 % 200 mM NaOH + 170 mM NaOAc between the runs. D (+) glucose, D (+) xylose, D (+) galactose, L (+) arabinose, L (+) rhamnose and D (+) mannose was used as standards. Samples were measured in duplicate.

3.1.2 Uronic acid analysis

Uronic acid analysis was done with a modified method of Liu et al. [72]. The samples were kept on ice and hydrolysed with H_2SO_4. The hydrolysed sample was then mixed with 12.5 mM borax in 96 % H_2SO_4 and 20 µl of 4 M sulfamic acid-potassium sulfamate (pH 1.6) to be heated to 95 °C for 20 minutes. After the samples cooled, 40 µl of 0.15 % (w/v) 3-phenylphenol in 0.5 % (w/v) NaOH was added and UV absorbance was measured at 525 nm (Cary 60 UV-Vis Spectrophotometer, Agilent Technologies, Santa Clara, USA). Samples were measured in duplicate.

3.2 Analysis of sample dispersions

3.2.1 Preparation of dispersions

To obtain 15 % (w/v) dispersions, 51.17 g of dry matter was mixed with 300 ml of deionized water. Different ratios of fibre and protein dispersions was prepared, see Table 3.1. The overall dispersibility was increased by homogenizing the dispersions using Silverson L5M-A (Chesham, UK) and T18 digital ULTRA-TURRAX (IKA Works GmbH & Co. KG, Staufen,

Germany). Thermally treated samples were further subjected to heating at 95 °C in a water bath for 30 minutes.

Table 3.1: Sample name and composition of prepared fibre and protein dispersions. Total solid content was kept at 15 wt %, with 85 wt % water.

Sample name	Fibre (% solid content)	Protein (% solid content)
F	100	0
F80	80	20
F60	60	40
F50	50	50
F40	40	60
F20	20	80
P	0	100

3.2.2 Particle size determination

The particle size distribution (PSD) was determined using Mastersizer; 2000 (Malvern Instruments Ltd, Malvern, UK) equipped with a 2000 Hydro-SM accessory, filled with 100 ml of deionised water. A few drops were dropped into the water for each sample. A refractive index of 1.47 was used to determine the particle size distribution. A model for irregular particles was selected, due to the irregular shape of the particles. The volume-based $D_{[4,3]}$ and surface area-based $D_{[3,2]}$ diameters for each sample were determined using the equation 1 and 2.

$$D_{[4,3]} = \sum_i n_i d_i^4 / \sum_i n_i d_i^3 \quad \text{eq. 1}$$

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

3.2.3 Flow measurements

Flow measurements were performed on fibre dispersions at six concentrations (1, 2, 4, 8, 10 and 15 % (w/v)). All measurements were carried out at 20 °C. The shear rate ranged from 0.01 s^{-1} to 100 s^{-1}. The measurements were done with a DHR-3 rheometer (TA Instruments, New Castle, USA) equipped with a 40 mm diameter parallel plate geometry. A 1 mm gap was used, and the temperature was controlled with a Peltier plate.

3.2.4 Oscillatory measurements

Rheological measurements were carried out at 15 % (w/v) for both fibre and protein. The sample was added to the DHR-3 rheometer at 20 °C. The temperature was then increased at 5

°C/min until it reached 95 °C. The sample was held at this temperature for a duration of 30 minutes to allow for thermal equilibration. The sample was cooled to the initial temperature of 20 °C, employing a cooling rate of 1 °C/min. After cooling, a frequency sweep analysis was performed, with the frequency varying from 0.01 rad/s to 100 rad/s. The strain amplitude used during the measurement was 0.5%.

3.3 *in vitro* colonic fermentation

3.3.1 Preparation of medium and substrate

The medium (1500 ml) was prepared containing basal solution phosphate buffer, bicarbonate, and vitamins, but with no added carbon source. The medium was aliquoted by adding 89 ml into 500 ml Schott bottles and then sterilised in an autoclave. The bottles were then bubbled with nitrogen to and 1 ml of reducing agent was added to remove oxygen.

The substrates used for *in vitro* colonic fermentation was F, FT, P, PT, F50 and F50T. The substrates were subjected to sterilization within a laminar airflow (LAF) bench utilizing UV-light exposure for a duration of 30 minutes. Thereafter, the substrate was carefully weighed and transferred into separate 50 ml falcon tubes, which were then sealed securely. 1.5 g was weighed for F, FT, P and PT, while 3 g was used for F50 and F50T. For each substrate, the preparation was performed in triplicate.

3.3.2 Faecal inoculum preparation

Faeces was donated by three human volunteers with no reported gastro-intestinal diseases and unrestricted diets. Preparation of the inoculum occurred within two hours of the faecal collection. The faeces were diluted to 20 wt % in 50 mM phosphate buffer solution under sterile conditions in a LAF bench.

3.3.3 Fermentation procedure

10 ml of faecal inoculum was added to each substrate and mixed. Mixture was then transferred to a Schott bottle containing the anaerobic media, to obtain a total volume of 100 ml. Experiments were run for 24 hours and sampling occurred at 0, 8 and 24 hours. After each sampling, the samples were centrifuged, and supernatant was separated from the pellet. pH was measured and the samples were then kept at -80 °C. By using the Gas Endeavor system (Bioprocess Control), the total gas production could be measured.

3.3.4 Short chain fatty acid (SCFA) analysis

To validate the *in vitro* colonic fermentation process, the levels of acetate, propionate, and butyrate at different time points (0, 8 and 24 hours) were measured. The analysis of short-chain fatty acids (SCFAs) was conducted using UHPLC-qToF-MS following the 3-nitrophenylhydrazine (3-NPH) derivatization method, as previously described by Dei Cas et al. [73]. To start, 90 µL of cold methanol containing internal standards (IS) at a concentration of 10 µg/mL each, which included acetic acid-d4, butyric acid-d8, and propionic acid-d2 was added to 50 µL aliquot of the fermentation extract. Mixture underwent ultrasonication for 5 minutes, followed by centrifugation (10,000 g, 5 minutes, 4 °C), then transferred 100 µL of the supernatant to an LC vial for further analysis. For derivatization, the sample was combined with 50 µL of 50 mM 3-NPH, 50 µL of 50 mM N-ethylcarbodiimide (EDC), and 50 µL of 7% pyridine. After an incubation period of 1 hour, the derivatization reaction was stopped by adding 0.2% formic acid.

The UHPLC system was equipped with an Acquity BEH C18 column (2.1 × 100 mm, 1.7 µm; Waters) with mobile phase A consisting of 0.1% formic acid in water and mobile phase B containing acetonitrile. The elution gradient, running at 0.4 ml/min, started at 10% mobile phase B (0–2 minutes), followed by a linear increase to 100% mobile phase B (2–4 minutes), maintained at 100% mobile phase B (4–6 minutes), and re-equilibrated with 10% mobile phase B for 4 minutes. The column temperature was set 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 spanning from 70 to 1500. The parameters for the electrospray ionization (ESI) source were configured as follows: collision energy 0 V, capillary voltage 3.6 kV, nozzle voltage 1500 V. N2 pressure at nebulizer, the flow rate and temperature of the sheath gas were set at 21 psi, 10 L/min, and 379 °C, respectively. The injection volume for the analysis was 5 µL. Data acquisition and processing were executed using MassHunter Workstation Software by Agilent.

3.4 Microscopy techniques

3.4.1 Light microscopy analysis

Frozen pellets of fibre were cut into 8 µm thick sections using a Leica CM3050S cryostat. Polysine® microscope slides (VWR International, Radnor, USA) was used for the sections. A drop of water was added to the slides before they were studied with an Olympus BX53 light microscope (Olympus Life Science, Tokyo, Japan). An x10 objective was used with differential

interference contrast (DIC) setting to increase contrast. A CMos SC50 camera (Olympus Life Science) and cellSense software was utilized for capturing micrographs and post-processing.

3.4.2 Confocal laser scanning microscopy analysis

Dispersions of pea protein at 15 wt % was set to pH 7 with 1 M NaOH and 1 M HCl. For thermally treated samples were 3 ml added to a metal cylinder and heated at 95 °C for 30 minutes. After cooling down overnight at 20 °C, the sample was mounted on a microscope slide. Texas Red at 0.2 % (w/v) was added to the sample and diffused for 20 minutes [74]. A Leica TCS SP5 (Leica Microsystems GmbH, Heidelberg, Germany) equipped with a HXC PL APO lambda blue 20 x 0.70 IMM UV objective was used to capture images. Excitation laser used had a wavelength of 594 nm and the emission wavelength was 610 - 694 nm. A minimum of 5 micrographs was taken of each sample.

3.5 Statistical analysis

Analysis of particle size and SCFA production was subjected to statistical analysis. A one-way analysis of variance (ANOVA) with a significance $p < 0.05$ was used to evaluate. Flow sweep and oscillatory measurements were done in duplicate, and the figure display the mean value.

4
Results and Discussion

4.1 Compositional analysis of pea hull fibre

Pea hull fibre consists of a variety of polysaccharides. Acid hydrolysis of the polysaccharides followed by monosaccharide analysis, gave a monosaccharide composition of glucose, xylose, arabinose, galactose and rhamnose (Table 4.1). Colorimetric assay revealed the presence of uronic acid as well. The pea fibre was analysed as a complete material (F), but also the supernatant of the pea hull fibre before (Soluble F) and after heat treatment (Soluble FT) was analysed, to determine percentage and composition of any soluble polysaccharides.

Table 4.1: The monosaccharide content of pea hull fibre (F), including solubilised fraction before (Soluble F) and after (Soluble FT) thermal treatment at 95 °C for 30 minutes. All values are given in wt % of total monosaccharide concentration.

Monosaccharide	F	Soluble F	Soluble FT
Arabinose	5.6	14.1	14.3
Galactose	2.0	12.9	10.5
Glucose	61.5	5.5	15.2
Mannose	tr	tr	tr
Rhamnose	1.8	4.1	4.9
Xylose	15.5	15.4	13.3
Uronic acids	13.6	47.9	41.7

tr: traces (<1 mg/ml)

The high glucose levels (61.5 %) in F primarily originated from cellulose, but also starch and hemicelluloses [75]. The total starch content was 1.3 g/100 g, resulting in only a small fraction of the glucose stems from starch. Xylose is the second largest fraction (15.5 %), indicating the presence of xyloglucans (which also contributes to the glucose concentration) and arabinoxylan [76, 77]. Existence of arabinoxylan is further supported by the moderate content of arabinose (5.6 %). Other neutrally charged monosaccharides, galactose (2.0 %) and rhamnose (1.8 %), were only detected in small quantity. These monosaccharides are most probable part of the sidechains of pectic-like polysaccharides, where the uronic acids (13.6 %) constitute the backbone. Pectins are a complex collection of polysaccharides with variation in both backbone

and the side chains. The common characteristic between the pectins is their charged backbone, which degrade into uronic acids. Rhamnogalacturonan-I and xylogalacturonan are two pectins which has earlier been suggested to be present in pea hull fibre [77, 78].

Pectins are generally soluble in an aqueous solvent, which is further supported by the increase in uronic acids proportion for the solubilised pea hull fibres. The ratio of uronic acid in the solubilised part of the pea fibres were high, independent of heating (47.9 % and 41.7 % respectively). The proportion of glucose was low in the soluble part, highlighting the probability that a majority of glucose originates from cellulose, which is not soluble in water. The glucose content in soluble F (5.5 %) and soluble FT (15.2 %) samples could be from partly solubilised starch, with higher solubilisation after thermal treatment. Arabinose, galactose and rhamnose concentrations were higher in the solubilised fraction, which could be due to them being part of pectin side chains. In total, only a small fraction of the pea hull fibre was solubilised, with 2 wt % for F and 3.1 wt % for FT.

4.2 Behaviour of fibre and protein dispersions

To get a better understanding of the behaviour of the material, zeta potential and particle size was analysed. Zeta potential indicate colloidal stability and potential for electrostatic interaction between fibre and protein. The particle size distribution gives an average particle size of the fibre and protein, to interpret the rheological behaviour more easily.

4.2.1 Electrostatic interactions between fibre and protein

The zeta potential was measured over a range of pH, from pH 3 to pH 10, see Table 4.2. Fibre (F) was negatively charged throughout the entire range of pH, with -8 mV at pH 3 down to -22.5 at pH 10. The zeta potential was kept constant between pH 7 to pH 10, ranging from -20 mV to -22.5 mV, to then increase from pH 6 to pH 3. This indicates weak electrostatic repulsion between fibre particles at all pH.

The protein displayed higher variation in zeta potential over the pH range. At pH 3 and pH 4, the protein was positively charged with values of -20 mV and -5 mV respectively. At circa pH 4.2, the protein had zero charge which is described as the isoelectric point (pI). Above the pI, the zeta potential became negative, and reached a plateau at pH 6 with a charge of -20 mV. Therefore, an electrostatic repulsion would occur between fibre and protein above the pI, but a possible weak electrostatic interaction could happen at lower pH.

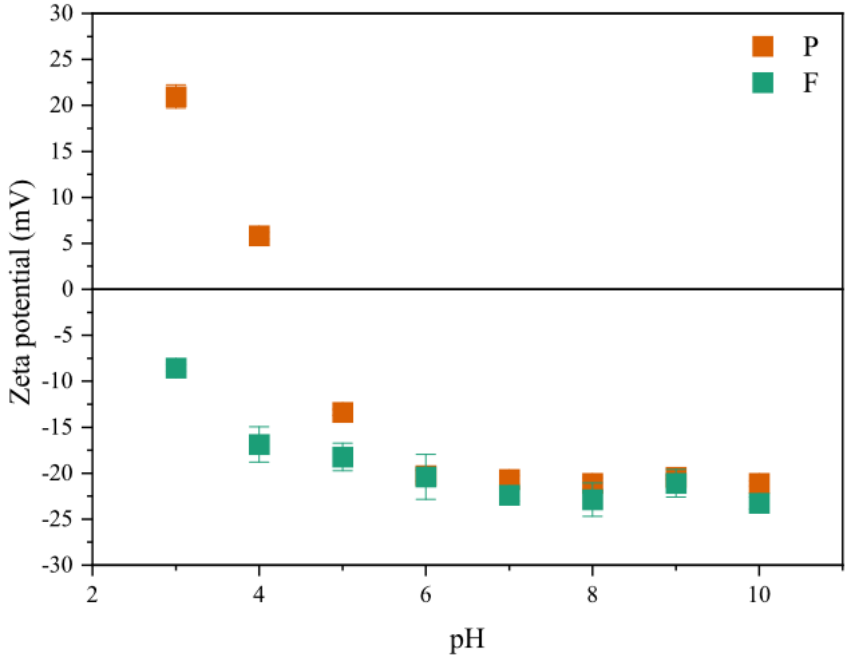

Figure 4.1: The charge (zeta potential) of fibre (F) and protein (P) dispersions at pH ranging from pH 3 to 10. The analysis was carried out at 20 °C and in 100 mM NaCl.

4.2.2 Effect on particle size of fibre and protein

The particle size was determined for two fibre samples and three protein samples, see Table 4.2. Fibre samples were non-thermally (F) and thermally (FT) treated dispersion at 95 °C for 30 minutes. For the F sample, the $D_{[3,2]}$ was 98.4 ± 2.6 µm and the $D_{[4,3]}$ value was 309.6 ± 8.0 µm. The large difference between the average particle sizes is due to $D_{[3,2]}$ takes smaller particle populations into considerations, while $D_{[4,3]}$ is more affected by large particles. Thermal treatment of the fibre lead to increased particle size due to swelling. FT samples had a $D_{[3,2]}$ of 116.2 ± 0.5 µm and the $D_{[4,3]}$ was 326.0 ± 0.8 µm, which was statistically significantly larger than the F samples.

Protein samples were analysed before thermal treatment. The $D_{[3,2]}$ value for the sample was 19.2 ± 0.1 µm. The volume mean values, $D_{[4,3]}$ was the size of 41.5 ± 0.3 µm for protein. Compared to the fibre, the protein particles were significantly smaller. Particle size determination using Mastersizer could not be done after thermal treatment due to aggregation. Heat set gelation of the protein caused inconsistencies in the measurement, reducing the reliability of the results.

Table 4.2: Average particle size values for non-thermally (F) and thermally (FT) treated fibres, and protein. The $D_{[3,2]}$ is the surface area moment mean and the $D_{[4,3]}$ is the volume moment mean values shown in μm.

Sample	$D_{[3,2]}$ (μm)	$D_{[4,3]}$ (μm)
Fibre		
F	98.4 ± 2.6	309.6 ± 8.0
FT	116.2 ± 0.5	326.0 ± 0.8
Protein	19.2 ± 0.1	41.5 ± 0.3

Micrographs show the difference in size and morphology between the fibres and proteins, see Figure 4.2. Protein particles displayed a smaller particle size with a spherical shape. The particles appear at times imploded. Based on the variation in $D_{[3,2]}$ and $D_{[4,3]}$, a rather large particle size distribution can be observed in the protein. Pea hull fibre has a large particle size distribution, and a large variety in the shape, ranging from rectangular, drop-sized to circular. Change in particle size upon heating was not visually observed using microscopy in the fibre dispersion, however swelling after heating was visible in the protein samples, see Paper 2.

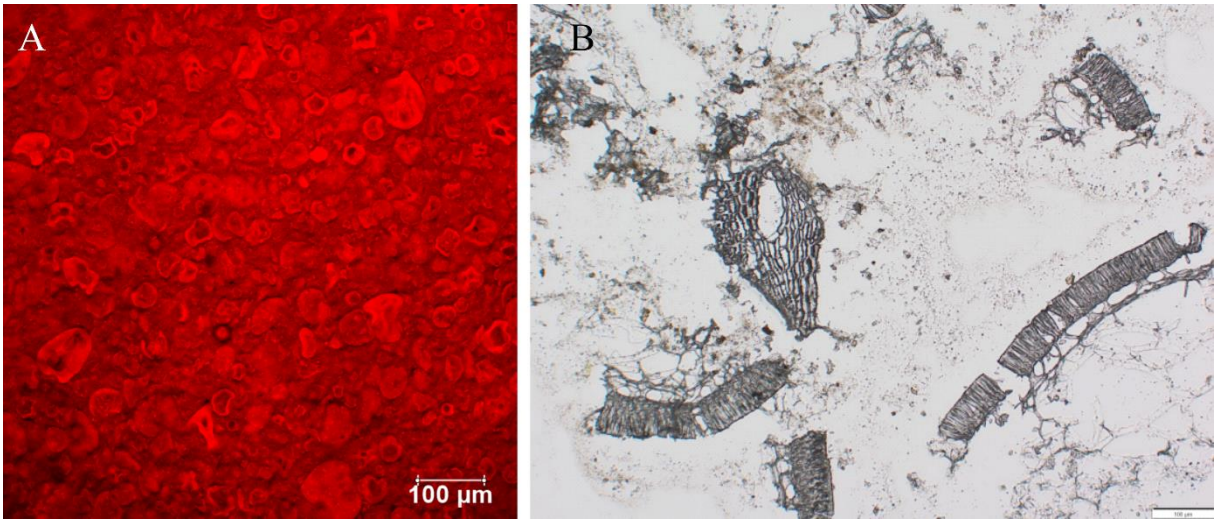

Figure 4.2: Confocal laser scanning microscopy (CLSM) micrograph of pea protein and Light microscopy (LM) micrograph of pea hull fibre. The scale bars represent 100 μm. A. CLSM micrograph of a non-thermally treated pea protein dispersion. B. LM micrograph with DIC imaging of non-thermally treated pea hull fibres.

4.3 Rheological observations of fibre and protein blends

Flow sweep measurements were done on the pea fibre dispersions to determine the viscosity over a concentration range (1 - 15 wt %). Two conditions were analysed, non-thermally (F) and thermally (FT) treated fibres to see if the viscosity changed with the treatment. Oscillatory measurements were done on fibre and protein dispersions at fixed solid content of 15 wt % and various fibre and protein ratios (80 % fibre and 20 % protein, 60 % fibre and 40 % protein, 40 % fibre and 60 % protein and 20 % fibre and 80 % protein).

4.3.1 Concentration effect on fibre dispersion viscosity

Six concentrations of fibre dispersions were analysed for the F and FT samples, see Figure 4.3. The viscosity increased as a function of fibre concentration, independent of heating history. At low concentrations (1 - 2 wt %) the system behaved as a Newtonian fluid, in the dilute region. With increasing fibre concentrations, the particles start to take up more space, leading to a larger volume fraction (ϕ). At higher fibre concentration (and thus larger ϕ), the Newtonian plateau is not reachable within the shear rates studied. The systems are shear-thinning over the shear rates studied, displaying the behaviour of a concentrated dispersion. The critical packing fraction (ϕ_c), of the system is determined to 5 wt % for F and FT samples. The ϕ_c is thus independent of heating history. At dispersion concentrations > ϕ_c, we observe a rapid increase in viscosity. The driving factor for a solid-like behaviour is particle-particle interaction. Because the pea fibres do have a relatively large size distribution with non-uniform morphology, steric hinderance will already occur at 5 wt %, causing the observed system. The FT samples display higher viscosity compared to F samples, albeit same viscosity- fibre correlation. It is tempting to believe this is related to the solubilisation of dispersible fibres. Instead, the higher viscosity after heating can be related to the swelling of the fibre particles as determines using Mastersizer.

Concentrated particle suspensions of hard spheres are a well understood system. At a certain concentration of hard spheres, the particles cannot pack any tighter and this is at the volume fraction of $\phi_{rcp} = 0.6435$ (called random close packing). At this volume fraction, the relative viscosity will increase indefinitely, and higher concentration does not affect the systems rheological behaviour. It is therefore possible to fit this system accurately with models, such as described by Quemada [79] and Krieger and Dougherty [80]. For soft particles, the packing becomes more complex as the particles themselves can change size and shape. At low volume fractions, soft particle suspensions behave similarly to hard particle systems, as there is limited interaction between the particles. However, when higher volume fractions are reached (ϕ >

0.6435), another model has to be used. The Mendoza model allows volume fraction above 1 and fits fairly well at highly concentrated soft particle suspensions [81]. The main problem is that the model does not predict an upper volume fraction limit [82]. However, an upper volume fraction limit in soft particle dispersions will be highly variable, due to soft particles having different structural integrity and morphology. In the case of pea hull fibre particles, we expect rheological soft particle behaviour coupled with some structural integrity (hardness) due to the high cellulose content and crystallinity [67]. It is also worth noting that predicting the volume fraction of particle dispersions is a challenge, and for these experiments, the concentration (w/v) was used instead of ϕ. This type of study has not been performed on pea hull fibre dispersions previously but display similar behaviour to that of a soft particle suspension.

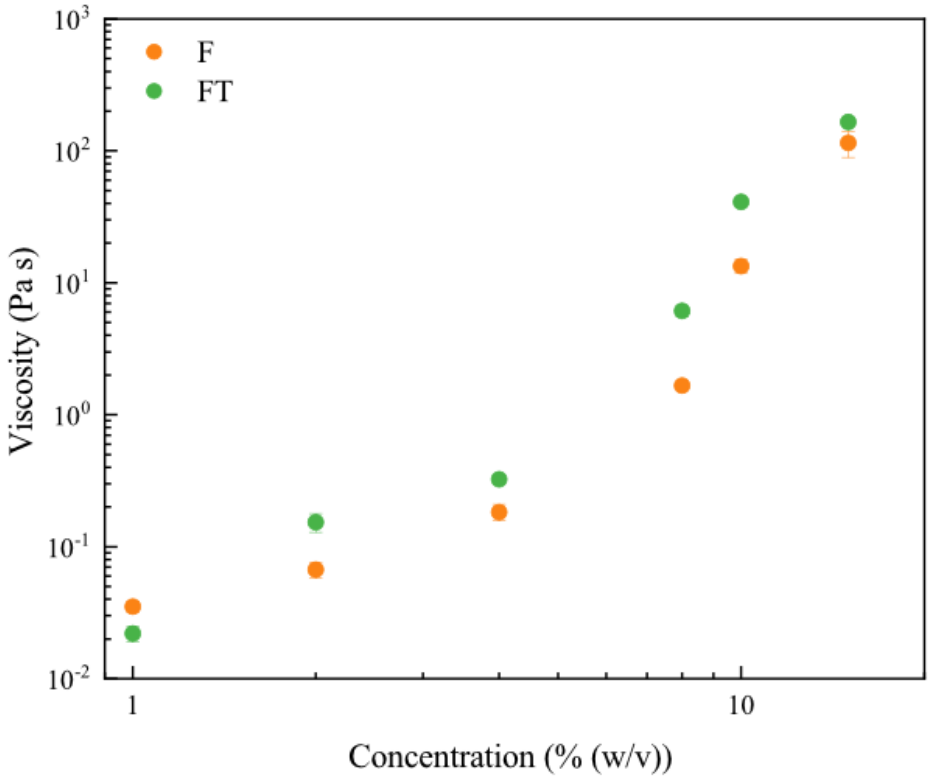

Figure 4.3: Viscosity of non-thermally (F) and thermally (FT) treated pea fibres at six concentrations. Measurements were done in triplicate at 20 °C and a shear rate of 10 s^{-1}. The values are an average and error bars not visible are smaller than the symbol.

4.3.2 Viscoelastic response to thermal treatment

To see the thermal properties of fibre and protein blends, six samples were subjected to a temperature sweep during oscillatory measurements. The samples were different ratios of fibre and protein, with a total mass content of 15 wt % for all dispersions. Protein (P) displayed the lowest G' with 0.3 kPa after the temperature ramp and cooling, see Figure 4.4. The G' increased

with higher fibre ratio, and the largest were observed in fibre (F) and 80 % and 20 % protein (80F). The measured values were 15 kPa and 10 kPa, respectively. Furthermore, the G' was larger for higher fibre content for all temperatures, indicating that the fibre dominates the rheological behaviour of the samples even before the temperature ramp. However, in the P sample, a steep increase in G' can be seen when the temperature increases from 20 °C to 95 °C, displaying the properties of a heat-set gel. This property is lost with the increased content of fibre, and the heat-set gelation is diminished in those dispersions. An explanation for this could be the loss of possibility to form a percolation network for the protein when fibre is present. With large fibre particles present, such a network would be disrupted.

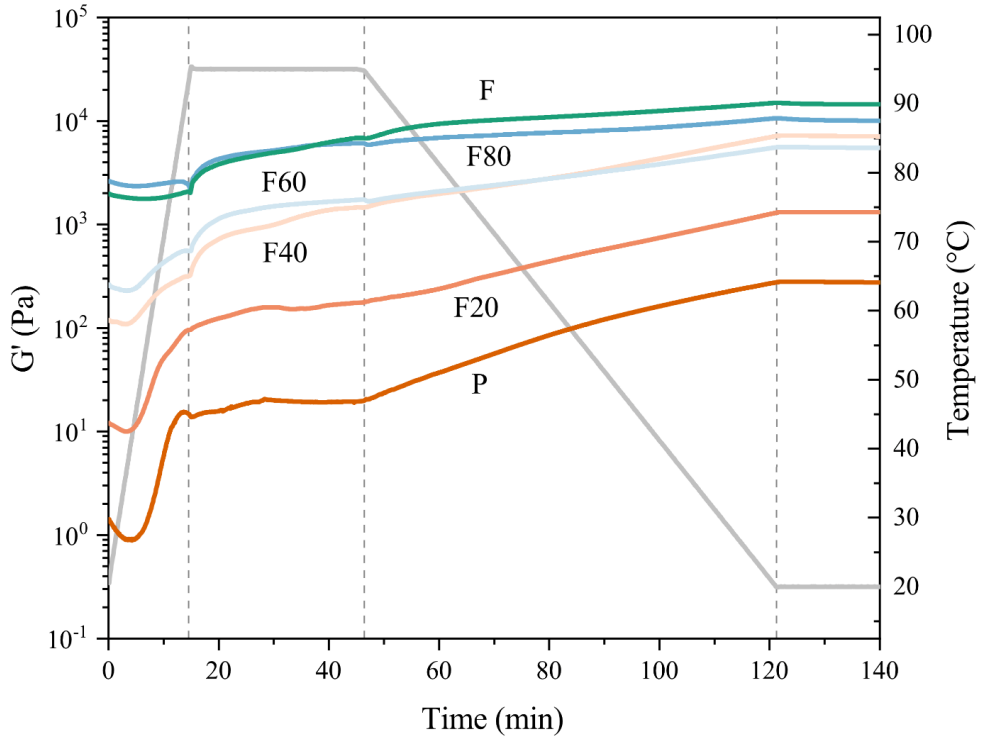

Figure 4.4: G' of six ratios of fibre and protein, where the sample were fibre (F), 80 % fibre and 20 % protein (80F), 60 % fibre and 40 % protein (60F), 40 % fibre and 60 % protein (40F), and 20 % fibre and 80 % protein (20F) and protein (P). The total mass content was 15 wt %, and the temperature ramp went from 20 °C to 95 °C, and then cooled to 20 °C.

4.4 Influence on *in vitro* colonic fermentation products

The end products from colonic fermentation are of high variety and depends largely on the substrate being fermented. Main products from carbohydrate (in this case fibre) fermentation are different gases, mainly CO_2 and H_2, but also short chain fatty acids (SCFA). Protein fermentation produces similar products but also additional nitrogenous compounds, including

ammonia. All these metabolites will affect the pH in colon, why pH measurements are used to directly follow fermentation, and give indication of formed metabolites.

There were no large differences in pH between the samples before fermentation, ranging from 7.09 for thermally treated 50 % fibre and 50 % protein (F50T) to 7.41 for protein (P), see Table 4.3. However, after eight hours of fermentation could deviations in the samples could be observed with protein (P) and thermally treated protein (PT) both having pH 6.92 – 6.95, while fibre (F) and thermally treated fibre (FT) had a pH of 6.56 and 6.39 (approximately pH difference of 0.5). The mixture of fibre and protein (F50 and F50T) had a pH of 6.72 and 6.67. Differences became larger at 24 hours with P at pH 7.13 and PT at pH 7.16, whilst F and FT ended at pH 6.05 and 5.84 (pH difference of > 1). The pH of F50 and F50T was 6.67 and 6.66 after 24 hours. Observed differences in pH is due to the production of mainly SCFA during fibre fermentation, resulting in a lower pH. Protein fermentation produces ammonia as well, resulting in a pH that is kept constant over 24 hours. The mixture of fibre and protein has a pH in between the pure samples, because the moderate production of ammonia compared to pure protein. However, no difference between the non-thermally and thermally treated samples could be seen.

Table 4.3: pH values of non-thermally and thermally treated samples of fibre (F and FT), blend of 50 % fibre and 50 % protein (F50 and F50T) and protein (P and PT). The measured values are after eight and 24 hours of *in vitro* colonic fermentation.

Sample	pH (8 hours)	pH (24 hours)
F	6.56	6.05
FT	6.39	5.84
F50	6.72	6.67
F50T	6.67	6.66
P	6.92	7.13
PT	6.95	7.15

4.4.1 Treatment and compositional factors on total gas production

The total gas production was measured over the 24 hours fermentation experiment. Protein had the highest gas production with approximately 50 ml/g for both P and PT sample, see Figure 4.5. A lower gas production was observed in both F and FT, which both produced 45 ml/g. The lower gas production from pea hull fibres could be lower susceptibility to fermentation compared to protein. High cellulose content in fibres has been reported to produce less gas,

which could also be the case for pea hull fibres. F50 and F50T samples produced the lowest total gas with 38 ml/g. This could be explained by a larger total mass was used for the F50 and F50T samples compared to fibre and protein. A total concentration of 3 g/ 100 ml was for the F50 and F50T samples, while the other samples used a concentration of 1.5 g/ 100 ml. Too high concentration of substrate could lead to inhibition of fermentation products, which could be the reason for reduced gas production. A difference between the treatments for the fibre, protein and mixture was not observed in the total gas production.

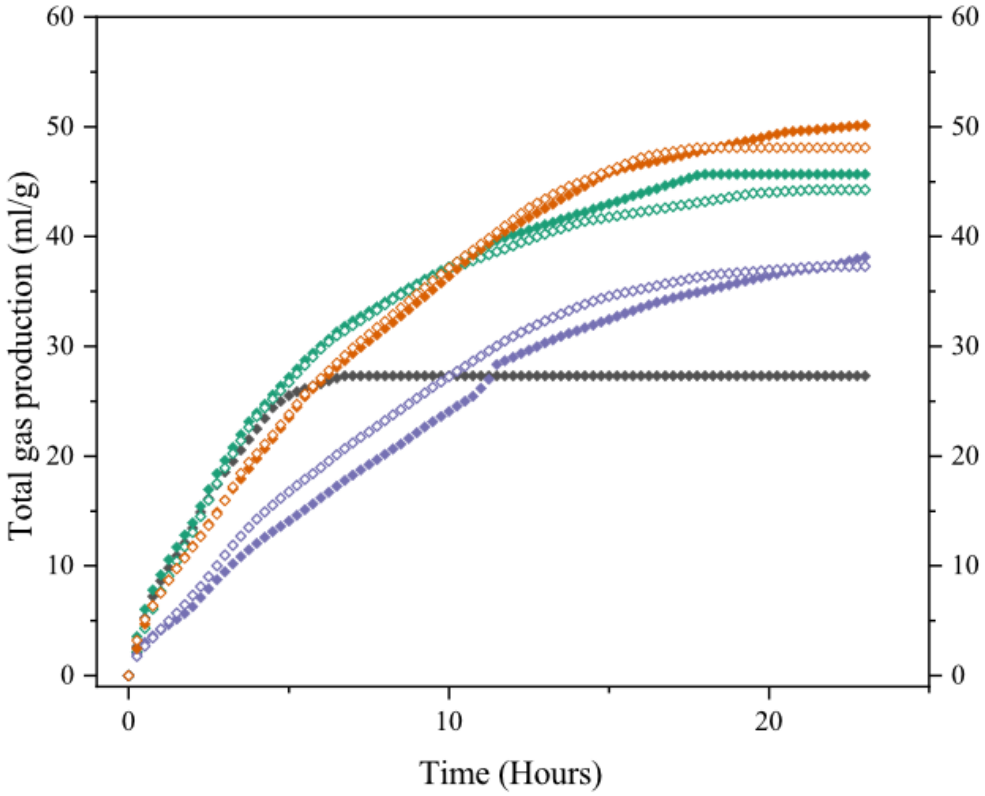

Figure 4.5: Total gas production over 24 hours for non-thermally (solid) and thermally (open) treated samples. The measured samples were protein (red), fibre (green), 50 % fibre and 50 % protein (purple) and control (black). The values are an average from three measurements.

4.4.2 Factors on SCFA production outcome

During fermentation of fibres and protein in the colon, the main end product is short chain fatty acids (SCFA). The highest total amount of SCFA was produced from the non-thermally treated protein sample P (3.8 mmol/g), see Figure 4.6. Thermally treated protein, PT, showed the second highest production of total SCFA with 3.0 mmol/g. Samples with fibre content (F, FT, F50 and F50T) produced similar amount of SCFA, from 1.1 mmol/g for F to 1.5 mmol/g for F50T, which were all significantly less compared to the P sample. Fibre fermentation is often correlated to higher SCFA production compared to protein, however in this case is the opposite

observed. One reason could be that pea hull fibre have low fermentability due to the high amount of cellulose. High variation within the samples stems from the differences in the volunteer's microbiota, which affects the fermentation outcome.

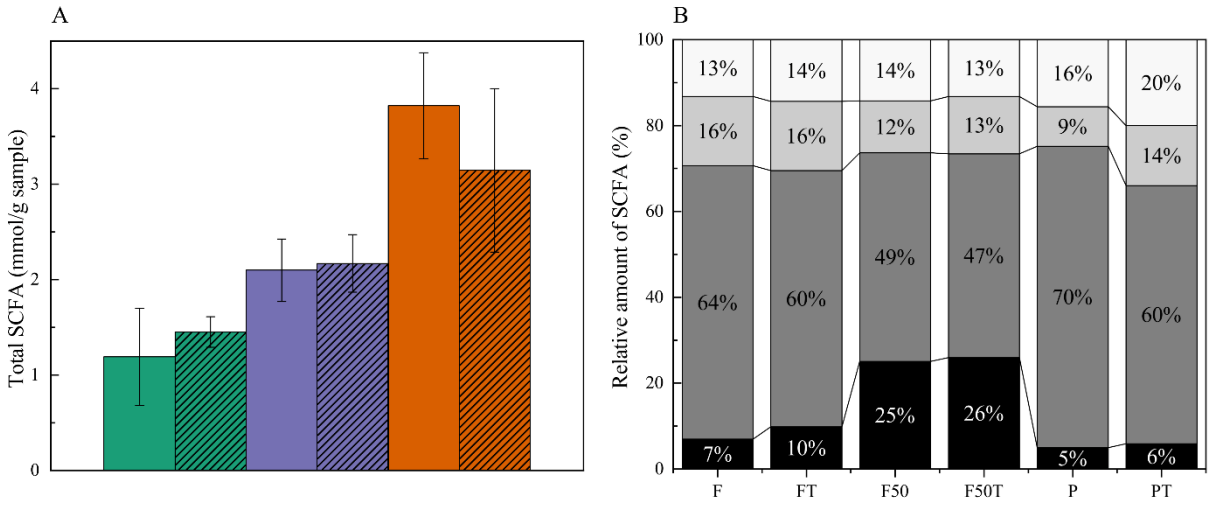

Figure 4.6: Total and relative amount of SCFA produced after 24 hours of colonic *in vitro* fermentation. A. The total amount of SCFA produced for non-thermally (filled) and thermally (dashed) samples. The samples were fibre (green), 50 % fibre and 50 % protein (purple) and protein (red). B. The relative fractions of SCFA produced for the six samples. Different SCFA produced were butyrate (white), propionate (light grey), acetate (dark grey) and other SCFA (valerate, i-valerate and caproate) (black).

Differences in the relative amount of SCFA produced could be seen between the samples. The P and PT sample produced higher amounts of butyrate (16 % and 20 %, respectively) compared to any sample with fibre. Butyrate can be produced from carbohydrates but also specific amino acids, for example glutamate and lysine, which are present in pea protein. Furthermore, butyrate comes in two forms, *n*-butyrate and *i*-butyrate, which are both included in the butyrate measurement. *i*-butyrate can only be synthesised from branched amino acids in protein, resulting in the higher butyrate ratio in the P and PT sample. Propionate production was similar in all samples, with slightly higher proportions in F and FT with 16 %. A large variation in the acetate production can be seen between the F50 and F50T samples compared to the pure fibre and protein. F50 and F50T generate less acetate but instead produces significantly more SCFA with longer carbon chain. The reason could be that with a higher nitrogen source compared to fibre, and a higher carbon source compared to protein, proliferation of the microbiota is advantageous resulting in different metabolic and cross-feeding pathways.

The substrate available for the microbiota to ferment will affect the end products, which is seen with the SCFA produced from fibre and protein. When fibre and protein was fermented by

themselves, a difference in total SCFA can be observed, but the relative amounts of SCFA are similar. However, when the mixture of fibre and protein is subjected to fermentation a shift in relative proportions of SCFA can be noted. Microbiota has a preference to use the carbohydrate source first if one is present, which will lower the pH and thereby promote the growth of bacteria commonly connected to fibre fermentation. The nitrogenous compounds produced from the protein fermentation will be used for proteosynthesis, increasing the growth of the microbial community, due to the readily available carbohydrate source. Therefore, with the presence of fibre there will be a lower accumulation of nitrogenous compounds in the colon and thereby reduce the risk of harmful effects.

Little effect of thermal treatment could be observed in all measured variables for the colonic *in vitro* fermentation. In the pH and gas production, no difference was observed between the treatment of the samples. The total SCFA production differed slightly, however due to the large deviation within the samples (interpersonal variation of microbiota) between the donors, no statistical difference could be seen. Similarly, only small variation was observed in the relative fraction of SCFA produced. Comparatively, difference was visible in both particle size measurements and during rheological measurements. Thermal treatment at 95 °C for 30 minutes did cause swelling in fibres, which affected viscosity of a fibre dispersion. Protein was also affected by thermal treatment and gelation occurred during heating. However, this change in rheological properties did not affect the outcome of fermentation. The interactions between pea hull fibres and pea protein were limited based on the physico-chemical characterisation. This interaction did therefore not have any effect on the colonic fermentation.

Harsher treatments to pea hull fibre could possibly invoke further differences in both rheological properties but also in colonic *in vitro* fermentation. Increasing the temperature or time during thermal treatment could be one option, but 95 °C is already a high temperature. Instead, looking into other mechanical treatments could be a possibility, for example homogenisation or microfluidisation [19]. An increase in solubilisation of fibres could be observed after heating, but with other mechanical treatments further solubilisation could be observed and a possible effect on colonic *in vitro* fermentation, as it appears not enough of soluble fibres are released after heating treatment employed here. Furthermore, a reduction in particle size and reduced crystallinity might have a similar effect. Worth to keep in mind is that even if pea hull fibre is not easily fermentable, the bulk and water-holding properties still contribute to beneficial colon health.

To gain a better understanding of what effects the fermentation outcome, it is not only valuable to analyse the end products but also what happens with the substrate during and after fermentation. When analysing how fibre affects the fermentation, observing how the structure changes over time could give insight in if some parts are more susceptible to fermentation. However, more specific analytic methods such as monosaccharide analysis to see if particular polysaccharides are preferably fermented, and which ones are easily accessible for the microbiota is of interest. This way it is also possible to determine if processing and treatment can affect the accessibility of the fibre more conclusively. This has previously been performed successfully, but with main concerns of removing all residual biological material [66]. Due to the mixture of the substrate and the faecal inoculum, it is hard clean the fibres fully. If the sample is not fully clean, variation in the measurements is to be expected and therefore no conclusive results. Similar analysis could be done with protein, but there instead study the amino acid composition before and after fermentation. Depending on the amino acid fermented, different product will be produced [83]. Connecting the harmful metabolites to specific amino acid and protein fermentation could help broaden the understanding how to avoid them. In addition, changes in microbiota composition are of interest.

5
Conclusion and Outlook

This thesis explored how composition and treatment of pea hull fibre and pea protein blends can affect rheology and *in vitro* colonic fermentation outcome. The effect of pH and temperature on pea hull fibre and pea protein interaction was studied using zeta potential and rheology. Effect on *in vitro* colonic fermentation outcome due to thermal treatment and compositional differences in pea hull fibre and pea protein blends was also investigated. Particle charge measured with zeta potential displayed that pea hull fibre and pea protein have a negative charge above pH 4.3. Thermal treatment of pea hull fibre dispersions increased the viscosity and storage modulus, due to particle swelling. Protein was shown to form heat-set gels, but this property was disrupted when fibre was introduced to the system. The rheology of the fibre dispersion dominated the rheological properties of fibre and protein mixtures.

The fermentation outcome was not affected by thermal treatment, but variation could be observed due to compositional differences. The total gas produced by protein and fibre separately was similar, but a mixture of fibre and protein showed less gas production. Differences in production of short chain fatty acids was observed, as protein had significantly higher production compared to any sample containing fibre. Relative amount of these fatty acids was dependent on the substrate composition, with a mixture of fibre and protein produced significantly higher fraction of valerate, *i*-valerate and caproate compared to pure fibre and protein.

Conclusively, the thesis shows that (*i*) modification of pH and thermal treatment did not affect the interaction of pea hull fibre and pea protein particles, and (*ii*) mainly compositional differences in pea hull fibre and pea protein blends affected *in vitro* colonic fermentation outcome, with thermal treatment displaying little effect.

The continuation of this work can take multiple paths. Observing how the monosaccharide composition changes during *in vitro* colonic fermentation could give us further knowledge how dietary fibres are fermented. Determine differences in the microbiota composition during *in vitro* colonic fermentation would also give insight in what affects fermentation outcome and

health. Looking into further treatments of the pea hull fibre and pea protein to benefit interactions and thereby rheological properties is one possibility. Applying similar methodologies on other dietary fibres and macromolecules are also of interest in order to understand the health effects from treatment of plant-based food systems.

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