Thesis for The Degree of Doctor of Philosophy

Relationships between physico-chemical properties and colonic fermentation in pea fibre and protein

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Cover: Photo of yellow peas with a pea hull in focus.

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Abstract

Heightened consumption and production of plant-based foods increase the amount of dietary protein reaching the colon. Excessive colonic fermentation of protein by the gut microbiota produces toxic metabolites. The demand for plant proteins results in a larger amount of side-streams. One such side-stream are pea hull fibres, which are generated during the extraction of pea proteins. Pea hull fibres need to be modified to improve their physico-chemical properties and their utilisation as food components. Modifications of pea hull fibres and pea protein can also affect how they are fermented in the colon.

The aim of this thesis is to explore how modifications of pea hull fibre and pea protein can prompt changes in their physico-chemical properties and *in vitro* colonic fermentation. Thermal and enzymatic treatments were applied to pea hull fibres and pea protein. The effect of these processes was studied through compositional, physical and rheological changes. Subsequently, colonic fermentation of modified fibre and protein was investigated using *in vitro* batch fermentation, and evaluated through the microbial and metabolic responses.

Enzymatic treatment of pea hull fibre by pectate lyase induced differences in cell wall structure and rheological properties. The rheological behaviour of the pea hull fibres could be predicted using soft particle models. Modifications to pea hull fibres did not alter the metabolite production during *in vitro* colonic fermentation. However, changing the pea protein degree of hydrolysis induced changes in the metabolic response and differences in the microbial community. Higher degree of hydrolysis of the pea protein resulted in a microbiota similar to those found during pea hull fibre fermentation. Combined fibre and protein fermentation gave a higher fraction of healthy metabolites compared to pure protein fermentation, but also increased proteolytic fermentation products. Overall, changes in physico-chemical properties can be related to differences in colonic fermentation outcome, but is dependent on substrate and treatment.

Keywords

Pea hull fibre, pea protein, rheology, *in vitro* colonic fermentation

List of publications

This thesis is based on the following publications and manuscripts.

[I] **Rheological behaviour of pea hull fibres treated with pectate lyase**

Jakob Ytterberg, Annika Krona, Pamela Freire de Moura Pereira, Amparo Jiménez-Quero, Patricia Lopez-Sanchez and Anna Ström
Submitted

[II] **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 **2024**, *147*, 109306

[III] **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
Food Hydrocolloids **2024**, *150*, 109732

[IV] **Influence of hydrolysed pea protein on *in vitro* colonic fermentation in presence and absence of pea fibres**

Jakob Ytterberg, Karin Ytterberg, Ratchawit Janewithayapun, Tatiana Milena Marques, Patricia Lopez-Sanchez and Anna Ström
Manuscript

Contribution report

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

[I] Conceptualisation. Methodology. Investigation except uronic acid content, particle size measurement, and light microscopy. Formal analysis. Writing - original draft, review and editing.

[II] Methodology. Investigation except particle size measurement, light- and confocal laser scanning microscopy, and short chain fatty acid analysis. Formal analysis. Writing - original draft, review and editing.

[III] Conceptualisation. Methodology. Investigation except particle size measurement, light microscopy, and short chain fatty acid analysis. Formal analysis. Writing - original draft.

[IV] Conceptualisation. Methodology. Investigation except except analysis of short chain fatty acids and microbiota. Formal analysis. Writing - original draft.

Publications not included in this thesis

Effect of starch and fibre on faba bean protein gel characteristics
Mathias Johansson, Daniel Johansson, Anna Ström, Jesper Rydén, Klara Nilsson, **Jakob Karlsson**, Rosana Moriana and Maud Langton
Food Hydrocolloids **2023**, *131*, 107741

Effect of cellulose-rich fibres on faba bean protein gels is determined by the gel microstructure
Mathias Johansson, **Jakob Karlsson**, Frans W. J. van den Berg, Anna Ström, Lilia Ahrné, Corine Sandström and Maud Langton
Food Hydrocolloids **2024**, *156*, 110295

Towards personalized drug delivery via semi-solid extrusion: Exploring poly(vinyl alcohol-co-vinyl acetate) copolymers for hydrochlorothiazide-loaded films
Karin Korelc, Bjarke Strøm Larsen, Anna-Lena Heintze, Åke Henrik-Klemens, **Jakob Karlsson**, Anette Larsson and Ingunn Tho
European Journal of Pharmaceutical Sciences **2024**, *192*, 106645

Contents

Chapter 1

Introduction

The food systems contribute to a significant impact on the local and global environment [1]. About a third of all greenhouse gas emissions come from the food system, with a majority stemming from land use and production. Animal protein production has a high environmental impact due to large greenhouse gas emissions, land use, and water requirements [2]. The shift towards a diet incorporating more plant-based protein sources would reduce environmental stress [2–4]. However, a shift in the food system should not only consider the environmental impact but must also account for the accessibility, healthiness, affordability, and safety of the food [5]. A way to improve some of these aspects is with a higher utilisation of side-streams in food processing, especially those generated after the extraction of plant proteins [6].

Legumes, such as peas and beans, are used today as a source of protein but produce a significant amount of side-streams [6]. Yellow peas (*Pisum sativum L.*) are one of the fastest growing plant protein alternatives on the market, mainly due to their sustainability and nutritional quality [7, 8]. Yellow peas consist of 20-30% protein, the rest mainly being starch and fibres [9, 10]. When protein extraction occurs, fibres (5-20%) are also produced as a side-stream. The extracted fibres can be split into two classes: the fibres originating from the hull of the pea, and the fibres originating from the cotyledon (inside the pea). The two fibre fractions are different in composition. The hull fibre consist mainly of cellulose, with smaller amounts of pectin and hemicelluloses, while the fibre originating from the cotyledon consist of mostly hemicelluloses and pectins [11, 12]. Today, the fibre fractions are used as animal feed, limited food industrial usages or disposed into landfills [6]. Incorporation of legume fibres in food can bring positive health benefits, as the dietary fibres bring nutritional value [13].

An increased intake of pea protein or other plant-based proteins could affect human health in various ways. High protein, fibre and carbohydrate content combined with minerals and vitamins have beneficial health effects [14]. However, digestibility is an aspect to consider when evaluating protein quality, in addition to amino acid composition. Plant-based proteins generally have a lower digestibility than animal protein, although it is dependent on the source and processing of the protein [15]. Low protein digestibility can result in reduced amino acid uptake in the human gastrointestinal tract, and thereby an increased fraction of dietary protein reaching the colon. The protein reaching the colon is then fermented by microbiota [16]. Colonic fermentation of protein is a complex process, with production of both beneficial and harmful metabolites. Protein produces short-chain fatty acids that act as an energy source for epithelial cells [17]. However, the fermentation of proteins further generates toxic metabolites, for example nitrogenous and sulphuric compounds, which are linked to diseases, such as colon cancer among others [18].

The thesis I put forward is that thermal and enzymatic treatments to pea protein and dietary fibres impacts their physico-chemical properties and hence their colonic fermentation. The objectives of the study are three-fold; i) investigating the effect of thermal and enzymatic treatment of pea hull fibres on their physico-chemical properties and *in vitro* colonic fermentation (paper I and II), ii) investigate changes in physico-chemical properties and *in vitro* fermentation of pea protein as a function of pH, thermal treatment and degree of hydrolysis (paper III and IV), and iii) investigate the combined presence of pea hull fibres and pea protein on physico-chemical properties and *in vitro* fermentation outcome (paper III and IV).

Chapter 2

Composition and physical properties

Yellow pea (*Pisum sativum L.*) is a widely grown legume crop in Europe, with an annual production of around two million tonnes each year [7, 19]. Yellow pea as crop and food ingredient is of high interest in Europe, due to its possibility to grow in temperate climates [19, 20]. Human consumption of pea protein products has increased over the last years, with an expansion of launched products and plant-based alternative market revenue [21]. However, production of yellow peas currently occupy less than 1% of EU crop land compared to cereals which cover 46%, leaving room for development [19].

With an increased production of peas, there will also be an increase in pea hull fibre as a side-stream. Today, there are limited uses for pea hull fibres in the food industry [22]. Therefore, to widen the applications of the hull fibres, we need to develop routes to functionalise and control the properties of pea hull fibres. The yellow pea hull has a microstructure with a protective outer layer (outer epidermis of macrosclereids) and a subepidemis of osteosclereids providing structural support, see Figure 2.1 [23, 24].

The pea hull fibres have a large total fibre content (90%) and are mainly insoluble, with around 1-2% solubility in water [25]. The poor solubility can partly be explained by the composition of the pea hull fibre, where approximately 50 to 60% is glucose originating from cellulose. Other large fractions are xylose with ∼15% and galacturonic acid with ∼15% [25]. There are also smaller fractions of arabinose, galactose, and, rhamnose that in combination with galacturonic acid suggests a substantial pectic fraction. The pectic fraction has been studied in more detail, where the extraction methods defines the chemical composition of the pectic fraction of the pea hull fibres [26]. However, it is worth noting that the extracted pectic fractions does still have low yields, ranging from 5 to 10% [27]. A pectic fraction with less branching and low methylation can be extracted using chelating agents, while a more branched pectin with higher methylation can be extracted by acidic extraction or sequential acidic-alkaline extraction. The pectins extracted using

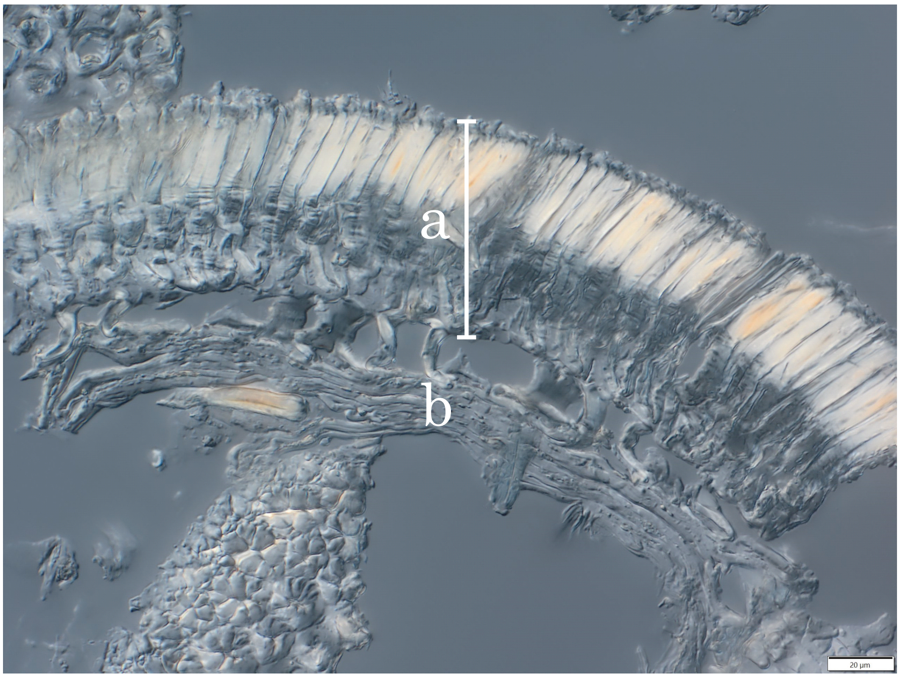


Figure 2.1: Yellow pea hull fibre visualised using light microscopy. The scale bar is 20 µm. Identified parts of the microstructure are a: outer epidermis of macrosclereids and b: osteosclereids

acidic extraction has especially large fractions of galacturonic acid, arabinose, rhamnose and xylose that has through enzymatic studies been determined to be constituents of arabinan, xylans, rhamnogalacturonan and xylogalacturonan [26, 28, 29]. Rhamnogalacturonan and xylogalacturonan fractions can also be extracted by sequential alkline-acidic extraction [27].

In addition to extracting specific polysaccharides, there has been improvements to the physico-chemical properties of the whole pea hull fibre. Various methods has been used, such as grinding [30], extrusion [25], chemical treatment [31, 32], microfluidisation [33] and enzymatic treatment [34]. Grinding of pea hull fibres decrease the particle size and subsequently increases the surface area, which results in a higher water-binding capacity [30]. A similar trend of increased water-binding capacity due to reduced particle size was observed when using extrusion and microfluidisation. Chemical and enzymatic treatment resulted in a looser cell wall structure and thereby a change in physical properteis. Observable differences in physical properties (particle size, water-binding, rheology, etc.) after treatments are dependent on two main factors. The first is the pea hull fibre particle, where it gains a looser cell wall structure and thus increases porosity, leading to a higher water uptake and swelling of the fibre. The second is treatment of the pea hull fibre could also release soluble high molecular weight polysaccharides, resulting in enhanced physical properties [34].

2.1 Modifications to pea hull fibres

The pea hull fibre used in this thesis was a ground powder containing 90% total dietary fibre as provided by the supplier (Vestkorn Milling A/S). Three different approaches were used to modify the pea hull fibres (F): thermal treatment (FT) or enzymatic treatment by pectate lyase (FP) or cellulase (FC). The pecate lyase (180 U/mg) and cellulase (50 U/mg) were acquired from Megazyme Ltd. The treatments started with dispersing the fibres in liquid media (water or aqueous buffer solution) and then sheared by Silverson L5M-A (7000 rpm for 2 min), which had previously shown to improve water-uptake. The thermal treatment was performed by heating the dispersion at 95 °C for 30 minutes in a water bath. The enzymatic treatment that used pectate lyase as an enzyme has previously been used in combination with other enzymes to modify pea hull fibres, but not by itself [34]. Pectate lyase plays an important role in the softening of plant tissue, and is being utilised in food, pulping and textile industry [35, 36]. The pectin is cleaved by pectate lyase at the α-(1,4)-glycosidic bond and primarily acts on low methyl esterified pectic acid [37]. Cellulase is an enzyme that targets the (1,4)-β-D-glucosidic linkages by *endo*-hydrolysis in cellulose [38].

2.1.1 Composition

Monosaccharide composition of the insoluble and solubilised fractions differed between the treatment methods, see Table 2.1. The fractions were determined by removing the moisture from the fibre by either freeze drying (FP and FC) or in an oven (F and FT), and then weighing the separate fractions. Unmodified pea hull fibre and the thermally treated fibre had similar solubilised fractions (2.0 wt% and 3.1 wt%), and the enzymatically treated samples with pectate lyase (8.0 wt%) and cellulase (6.0 wt%) had the highest in solubilised mass. A higher solubilised fraction is expected after treatment, but it is worth noting that the yield is still small compared to the whole sample. Neutral monosaccharide composition was determined by degrading the fibre using H_2SO_4 and then detecting the individual monosaccharides with high performance anion exchange chromatography (HPAEC) with a pulsed amperometry detector (PAD) [39]. Uronic acid content was determined using two different methods: a colorimetric method [40] (F and FT) and HPAEC-PAD (FP and FC) using trifuoracetic acid (TFA) to degrade the fibre [41]. The main component of the solubilised fraction was uronic acids, 27.2 g $100g^{-1}$ in F-S, increasing to 41.7 g $100g^{-1}$ in FT-S, 53.8 g $100g^{-1}$ in FC-S and 62.5 g $100g^{-1}$ in FP-S. The highest uronic acid content in FP-S supports that the pectate lyase acts on the pectin in the pea hull fibre. There are minor differences in neutral monosaccharides as well, with F-S having high levels of xylose and FP-S only having traces of glucose. The insoluble fractions F-I and FP-I did not have any differences, highlighting that the treatments cause changes in the solubilised fraction, but the majority of the sample stays the same from a compositional point of view. The FC-I sample had a lower content of glucose, which indicates that the cellulase can degrade the cellulose into glucose. However, there is not an increase of glucose in the FC-S sample which would be expected. The reason for this could be that H_2SO_4 was used to analyse the neutral monosaccharides in the solubilised samples, which can lead to excessive degradation of glucose molecules, resulting in an underestimation of the neutral monosaccharide content. The lower glucose content also inflates the uronic acid content in the FC-I sample. The FT-I fraction was not analysed, and due to small differences in the F-I, FP-I and FC-I samples, there is nothing indicating a change in the FT-I fraction. The monosaccharide composition was analysed by a single measurement, introducing error to the method. However, the insoluble fractions analysed show similar values in monosaccharide composition compared to previous reported values [25, 26].

Table 2.1: Fraction percentage in wt% and monosaccharide composition of insoluble (I) and solubilised (S) fractions of unmodified pea hull fibres (F), thermally (FT) and enzymatically treated with pectate lyase (FP) and cellulase (FC) in g 100g^{-1} monosaccharide.

	F-I	**F-S**	**FT-I**	**FT-S**	**FP-I**	**FP-S**	**FC-I**	**FC-S**
Fraction of total sample (wt%)	98.0	2.0	96.9	3.1	92.0	8.0	94.0	6.0
Arabinose	5.4	16.5	n.d	14.3	4.4	15.9	4.5	10.8
Galactose	1.7	11.7	n.d	10.5	0.3	6.7	1.5	5.0
Glucose	55.9	12.6	n.d	15.2	57.1	tr.	47.3	3.0
Rhamnose	0.3	1.9	n.d	4.9	1.3	1.8	0.3	0.8
Xylose	20.9	30.1	n.d	13.3	21.5	13.1	18.5	9.6
Uronic acids	15.8	27.2	n.d	41.7	15.4	62.5	27.7	53.8

n.d - not determined. tr. - traces (<1mg/L).

A larger solubilised fraction does not necessarily result in a change of physical properties. The pectic fraction of the pea hull fibre that was released after thermal and enzymatic treatment will affect the physical properties, depending if the pectin is released as polysaccharides, oligosaccharides or monosaccharides [34]. Solubilisation of high molar mass polysaccharides increase rheological properties, such as viscosity, while monosaccharides would not. The increase in solubilised uronic acid does not correlate to a decrease in uronic acid in the insoluble fraction. The insoluble fraction is substantially larger than the solubilised one, causing a change in the solubilised fraction to be negligible in the insoluble fraction. Using pectate lyase to modify the pea hull fibres does specifically target the pectin, but only a minor change was detected in uronic acid content between F-I and FP-I, suggesting that a large part of the pectin in the pea hull fibre is inaccessible to the enzyme. Further modifications prior to the enzymatic treatment, for example steam explosion [42], could improve the pectin availability by loosening the cell wall structure and thereby induce a larger change in the composition.

2.1.2 Physical properties

There was an observable difference in physical properties for the thermal and enzymatic treatments, see Table 2.2. Particle size distribution was determined using laser diffraction and the water retention capacity (WRC) by soaking the fibres in water overnight and removing the excess water to weigh the water retained in the fibres. Statistical differences were determined in the particle size and WRC for the enzymatically treated samples compared to the unmodified and thermally treated. Particle size of the FP-I and FC-I samples was significantly larger than the F-I and FT-I samples, probably from increased water uptake and subsequent swelling of the particles. An increased particle size in the FP-I sample agrees with the differences seen in the compositional changes. Pectin is proposed to have a loadbearing function in the cell wall structure, and degrading the pectin with pectate lyase would weaken the support [43, 44]. In turn, that would result in a less rigid and more porous structure that can swell more easily [45, 46]. Therefore, even a small change in the composition of the pea hull fibre can modify the physical properties. Swelling was also observed after cellulase treatment, which implies that reduction of cellulose content results in a less rigid fibre. An improved water uptake is further supported by a significant increase in WRC after pectate lyase or cellulase modification. The calculated Brunauer-Emmett-Teller (BET) surface area was 0.66 m^2/g for FP-I, 0.56 m^2/g for FC-I and 0.53 m^2/g for F-I, indicating that pectate lyase has the most effect on surface porosity. However, there was no statistical difference between the samples. An increased BET surface area has been observed previously in green pea peels after enzymatic treatment by cellulase and xylanase [47].

Table 2.2: Particle size ($D_{[4,3]}$), water retention capacity (WRC), BET surface area and crystallinity index of the F-I, FT-I, FP-I and FC-I samples.

	F-I	**FT-I**	**FP-I**	**FC-I**
$D_{[4,3]}$ (µm)	309.6 ± 8.0^a	326.0 ± 0.8^a	368.0 ± 11.1^b	379.1 ± 7.6^b
WRC (ml g^{-1})	5.1 ± 0.1^a	n.d	5.9 ± 0.2^b	5.8 ± 0.3^b
BET surface area (m^2 g^{-1})	0.53 ± 0.01^a	n.d	0.66 ± 0.10^a	0.56 ± 0.02^a
Crystallinity index (%)	61.4	n.d	61.7	61.6

n.d - not determined. Written values are mean ± standard deviation with n=3. The superscript letters indicate statistically significant differences between the samples at $p<0.05$. Values with no letters showed no significant difference in a one-way ANOVA assay.

The BET surface area is calculated from an adsorption isotherm using nitrogen gas at -196 °C and the samples had been completely degassed at 60 °C under vacuum prior to adsorption. Because the pea hull fibres where treated under vacuum, a less rigid sample and possibly weaker cell wall structure present in the FP-I and FC-I samples could cause the the structure to partly collapse. A collapsing cell structure would not show a noticeable increase in surface area, even if the structure would remain intact under ambient conditions. The scenario would be opposite that of the pea hull fibre in liquid media, where it can absorb the liquid and swell, increasing the available surface area.

Crystallinity index was measured using X-ray diffraction [48]. Pectate lyase targets the pectin, and not the cellulose, therefore no change is to be expected for the FP-I sample. However, no change in crystallinity was observed with cellulase either. Similarly, a previous study using cellulose and xylanase to treat green pea peels observed only small or no changes in crystallinity after treatment [47].

2.1.3 Fibre microstructure

The microstructure of the pea hull fibre was visualised using light microscopy, see Figure 2.2. The pea hull fibre mainly consists of outer epidermis, showing that a possible degradation could be important to affect the physico-chemical properties. Outer epidermis and osteosclereids are visible in all treatments, but there are visual differences in the microstructure between the samples, especially for the FP-I. The outer epidermis is visibly more degraded, being more rugged compared to the three other samples, supporting that the cell wall structure has been affected. For the F-I, FT-I and FC-I samples, the outer epidermis is more intact and ordered. Interestingly, the pea hull fibre does not look more swollen in the FP-I sample, but the looser cell wall structure can possibly cause the fibre to expand [49], which could be hard to observe with light microscopy.

2.2 Modifications to pea protein

Compared to the pea hull fibres, the extracted pea protein is highly utilised for food products, for example as texturisers [50, 51], stabilisers [52] and foaming agents [53, 54]. When pea protein is used as a texturiser, the main property that contributes is the ability of pea protein to gel. Protein gelation is dependent on parameters, such as pH and salt concentration, but also the source of the protein [55, 56]. A tougher extraction process of the protein, for example high temperature or pH, tend to denature the protein, which will greatly affect the gelation [57]. A denatured protein leads to aggregation which, in turn reduces the solubility of the protein and thus the techno-funcational properties [58].

Gelation of pea protein is induced after heating, where at a specific temperature the protein starts to denature [58]. Pea protein denatures between 75-95 °C, and the gelation temperature of pea protein is between 82-86 °C [59]. Protein gels are typically split into two groups, fine-stranded gels and particulate gels [60]. Fine-stranded gels are formed when there is low attraction between the protein chains, producing strands that are less than 10 nm in diameter. Fine stranded gels form strong gels by first going through denaturation, and then association into dense structure by weaker physical forces, e.g., electrostatic interactions, hydrophobic interactions and hydrogen bonding, but can also form covalent bonds via disulphide bonding [61, 62]. The dense structures will then self-form into a stranded network *i.e.* a gel. On the other hand, particulate

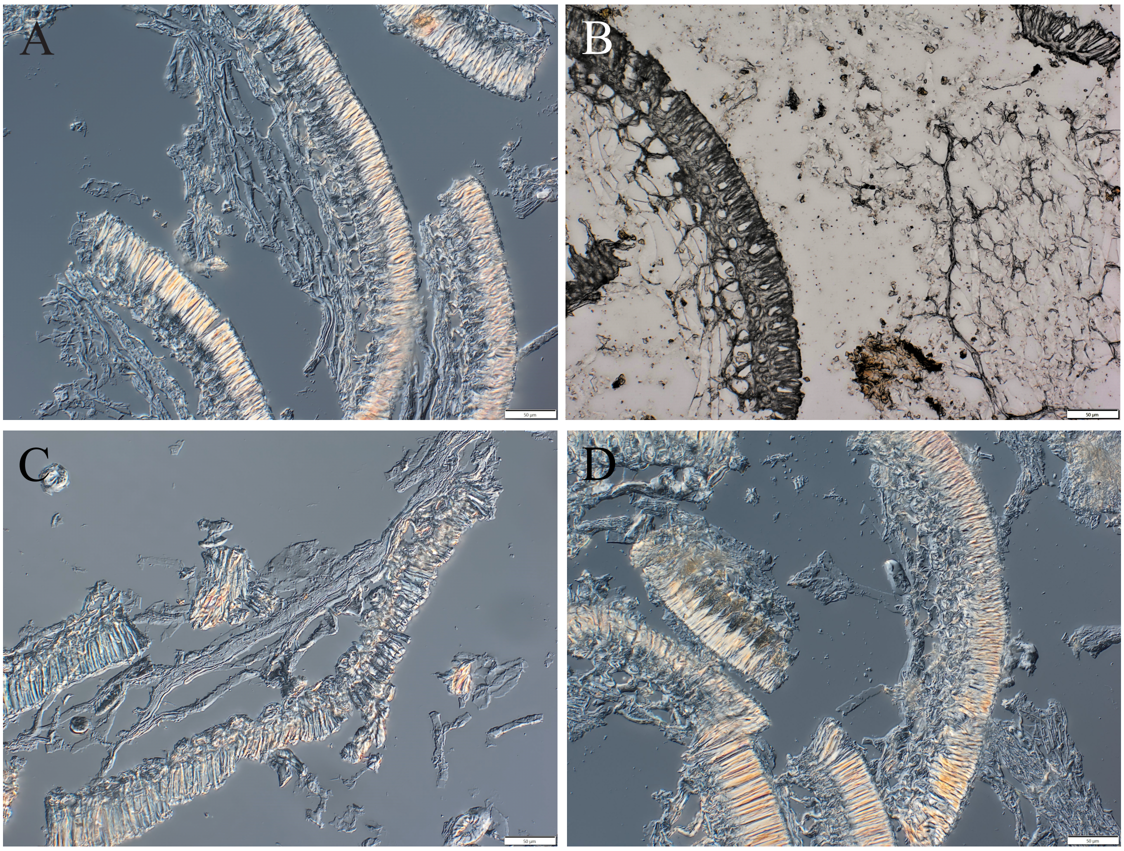


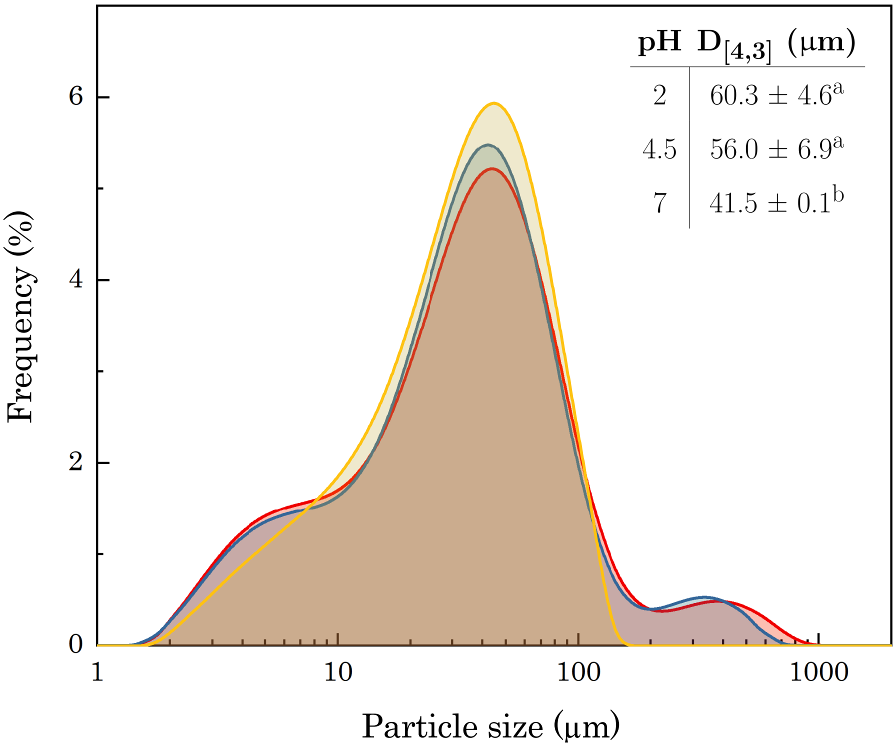
Figure 2.2: Images of the microstructure of the pea hull fibres. A: F-I, B: FT-I, C: FP-I and D: FC-I. The scale bar is 50 µm

gels are formed when there is a larger attraction between the protein and they form spherical protein particles that have diameters spanning from 50 nm to a couple µm. The spherical particles are denatured proteins that have already aggregated and have low solubility, resulting in the protein behaving like soft particles in suspension. During heating, the particles will then swell and form a percolating network. The particulate gel is supported by weakly interacting soft particles but also steric hindrance, causing elastic properties [63, 64].

The pea protein used in this thesis was a protein isolate containing 88% protein as given by the supplier (Cosucra Groupe Warcoing S.A.). The pea protein also consisted of <1% starch and <2% total carbohydrates. The physical properties and heat-induced gelation of the pea protein isolate was investigated at three different pH: 2, 4.5 and 7. pH values studied here was chosen according to relevance for food processing (4.5 and 7) as well as pH values of relevance for food digestion (2 and 7). Furthermore, pH 4.5 is close to the isoelectric point (pI) of pea protein [52]. Pea protein suspensions were prepared at 15 wt% based on dry matter in water and sheared by Silverson L5M-A (7000 rpm for 2 min) to improve the dispersibility of the protein. The pH was then adjusted using 1 M HCl and 1 M NaOH.

2.2.1 Physical properties

Particle size distribution (PSD) of the pea protein was determined in a similar manner to the pea fibre, using laser diffraction. The particle size distribution and $D_{[4,3]}$ of the pea protein was affected by the pH, see Figure 2.3. By lowering the pH to 4.5 and 2, the protein became significantly larger. At pH 7 the particle size is 41.5 µm, thus indicating that the protein are aggregates and forming larger particles. At pH 7, the PSD is centred around 40 µm, while at pH 2 and 4.5, there are three distinct populations. The population above 200 µm in pH 2 and 4.5 is probable due to aggregation that occurs around pI of the pea protein, which is 4.6 [52]. During addition of acid to reach pH 2, irreversible aggregation occurs and that causes the large population above 200 µm in the pH 2 sample. At pI, the repulsion between the protein is reduced causing them to aggregate. However, an increased population below 10 µm in pH 2 and 4.5 is not as intuitive to explain. One reason could be that repulsion of individual proteins in the aggregated particles are reduced, and thereby forming a more compact particle, and thus reducing the size [65]. In other words, the particles increases in density close to pI.



pH	**$D_{[4,3]}$ (µm)**
2	60.3 ± 4.6^{a}
4.5	56.0 ± 6.9^{a}
7	41.5 ± 0.1^{b}

Figure 2.3: The particle size distribution (PSD) and $D_{[4,3]}$ values of pea protein suspensions at pH 2 (blue), 4.5 (red) and 7 (yellow). $D_{[4,3]}$ values are mean ± standard deviation with n=3. Different superscript letters indicate statistically significant differences between the samples at $p<0.05$. Reproduced from Karlsson, J. et al., *Food Hydrocolloids*, 2024 under the CC-BY license.

2.2.2 Protein microstructure

Pea protein size dependence on pH was visualised by utilising confocal laser scanning microscopy (CLSM), see Figure 2.4. The pea protein was stained using Texas Red as fluorescent dye, with a excitation wavelength of 594 nm and emission wavelength of 610-649 nm [66].

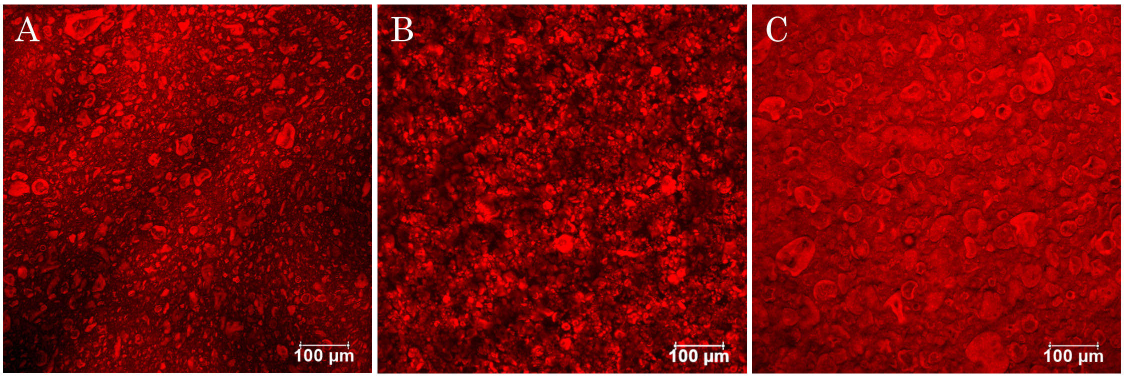


Figure 2.4: Microstructure of pea protein at three different pH. A: 2, B: 4.5 and C: 7. The scale bar is 100 µm. Reproduced from Karlsson, J. et al., *Food Hydrocolloids*, 2024 under the CC-BY license.

Observing the images, pH 2 and 4.5 seems to have smaller particle size compared to pH 7, which does not agree what was measured for the particle size measurements (Figure 2.4.A and B). The smaller particles in pH 2 and 4.5 can be related to a density increase of the protein particles close to pI, which does not occur for pH 7 (Figure 2.4.C). The samples were prepared at 15 wt% pea protein in water, which is a concentrated suspension of particles, and thus hard to observe aggregation of larger protein cluster for pH 2 and 4.5. There is a possibility that more dilute samples would show larger individual aggregates of protein particles for pH 2 and 4.5, but not for pH 7, that are seen in the PSD.

2.2.3 Protein hydrolysis

In addition to investigate the effect of pH, the pea protein was hydrolysed to different extents using digestive enzymes (paper IV). Pepsin and pancreatin acquired from Sigma-Aldrich were enzymes used at two concentrations to obtain two samples with different degree of hydrolysis (DH). The hydrolysis of the protein was determined using the o-phthalaldehyde (OPA) method [67] and resulted in two DH, 38% and 57%. To see how the hydrolysis affected the molar mass, size exclusion chromatography with a multi-angle laser scattering detector (SEC-MALS) was used. After hydrolysis, the chromatograms were different compared to an unhydrolysed sample (P), see Figure 2.5. The pea protein used in this thesis is highly insoluble, causing the P sample to have low signal to noise ratio resulting in no molar mass determination. The DH38 and DH57 samples had one large peak each around six to seven minutes, however this peak is most probable aggregation of proteins and not solubilised peptides. At a later elution time, between 11 to 14 minutes, a small peak is observed for both samples (see insert) which corresponds to majority of the sample based on the refractive index (RI) signal. However, these peaks have low signal to noise ratio so the determination of absolute molar mass is not possible, but an estimation of the molar mass can be obtained. For the DH38 sample, the molar mass (M_W) was determined to <3.4 kDa and for the DH57 sample <1.4 kDa. A lower molar mass for the sample with higher degree of hydrolysis is to be expected as the protein is believed to be digested to a larger extent.

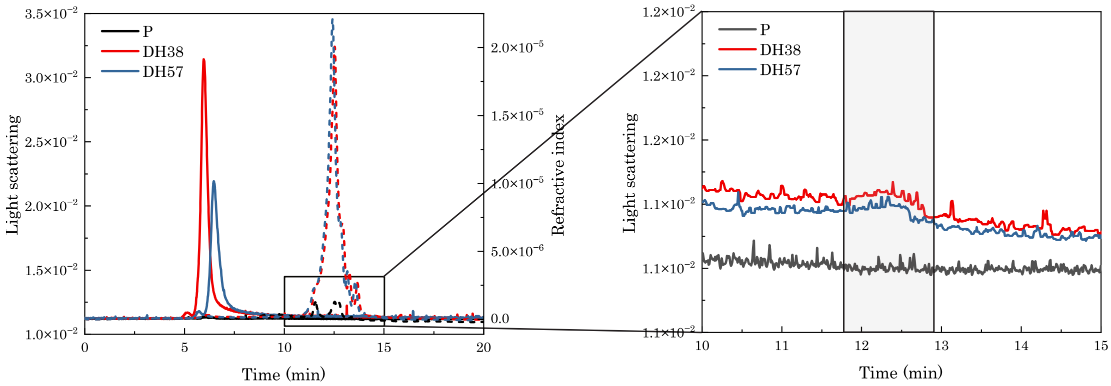


Figure 2.5: Chromatograms from SEC-MALS of P (black), DH38 (red) and DH57 (blue) samples. The insert shows the light scattering peak for the DH38 and DH57 sample between 11.8 to 12.9 minutes. Solid line corresponds to the light scattering at 90 ° and the dashed line corresponds to the refractive index.

Chapter 3

Suspension properties

During development of food products or extraction of pea protein and pea hull fibre, suspensions of plant particles are normally formed in aqueous solution. It is therefore of importance to understand how the plant particle suspensions behave at liquid state, at a solid-like state at higher concentrations and in the case of protein gelation. One way to get a better understanding of flow properties is to use rheology, which is a way to describe liquid-like and solid-like properties of a system (*i.e.* particle suspension) [68].

Viscosity is a key parameter to understand the liquid-like properties of a suspension. Viscosity determines for example how easy a liquid is to pump and flow, and gives the possibility to quantify frictional force between liquid layers in flow [69]. In a liquid with high friction, the viscosity will be high and more force is needed to set the liquid in motion (shear). In most cases, the force applied is dependent on the speed, the area of the moving plane and the distance between the planes, see Equation 3.1:

$$F = \eta A \frac{\mathrm{d}v}{\mathrm{d}y} \tag{3.1}$$

where F is the force, η is the viscosity, A is the area of the plane, $\frac{\mathrm{d}v}{\mathrm{d}y}$ is the shear rate. An easy way to illustrate flow is by two planes that are enclosing liquid, where the upper plate starts moving and thereby the liquid starts to flow [70]. The liquid flows because the adhesive forces between the plane and the liquid is stronger than the cohesive force between liquid molecules. Liquid "stuck" to the moving layer will impart movement on the next layer and so on, creating a flow in the entire liquid, see Figure 3.1.A.

Depending on how the liquid responds to increased shearing, it can be categorised into one of three groups, see Figure 3.1.B. Newtonian fluids, for example water, are independent of shear rate and will have a constant viscosity. However, most liquids are non-Newtonian and are instead dependent on the shear rate. Shear-thinning liquids have reduced viscosity with higher shear, which is common in for example polymer or particle suspensions. The opposite behaviour is shear-thickening, where the viscosity increases with shear. This behaviour is rare but can for e.g., form when there are strong interactions

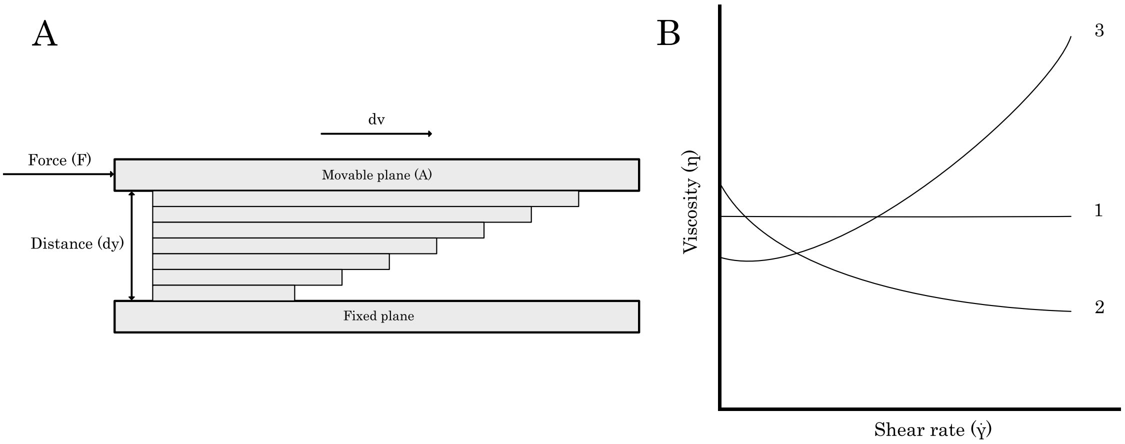


Figure 3.1: A: Model of flow to describe viscosity and B: Viscous behaviour of different liquids versus shear rate where 1. Newtonian, 2. shear-thinning and 3. shear-thickening.

between particles. There are also specific cases, for example, plastic liquid, or Bingham plastic, which starts to flow over a specific shear stress, but exhibit solid-like properties below [70].

When the analysed system display solid-like properties, only analysing viscosity is insufficient. To characterise a system which has solid-like properties, small and/or large deformations are used. Small deformation properties are measured by oscillatory rheology, which is done by applying a sinusoidal strain or stress to the system, generally between two plates. The deformation is small so the system is not irreversibly damaged, *i.e.* measurements are done in the linear viscoelastic region (LVR). The rheological properties are then presented as two different parameters, storage modulus (G') and loss modulus (G''). Storage modulus is a measurement of the reversible, elastically stored energy in the system and the loss modulus measures the energy dissipated during viscous flow. Therefore, a comparison between the storage modulus and loss modulus can reveal the properties of the system, where $G'>G''$ resulting in a system behaving like a viscoelastic solid. A third parameter is the loss factor (tan δ), which is the ratio between G'' and G'. A loss factor near zero implies a elastic solid, while a infinite loss factor describes a viscous fluid [70]. Large deformations are for example carried out by a compression test, where the system is put between two plates and deformed until break. During the test, parameters such as fracture stress, which measures the amount of stress can be applied until fracture and fracture strain, which is the strain at fracture and gives insight in how brittle the material is.

Plant cell wall particles, which is the case for pea hull fibres, are highly polydisperse and irregular particles. Due to the large variation in particle size, shape and deformability, there are challenges when characterising these systems [71]. Plant particles are generally soft in nature, as they have the ability to absorb water and swell. The softness of the particle contributes to complexity, and becomes more prominent at higher concentrations where the interactions have higher elasticity. Thus, at high particle concentrations the systems often show viscoelastic properties at low shear but starts to flow at a certain stress [72, 73]. The rheological behaviour of soft plant particles, for example on broccoli, tomato, apple and carrot cells, has been investigated [74–79]. The focus of these studies is on specific concentration regimes for the plant particles, and the viscoelastic behaviour that can be observed at higher concentrations.

3.1 Fibre suspensions

The modified pea hull fibre samples discussed in the previous chapter were analysed from a rheological point of view, to examine if the differences in composition and physical properties affected the rheology, see Figure 3.2. The rheological measurements were performed using a plate on plate geometry with a 1 mm gap. All samples where prepared in water, and have the same concentration based on dry mass. The normalised viscosity is the measured apparent viscosity for the sample normalised against the viscosity of the unmodified pea hull fibre (F).

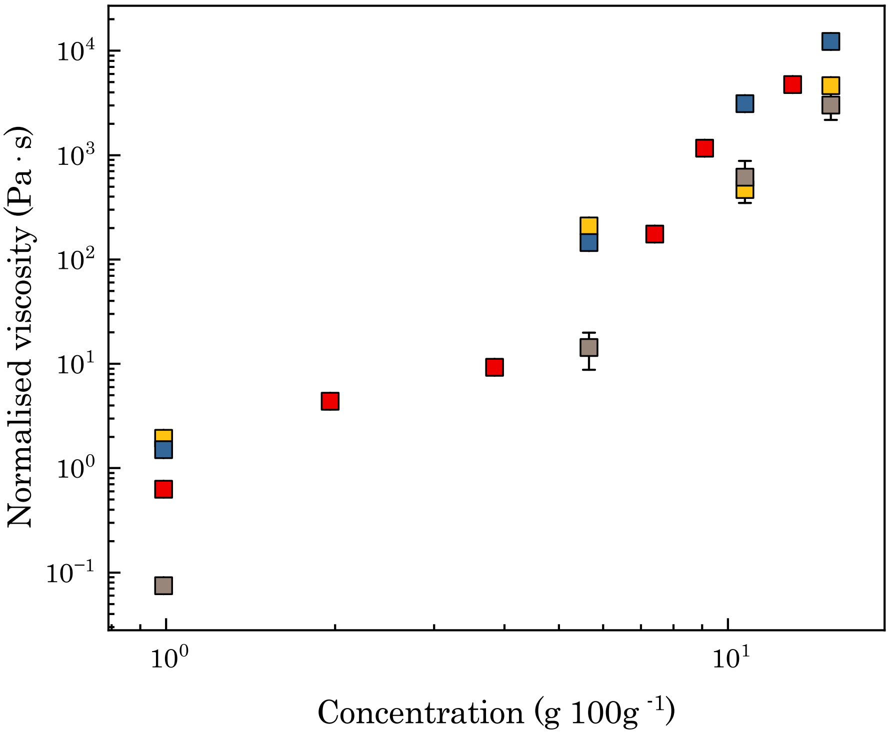


Figure 3.2: Normalised viscosity of FT (red), FP (yellow), FP-I (blue) and FC-I (brown) samples at 10 s^{-1} at concentrations ranging from 1 to 15 g 100g^{-1}. All samples were analysed at 25 °C.

All samples exhibited Newtonian behaviour at concentration below 1 g 100g^{-1} and shear-thinning behaviour at concentrations above 1 g 100g^{-1}. Based on the viscosity measurements, FP-I had the highest viscosity while FC-I had the lowest at concentrations above 10 g 100g^{-1}. The FC-I sample displayed increased swelling after treatment, but that does not seem to be significant enough to affect the viscosity (Table 2.2). The microstructure was also visibly unaffected by cellulase, hinting that the modifications might not be sufficient to influence viscosity (Figure 2.2). The FP and FP-I sample had similar viscosity at lower concentrations, but differences was observed at concentrations above 10 g 100g^{-1}. At first, it might be counter-intuitive that the enzymatically modified sample (FP) differed markedly from its purely insoluble fraction (FP-I). FP consists of both soluble and insoluble solids making up the total concentration, whilst FP-I consists on only insoluble solids. Therefore, the lower viscosity is due to higher soluble fraction in the FP sample that does not have an effect on viscosity. The solubilised fraction consists out of monosaccharides or lower molecular weight polysaccharides which are not expected to influence the viscosity, based on viscosity measurements on the solubilised fractions (not shown). On the other hand, FP-I consists of only insoluble pea hull fibre particles which have an effect on viscosity, and that means at a specific concentration the FP-I sample will contain more particles than the FP sample. The results presented show that the insoluble part of the treated pea hull fibres dominate the viscosity increase, and no or too few polysaccharides are released to impact viscosity. The thermal sample (FT) has a lower solubilised fraction compared to FP, and is therefore not as effected by the reduced particle concentration. Nonetheless, the viscosity is still lower than the FP-I sample which gives an indication that the particles swelling is not as prominent in the FT sample. Overall, the viscosity comparison clearly shows that it is the insoluble fraction that dominates the rheological properties of the sample. The system can be seen as a particle suspension in a liquid media (in our case water), and is not affected by the solubilised fraction in the continuous phase.

Oscillatory measurements of the pea hull fibre (F) at 15 g 100g^{-1} was done during a temperature ramp up to 95 °C, to measure the viscoelastic properties of the system (paper II). The measurement practically visualises the transition from F to FT sample. The G' was larger than the G'' for the full temperature ramp, and G' increased with the temperature. Thereby, a more elastic system is formed during heating, which is most probable due to the swelling of the fibre particles. However, there is no significant difference in the $D_{[4,3]}$ values for F and FT samples, but there is a slight increase after thermal treatment.

3.1.1 Modelling of rheological properties

An extended analysis of the pea hull fibre suspensions was performed by investigating the FP-I sample at a range of concentrations. The FP-I sample was chosen due to the largest increase in viscosity after modification. During the widened concentration range, three distinct regimes was observed in the flow measurements, see Figure 3.3. These regimes can be fitted to existing particle suspension models, where the simplest one is by Einstein where he described the rheology behaviour of a dilute hard sphere suspension [80]. Einstein's model was later expanded to cover the semi-dilute (intermediate) regime by Krieger-Dougherty and later Quemada [81, 82]. The limitations with these models are that they do not consider the deformation of particles and does therefore not work in the concentrated regime where particle deformation would occur [83]. To tackle this problem, Mendoza used star polymers as a soft particle system to develop a model that predict particle suspension behaviour in the concentrated regime [84]. Mendoza's model was later adapted to apple cells by Leverrier and colleagues who showed that the model can predict the viscous behaviour of apple purees [83]. The viscosity is normalised relative to the continuous phase (water), see Equation 3.2, where a η_{relative}=1 signifies the added particles do not contribute to viscosity at that concentration.

$$\eta_{\text{relative}} = \frac{\eta_{\text{apparent}}}{\eta_0} \tag{3.2}$$

Where η_{relative} is the relative viscosity, η_{apparent} is the absolute measured viscosity and η_0 is the viscosity of the continuous phase.

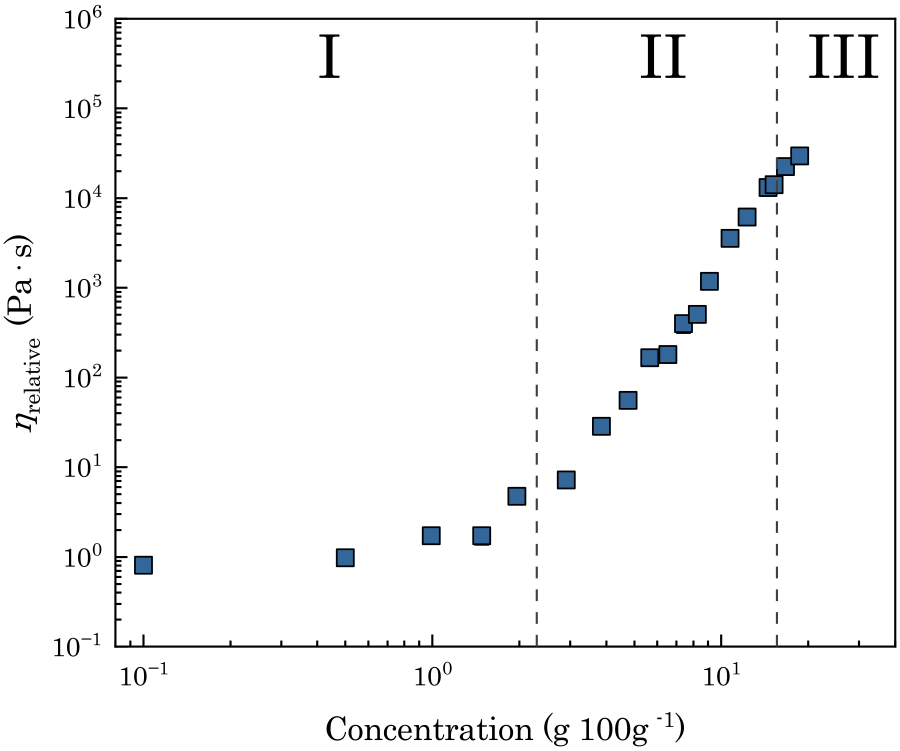


Figure 3.3: Flow sweep measurements at a concentration range of 0.1 to 18.7 g 100g^{-1} for the FP-I sample. The three regimes shown are the dilute (I), intermediate (II) and concentrated (III) regime.

The first regime stretches up to a concentration of 2.3 g 100g^{-1} for the pectate lyase modified pea hull fibres. The regime is typically referred to as the dilute regime, and for the pea hull fibres, there is no dependence on the particle properties, and the system behaves solely as the continuous phase (in this case water). Newtonian behaviour of the suspension is observed in regime I, and the particles are too far apart to have a significant interaction. The regime can be modelled according to Einstein's equation, see Equation 3.3. For hard spheres, the volume fraction relative to the viscosity results in k=2.5, and a larger k would imply stronger interactions between the particles. The k value obtained for the dilute regime was 0.3 indicating that sedimentation takes place [77].

$$\eta(c) = \eta_0(1 + kc) \tag{3.3}$$

In regime II, which occur between 2.3 g 100g^{-1} to 11.6 g 100g^{-1} of pea hull fibres, there is a sharp increase in the viscosity and has previously been modelled using a power law equation [72, 76], see Equation 3.4. The steep increase in viscosity is mainly related to long-range hydrodynamic interactions stemming from particles interacting, but is additionally affected by lubrication forces and friction between particles. The power law term b is dependent on particle polydispersity, with a larger b implying higher polydispersity. Earlier values for carrots (2.3), tomato (2.5) and apple (2.0) are markedly lower than the observed value for pea hull fibre (b=4.7) [76, 77]. Pea hull fibres do have high polydispersity (Figure 2.2 and Table 2.2), so a large b value is expected. Similarly, broccoli also had a larger b=6.9, which was motivated by stiffer particles, as a larger force is required to deform the particles, and can thus be the case for pea hull fibres as well [76].

$$\eta(c) = ac^b \tag{3.4}$$

Above 11.6 g 100g^{-1}, the third regime, or the concentrated phase, is reached, where the particles are tightly packed and soft particles start to deform to fit into available space. Regime III is heavily dependent on intra particle properties, where a large polydispersity and size results in reaching the third regime at a lower concentration [85]. With smaller particle sizes present in the suspension, small particles can fit into voids between the larger particles further increasing the packing efficiency resulting in a higher concentration required to reach regime III. To model this regime, a logarithmic approach has been used previously, adapted from clay and cement, which accounts for the disappearance of space between the particles [77, 86], see Equation 3.5.

$$\eta(c) = \eta_0(a + b \ln c) \tag{3.5}$$

In the same concentration range, viscoelastic properties of the pea hull fibre suspension was also analysed, see Figure 3.4. The same three regimes (dilute, intermediate and concentrated) observed in the flow measurements was also noticed in the oscillatory measurements, however the change from regime I and II are less pronounced. The lack of of particle interaction at lower solid concentrations (<2 g 100g^{-1}) will not develop a percolating particle network with the consequence of oscillatory measurements not being suitable at that concentration range. At so low concentrations the elastic contributions are negligible. The pea hull fibre concentration, at which a shift from regime I and II in the viscous data occur is then used for the viscoelastic data as well, which has previously been shown to align [77]. The intermediate regime can be modelled using the same procedure that was used for the viscous data, *i.e.* a power law equation, Equation 3.4. The b value for the oscillatory data was 8.1, which is almost double the value observed in the viscous data. As mentioned before, the power law term is affected by particle size and shape, and due to the large size of the pea hull fibres and their irregular shape, a large value is expected. The large particles will form a percolating network at lower concentration due to steric hindrance, and the smaller particles have the possibility to fill voids in the the network, promoting the rigidity [87, 88]

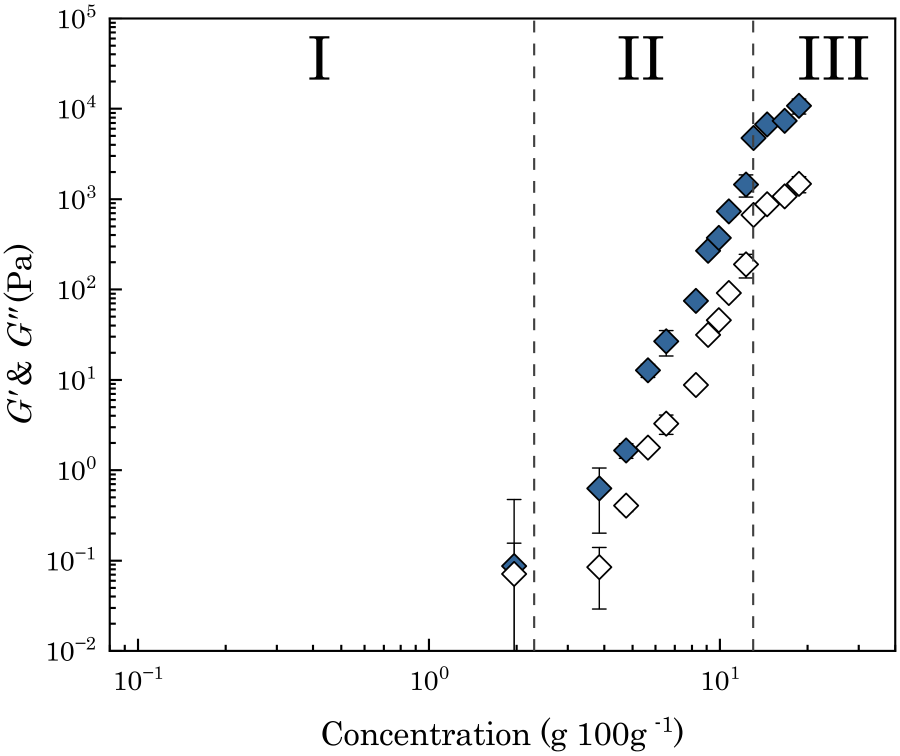


Figure 3.4: Oscillatory measurements at a concentration range of 0.1 to 18.7 g 100g^{-1} for the FP-I sample. The three regimes shown are the dilute (I), intermediate (II) and concentrated (III) regime.

The last and third regime marks the start of the concentrated regime, which is at 10.8 g 100g^{-1} based on the oscillatory data. This value is close to the predicted value from the viscosity data, 11.6 g 100g^{-1}. At regime III, (G') is much larger than (G''), revealing that the elastic contribution is significantly larger than the viscous contribution. Previously, Adams and colleagues proposed and used a model to predict the viscoelastic behaviour of the concentrated regime [89], see Equation 3.6:

$$G'(\phi) = A\left(1 - \frac{\phi_c}{\phi}\right)^{\frac{1}{3}} \tag{3.6}$$

where A is a variable constant, ϕ_c is the critical volume fraction and ϕ is the volume fraction. The critical volume fraction is where the particles are as tightly packed as possible. To be able to employ the Adams model to the pea hull fibre, a simplification was made by converting the volume fraction to solid concentration (c and c_c) [77].

The viscosity and the viscoelastic data was successfully modelled if the different regimes are split apart, however if one would want to model the entire concentration range with one model, the complexity increases. The first problem one encounters is that most models use volume fraction instead of total particle concentration. For hard spheres, this concern is not as major as it is possible to recalculate, however when it comes to soft or semi-soft particles that deforms, it posses a problem [90]. Generally, volume fraction is measured by separating the particles by centrifugation or measuring the particle volume after sedimentation occurs [91]. When centrifugation is used, the measured volume fraction is therefore dependent on the applied force and will not measure the correct volume fraction for the soft particles [76]. Therefore, experimental determination of volume fraction of soft particles is difficult as the methods used deform the particles leading to underestimation of the volume fraction [75, 77]. Theoretical approaches to determine volume fraction are based on the particles ability to deform and fit into available space, and has therefore practically a higher packing efficiency than random close packing for hard spheres (0.64). Based on the ability to deform, the concentration at which the regime goes from II to III is assumed to be a volume fraction of 1. At this concentration, all particles are as tightly packed as possible but has not yet started to deform. For the pea hull fibre suspension, based on the viscosity data the regime shift is at 15.6 g 100g^{-1} and for the viscoelastic data 13.0 g 100g^{-1}, showing that the estimated volume fraction is dependent on which rheological property that was analysed. From there, the apparent volume fraction is calculated according to Equation 3.7:

$$\phi_{\mathrm{app}} = \frac{c}{c^{**}} \tag{3.7}$$

where ϕ_{app} is the apparent volume fraction, c is the concentration and c^{**} is the concentration where regime shifts from II to III. From here, it is possible to transform the viscous and viscoelastic data to be dependent on apparent volume fraction instead of total particle concentration, and then fit it to established models, see Figure 3.5. The first relevant and simplest model to use is developed by Krieger-Dougherty, which introduced a maximum packing density parameter to Einstein's model for dilute systems [81], see Equation 3.8:

$$\eta(\phi) = \eta_0 \left(1 - \frac{\phi}{\phi_{\mathrm{rcp}}}\right)^{-k\phi_{\mathrm{rcp}}} \tag{3.8}$$

where ϕ_{rcp} is the volume fraction for random close packing. The random close packing is based on hard spheres, and is able to predict viscous behaviour in the dilute and intermediate regime for those systems. Because the model covers both Newtonian and non-Newtonian behaviour, it can be applied to the viscous pea hull fibre data, resulting in k=3.9. This k value is close to the theoretical value for hard spheres (k=2.5), and if we consider the pea fibre as hard particles, the dilute regime can be fitted well with the Krieger-Dougherty model because particles are far apart from each other. However, when volume fraction increases and particles interactions occur, there is a clear deviation from the model, due to the relevancy of particle shape and hardness, which the model does not account for. This causes the model to deviate markedly in the intermediate region compared to experimental data, see Figure 3.5.

A model of higher complexity was proposed by Mendoza. In the Mendoza model, the critical packing parameter is not limited by random close packing but also introduces terms that account for hydrodynamic interactions and

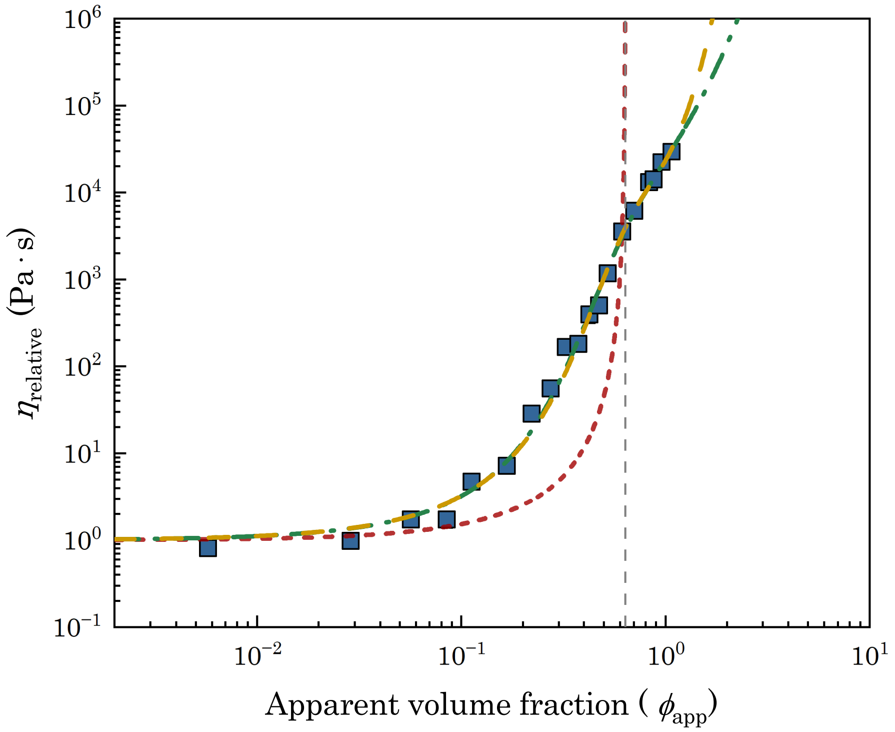


Figure 3.5: Experimental data points for the pea hull fibres and three particle suspension models showing the relative viscosity versus apparent volume fraction. Blue symbols are experimental data and the line are theoretical models with Krieger-Dougherty (red), Mendoza (green) and Leverrier (yellow). The grey dashed line corresponds to the volume fraction of random close packing (ϕ_{rcp}).

crowding effects [84], see Equations 3.9, 3.10 and 3.11. The model by Mendoza predicts the experimental data for the pea hull fibre in the entire volume fraction range.

$$\eta(\phi) = \eta_0 \left(1 - \frac{\phi}{1 - k\phi}\right)^{-[\eta]} \tag{3.9}$$

$$k = \frac{1 - \phi_{\text{c}}}{\phi_{\text{c}}} \tag{3.10}$$

$$\phi_{\text{c}} = \phi_{\text{rcp}} + \beta\phi^{\alpha} \tag{3.11}$$

The power law term $[\eta]$ can be connected to particle shape, where irregular particles would results in a larger $[\eta]$, and is therefore expected to be comparatively large for the polydisperse pea hull fibre sample [92]. The α is harder to interpret, but is correlated by Mendoza to the particles ability to deform, where particles with high deformability would result in a small α. For star polymers, the α value differs between 1.74 to 1.90, which is lower than what is obtained for the pea hull fibres (2.2), see Table 3.1. The fibres are expected to be less deformable than star polymers, so the larger value obtained here for the pea hull fibre is in line with expectations. In addition, Leverrier and colleagues applied Mendozas model to apple purees and obtained values of 2.10 to 2.16, values that are comparable to pea hull fibres, even if the fibres are substantially larger and have higher polydispersity. Leverrier and colleagues propose that α also is dependent on surface irregularities and sphericity of the particles, where high sphericity and few irregularities would yield a large α. The β value has been connected to particle softness but is hard to interpret, where a large β would imply softer particles. Values for star polymers (0.244 and 0.966) and apple puree (0.913-1.177) was substantially smaller than that obtained for the pea hull fibres (1.9), which would indicate soft particles for the fibres. The rigidity of the fibres is expected to be large, which would result in a small β, but because of the irregular shape of the pea hull fibres, and the high variety in the structure (macrosclereids and osteosclereids) the interpretation is complex. One reason for the large β could be that the effective volume fraction occupied by each fibre is larger compared to apple cells, due to their irregular shape (low sphericity) and the open structure observed in the microscopy images (Figure 2.2).There, large empty spaces or low-density regions can be seen within the pea hull fibre treated by pectate lyase. Such particle characteristics could result in softer particles.

Lastly, Leverrier and colleagues added additional terms to Mendozas model to account for a maximum packing fraction parameter ϕ_{max}, so that the volume fraction can not reach infinity [83], see Equation 3.12, 3.10 and 3.13.

$$\eta(\phi) = \eta_0 \left(1 - \frac{\phi}{1 - k\phi}\right)^{-S} \tag{3.12}$$

$$\phi_{\text{c}} = \phi_{\text{rcp}} + \frac{\phi_{\text{max}} - \phi_{\text{rcp}}}{1 + \exp\left[-\Lambda\left(\phi - \phi_{\text{i}}\right)\right]} \tag{3.13}$$

The ϕ_{max} is added to avoid the relative viscosity going to infinity. The ϕ_{max} is calculated together with other additional parameters ϕ_{i} and Λ, where ϕ_{i} is at which volume fraction the particles are at maximum deformability and Λ is the slope after that inflection point. The fibre sample obtained a ϕ_{max}=5.0, which is smaller than was observed for apple puree (16.0), implying that the particles are compressible and can fit into available space but not to the same extent as apple cells. The ϕ_{i} value was also lower for pea hull fibre at 1.0 compared to 2.4 for apple puree, which further signifies that the compressibility of the fibres are lower compared to apple cells, and the packing maximum of pea hull fibres is reached at a lower concentration. The slope at the inflection point Λ was markedly larger for pea hull fibres, 4.2, while it was 1.9 for apple puree which once again support that ϕ_{max} is reached at a lower volume fraction. For ϕ_{rcp}, a value of 0.637 was used because it has been shown to a have a negligible effect on the model outcome [78, 83]. All calculated model parameters can be seen in Table 3.1.

The models by Mendoza and Leverrier can successfully predict the viscous behaviour of the pea hull fibre suspension in the entire concentration range. Even if the models are based on model or simplified systems, the applicability transfer to a very complex system, such as pea hull fibres. The pea hull fibres are highly polydisperse and irregular in shape, however their rheological behaviour

Table 3.1: Parameters obtained from theoretical fits to pea hull fibre viscosity data using models from Krieger-Dougherty, Mendoza and Leverrier.

Model					
Krieger-Dougherty	$\phi_{\mathrm{rcp}}=0.637$	$k=3.9$			
Mendoza	$\phi_{\mathrm{rcp}}=0.637$	$[\eta]=10.4$	$\alpha=2.2$	$\beta=1.9$	
Leverrier	$\phi_{\mathrm{rcp}}=0.637$	$S=10.7$	$\phi_{\mathrm{max}}=5.0$	$\phi_{\mathrm{i}}=1.0$	$\Lambda=4.2$

can still be modelled, but the shortcomings lie in the ability to interpret the parameters from a physical point of view. It is valuable to discuss around what physical properties the parameters are related to, however due to the complexity of the systems and thus the complexity of the models, interpretation of the parameters should be done with caution. Applying these models to other systems, to evaluate if they are applicable, and give better understanding of what the parameters represent would be valuable for future interpretation of said parameters.

3.2 Protein suspensions

In addition to the extensive characterisation of the rheological properties of pea hull fibre suspensions, pea protein suspensions were investigated. Similar to the pea hull fibres, the effect of thermal treatment of pea protein is of interest, due to the gelation of the protein. Therefore, oscillatory measurements during a temperature ramp were performed to examine how viscoelastic properties changed with heat, see Figure 3.6.

The temperature rate was the same as for the oscillatory measurements for the pea hull fibre suspension. The temperature started at 25 °C, then increased to 95 °C and lastly went down to 20 °C again. At start, G'' was larger than G' for all samples, but a shift happened when heat was applied. G' rapidly increased from 50 °C, which is similar what has been observed previously for pea protein [61]. After cooling, G' was larger than G'' for all samples, indicating that a solid-like system had been formed. The largest G' could be observed for pH 4.5, which is to be expected as the repulsion between the protein particles at that pH is at its lowest. The lowest G' in absolute value was observed at pH 7, which can be explained by higher repulsion between the particles. More swelling and increased interaction between the particles was observed in the particle size measurements, which agrees with the viscoelastic data. Even if there are differences in G' at different pH values, the gelation process follows a similar trend for all samples.

The gelation mechanism in the pea protein is related to particle swelling and the formation of a particulate gel. This is the same phenomenon that is seen in the pea hull fibres. However, the protein swelling is larger relative to their size compared to the pea hull fibre because we measured a larger increase in the viscoelastic properties with heating for the protein [63]. The formation of particulate pea protein gels has previously been observed for commercial pea protein, displaying similar behaviour to the pea protein reported here [50].

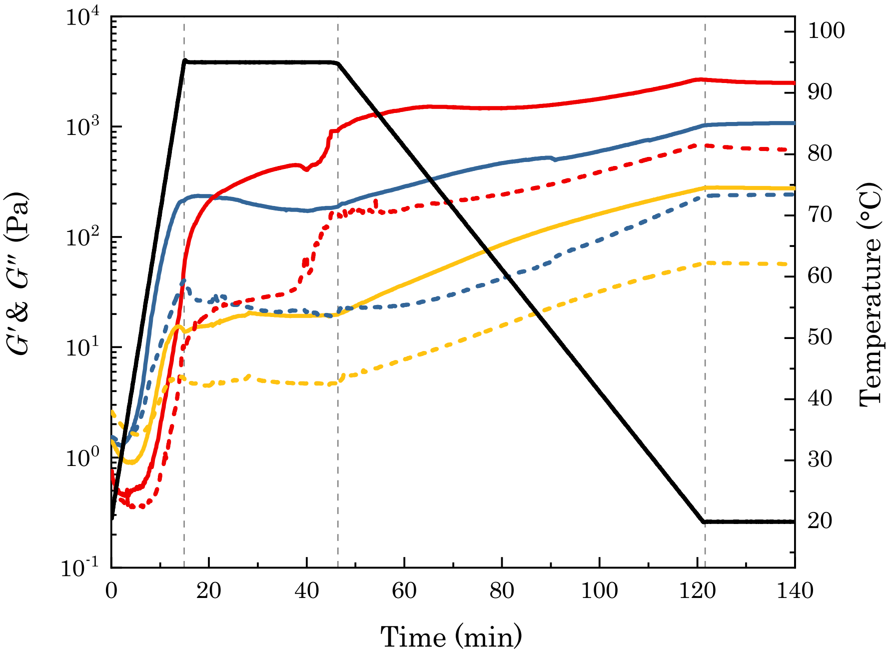


Figure 3.6: Oscillatory measurements as a function of temperature and time for pea protein suspensions at different pH: pH 2 (blue), pH 4.5 (red) and pH 7 (yellow). The solid line correspond to the storage modulus (G') and the dashed line correspond to the loss modulus (G''). The black line corresponds to the temperature. Reproduced from Karlsson, J. et al., *Food Hydrocolloids*, 2024 under the CC-BY license.

3.3 Fibre and protein blend suspensions

After the individual characterisation of the pea hull fibre and protein, the combination of the two was explored. From a food technology point of view, combining the fibre and protein give insight in possible interaction between them and how those can affect a food product. Blends of protein and fibres are used not only to form a more healthy product but also to texturise. Food products has been studied in combination with fibres to produce e.g., meat analogues [93–95] or protein gels [96, 97].

Possible electrostatic interaction between the pea hull fibre and protein was studied using zeta potential, see Figure 3.7. The charge of the pea hull fibre was slightly dependent on pH, ranging from -8 mV at pH 3 and decreasing to -20 mV at pH 6, but was negative for the entire pH range. Pea protein had a higher dependency of pH, being positively charged above pH 4.2, which is the expected pI for pea protein [52]. At higher pH, the pea protein becomes negative and at pH 6 it has the same charge as pea hull fibre (-20 mV). Therefore, there can be electrostatic interactions at low pH (<pH 4), but at higher pH, repulsion between the pea hull fibre and pea protein is expected.

Even if there are no significant electrostatic interactions between the pea hull fibre and pea protein, there could still be other interactions present (hydrophobic, etc.), and therefore combined oscillatory measurements were performed, see Figure 3.8. The blends of pea hull fibre and protein were all

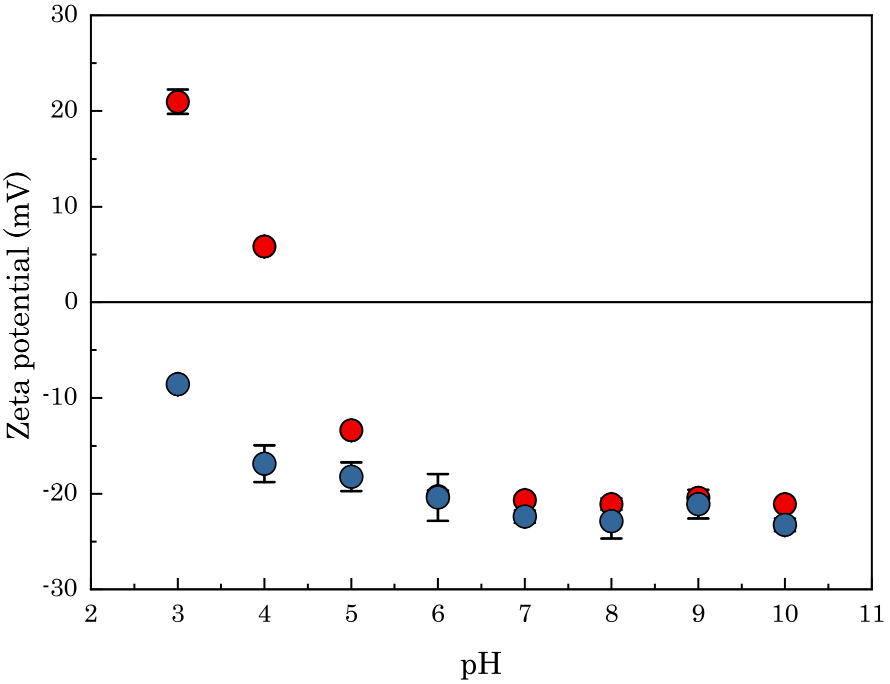


Figure 3.7: Zeta potential of pea hull fibre (blue) and pea protein (red) at pH ranging from 3 to 10. Reproduced from Karlsson, J. et al., *Food Hydrocolloids*, 2024 under the CC-BY license.

performed at 15 wt% in water, but at six ratios of fibre to protein, see Table 3.2. All combined measurements were performed at pH 7. The same temperature ramp sequence was used for the combined oscillatory measurements as had been used for the individual fibre and protein measurements.

Table 3.2: Pea hull fibre and pea protein ratios used for oscillatory measurements in combined suspensions. All samples had a total dry mass of 15 g 100g^{-1} in water.

Sample name	**Fibre (g 100g^{-1})**	**Protein (g 100g^{-1})**
F	15	-
F80	12	3
F60	9	6
F40	6	9
F20	3	12
P	-	15

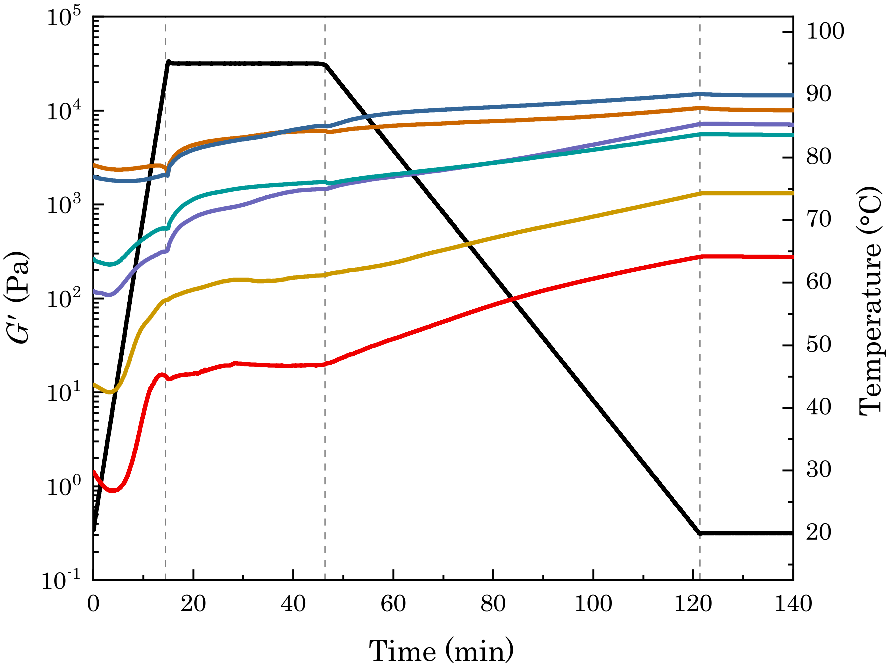


Figure 3.8: Oscillatory measurements at pH 7 of pea hull fibre and pea protein blends during a temperature ramp. There are six blends that were analysed: F (blue), F80 (orange), F60 (teal), F40 (purple), F20 (yellow) and P (red). The black line corresponds to the temperature. Reproduced from Karlsson, J. et al., *Food Hydrocolloids*, 2024 under the CC-BY license.

In Figure 3.8, the pea hull fibre suspension and pea protein suspensions exhibit different properties upon heating. The pure fibre (F) has the highest G' in absolute values, indicating that it forms a stiffer system less inclined to deform, while the lowest G' can be found in the pure protein (P). However, the protein has a sharp increase when temperature rises due to gelation that is not present in the fibre sample. Increasing the fibre ratio by 20% reduces the sharp increase in G', but it is still prominent. Twenty percent of fibre in the sample is a substantial amount for a food product, and it shows that at such high concentrations, the protein still contributes to the system's sol-gel transition. Nonetheless, when the fibre ratio is increased further, the gelation properties diminish, and the pea hull fibre is the dominant factor from a rheological point of view. The fibre produces a thicker system compared to the pea protein, but not necessarily a more viscoelastic one. The thicker pea hull fibre system is due to large particle size and irregular shape, but it does not form as viscoelastic system as the protein, because the increase in G' is smaller during heating. This is because the interaction between the fibre particles are weaker than between the protein particles. The reduction of thermal gelation at high fibre concentration is most probable due to the low concentration of protein causing the protein particles to no longer aggregate and swell to the same extent, and thereby not forming a strong percolating network. There is also no contribution from the pea protein at high fibre ratios (F80), showing that there are no or limited interactions between the ingredients or that the pea protein has no water available to swell. This is also apparent when the microstructure is analysed using CLSM (Figure 3.9). When the protein concentration is high (F20), the protein particles are clearly visible and have the ability to form an percolating network. However, for F80 there are no protein particles visible, and can therefore no longer form a particulate gel after thermal treatment. The tan δ values for the blended samples were all between 0.2 to 0.3, which tells us that the system behaves solid-like, but a strong gel is not formed. A strong solid is generally defined as tan $\delta << 1$, which has previously been shown to not be formed by commercial pea protein isolates [50].

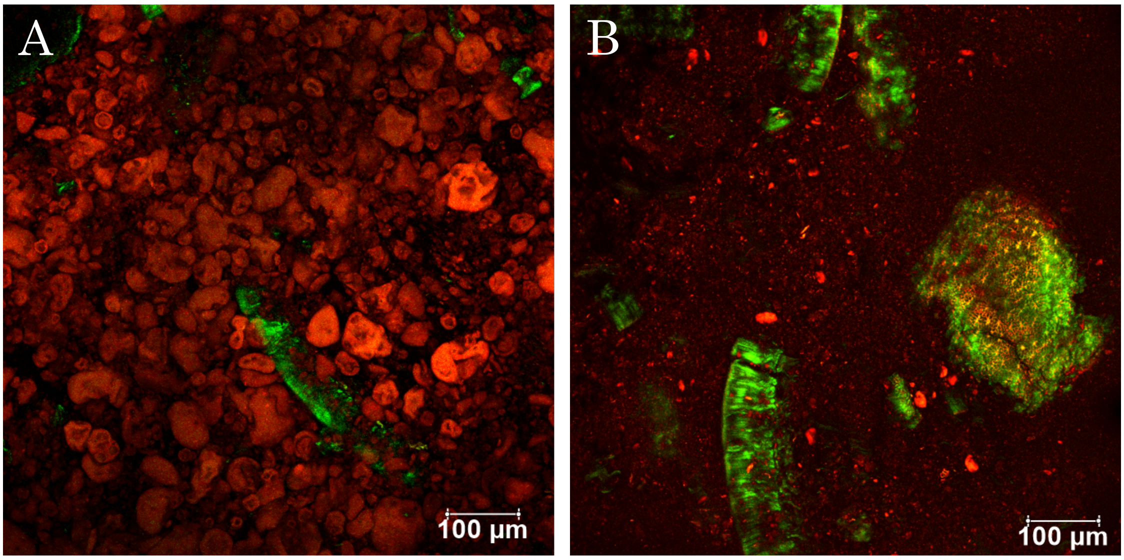


Figure 3.9: Microstructure of pea hull fibre (green) and pea protein (red) blends visualised using CLSM. Micrographs of two different ratios were taken. A. F20 and B. F80. The pea hull fibre was stained with Direct Red 23 and the pea protein with Texas Red. The scale bar is 100 µm

Chapter 4

Colonic fermentation

Modifications to plant cell wall structures and particles does not only affect their physico-chemical properties, but can also influence how they are digested and fermented in the gastrointestinal tract (GIT). Consumption of a healthy diet will result in plant particles with different microstructures and compositions reaching our gut. Intake of dietary fibres should reach 25 to 35 grams per day [98], an amount that few people meet [99]. When dietary fibres are ingested, they are not digested by the human body, but instead fermented by the microbial community in the colon. The dietary fibres are naturally occurring and originates from vegetables, fruits, cereals and grain, and in our specific case pea hull. There are numerous health benefits of consuming adequate amounts of dietary fibre, for example improved gut motility, body weight control, reduced risk of several diseases such as depression, rectal cancer and cardiovascular diseases, and of course the effect on the microbial community and metabolites [13, 100–104].

The main metabolites produced during dietary fibre fermentation are short chain fatty acids (SCFA), consisting of two to six carbons. Acetate, propionate and butyrate are the shortest of the fatty acids produced and are also the most abundant. SCFA has important functions in the gut, working as an energy source for colonic epithelial cells and acts as signalling molecules [105, 106]. Examples of what SCFA can regulate is insulin secretion from the pancreas, insulin sensitivity and satiety, but also reduce risk of inflammation or asthma [105]. Lactate is another metabolite produced by the microbiota but rarely accumulates in the gut, because it is further converted into other metabolites, for example SCFA [107].

Pea hull fibres, which we have established are highly insoluble, can contribute to improved motility but also adsorb harmful molecules and bile acids if they have sufficient water retention capacity [108]. Fermentation of pea hull fibres do produce SCFA, but only 25% of the pea hull fibres are fermented [109]. Pea hull fibre has therefore low fermentability compared to other dietary fibres [110]. As previously mentioned, the fermentability is not only dependent on the composition of the dietary fibre but also its structure. Particle size, solubility and interactions between components in the plant particle has major effects on the colonic fermentation outcome [110–112]. For example, large and dense plant particles are difficult to be fermented by the microbiota, which is aligned with the fermentability of pea hull fibres.

It is not only dietary fibres that reach the colon, it is also a significant amount of protein. The protein is mainly digested in the upper GIT, but a significant amount escape absorption in the small intestine [113]. Partly digested protein reaches the colon and is fermented by the microbiota. Depending on the source of protein and preparation, the digestion and absorption of protein will differ. For example, protein digestion of raw egg was only 65% and the rest reached the colon [114]. When cooking the egg, the absorption increased to 95%. Normally, plant proteins are considered to have lower digestibility than animal-based protein [115]. Pea protein isolates have been shown to have a digestibility between 60-80% *in vitro* [116]. The lower digestibility of protein can result in increased amounts of non-absorbed proteins reaching the colon. It is not only dietary protein that reaches the colon, endogenous proteins such as digestive enzymes also passes through the GIT, but a major part of the nitrogen that passes the small intestine are from unabsorbed dietary proteins [113]. The partly digested protein reaching the colon will result in prevalent proteolytic fermentation in the colon.

Compared to dietary fibres, protein fermentation produce metabolites of a higher variety. In addition to SCFA, protein fermentation also produces branched chained fatty acids (BCFA), ammonia and other nitrogenous compounds [64, 117]. BCFA, for example isobutyrate and isovalerate, are produced when branched chain amino acids (leucin, iso-lecuin and valine) are fermented [118, 119]. Even if the SCFA and BCFA are similar in molecular structure, it has been shown that they have different effects on metabolic regulation [120]. For example, BCFA can act as an energy source when the supply of SCFA are low [121]. Other metabolites produced from protein fermentation, namely nitrogenous compounds, can be toxic for the body. Increased ammonia levels in the colon is harmful to epithelial cells, but also correlated to hepatic coma and cancer [18, 122, 123]. Indoles and phenols that are formed from aromatic amino acids can also be carcinogenic [124, 125].

The present nitrogenous source can also be utilised differently, where larger peptides and ammonia is important for bacterial growth while smaller peptides and amino acids are mostly metabolised [126]. This was further demonstrated in a study where amino acids were poorly incorporated into the cellular matrix, and instead produced ammonia [127]. However, the ammonia could then be utilised by the bacteria to grow. The metabolism of the amino acids is also dependent on its structure, where aromatic, branched and sulphur-containing amino acids are generally fermented slower than other amino acids [127]. Pea protein have low amounts of sulphur-containing amino acids, but are abundant in some amino acids that are fermented quickly, for example lysine [127, 128].

Having dietary fibres and proteins being fermented together does alter the metabolic process that takes place in the colon [64, 124, 129–131]. Toxic compounds that are produced during protein fermentation are reduced when there is also dietary fibre present [124, 130], but the overall change in metabolites is hard to predict. However, increased fibre intake can promote formation of butyrate and butyrate-producing microbiota[132], but also, conflictingly, that addition of protein can enhance butyrate production [131]. The dietary fibres are generally fermented in the proximal colon, when the supply of fibre is good. However, the further along the GIT, the dietary fibres start to deplete and proteolytic fermentation becomes the main type of fermentation. In the distal colon, protein is the main substrate and therefore is the concentration of toxic metabolites the highest there [133].

The microbiome is suggested to consist of several thousands of different bacterial species and subspecies, where some are permanently present in the colon while other are transiting through originating from the environment or upper gastric tract [133]. The problem with investigating these bacterial species is that most are obligate anaerobe, which results in them dying in the presence of oxygen. The bacteria are therefore often hard to cultivate and analyse [134]. Even if the microbial community is hard to analyse, we do know that its composition is dependent on a variety of factors originating from the host (genetics, disease, etc.) and environment (substrate, pH, etc.). As discussed earlier, some substrates available for the microbiota to ferment are fibres and proteins, where different bacterial species exhibit specific pathways of how they utilise the substrate and what type of metabolite they produce. The catabolite regulation of the bacteria controls which digestive enzymes to secrete to selectively target the preferred carbon source, and thus control what type and amount of metabolite produced [135]. Bacteria from several genera has therefore been connected to specific metabolites, such as *Bacteroides* are known produce acetate and propionate, *Bifidobacteria* and *Lactobacilli* produces acetate and lactate [133], while the class *Clostridia* produces acetate, propionate and butyrate. In addition to the main SCFA, *Clostridia* also produces significant levels of BCFA when protein is utilised as a substrate [136, 137] and has been shown to increase in abundance in the presence of protein [131]. Other examples of bacteria that utilise protein and peptides as a carbon source are *Enterobacteriaceae*, *Escherichia* and *Fusobacterium* [126, 131, 138].

4.1 Colonic fermentation samples

Modified samples based on differences in physico-chemical properties were selected for *in vitro* colonic fermentation, see Table 4.1. For the pea hull fibre sample, the unmodified samples (F) were fermented, but also the thermally treated (FT) and the enzymatically treated by pecate lyase (FP) to see if the modification would affect the *in vitro* colonic fermentation. Two F samples were analysed (F-III and F-IV), connected to either paper III or paper IV. For the pea protein sample, the unmodified sample (P) at two concentrations (0.5g (IV) and 1.5g (III)) and the thermally treated sample was fermented (PT). In addition, the protein samples that had been subjected to hydrolysis (DH38 and DH57) were also fermented to see how protein and peptide size affects the *in vitro* colonic fermentation. Combinations of pea hull fibre and pea protein was also fermented, where non-modified fibre and protein (F+P) and thermally treated fibre and protein (FT+PT) was fermented. Combinations of fibre with

DH38 and DH57 was also conducted, resulting in the samples F+DH38 and F+DH57.

Table 4.1: The composition of the samples subjected to *in vitro* colonic fermentation, the amount of pea hull fibre and pea protein in grams for each sample and the fibre to protein ratio.

Sample name	**Fibre (g)**	**Protein (g)**	**Fibre to protein ratio (%)**
F-IV	1.5	-	100
F-III	1.5	-	100
FT	1.5	-	100
FP-I	1.5	-	100
P-IV	-	0.5	-
P-III	-	1.5	-
PT	-	1.5	-
DH38	-	0.5	-
DH57	-	0.5	-
F+P-IV	1.5	0.5	75
F+P-III	1.5	1.5	50
FT+PT	1.5	1.5	50
F+DH38	1.5	0.5	75
F+DH57	1.5	0.5	75

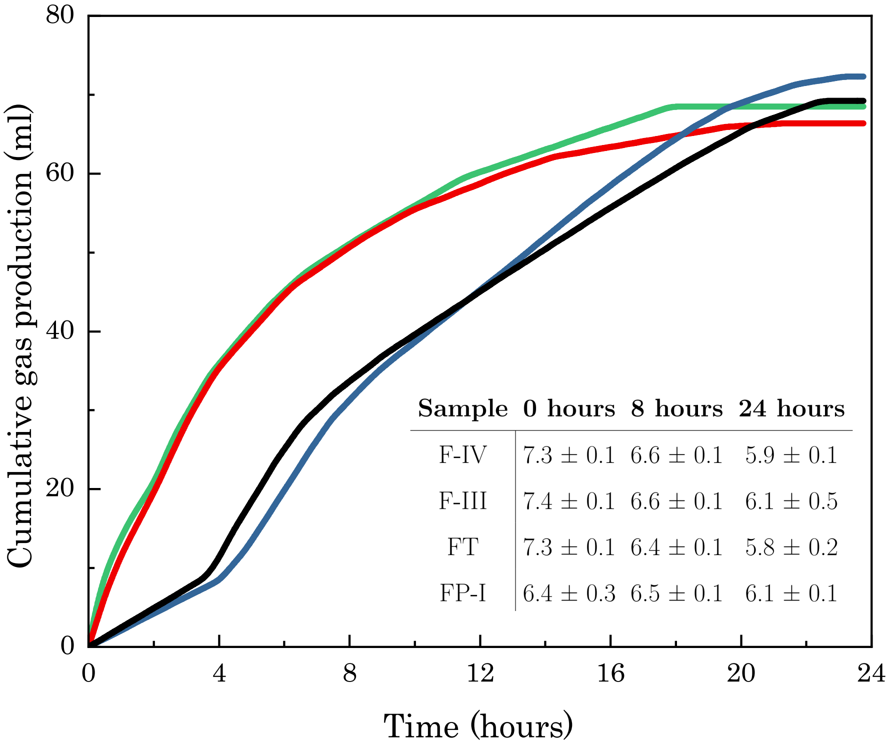
The amount of fibre was 1.5 grams for all samples containing fibre. The amount of protein varied, where P-III and PT used 1.5 grams of protein (paper III) while P-IV, DH38 and DH57 used 0.5 grams of protein (paper IV). The reduction in protein for the P-IV, DH38 and DH57 samples was to obtain a closer fraction of fibre and protein in the blends that is present during colonic fermentation and the new fraction was calculated based on the recommended daily intake [98]. All the samples were prepared in a similar manner, where they were sterilised using UV-light in a laminar airflow (LAF) bench for 30 minutes. Samples were then mixed with prepared anaerobic basal medium and faecal inoculum, where the preparation of the faecal inoculum differed between the samples. Samples in paper III (F-III, FT, P-III, PT, F+P-III and FT+PT) were ran in triplicate, using a separate donor for each run. Paper IV (F-IV, FP-I, P-IV, DH38, DH57, F+DH38 and F+DH57) used a pooled inoculum from seven donors where the experiment used the same pooled inoculum in triplicate runs. The *in vitro* fermentation used a batch setup, and therefore there was no influx or efflux of substrates or metabolites after the initial inoculation. After mixing, all bottles were put into a water bath at 37 °C under stirring for 24 hours, allowing for sampling at different time points.

4.2 Variation in pH and gas production during fermentation

The pH value changes during *in vitro* colonic fermentation due to the metabolites produced, e.g., SCFA and ammonia. This is especially the case when the experiments are performed in batch with no efflux of metabolites or continuos influx of substrate. Production of SCFA lowers the pH while an excessive amount of ammonia increases the pH. Observing the pH can therefore give a indication of metabolites produced. However, the pH is not just an indication of what happens during fermentation, but the pH also affects the outcome of the the fermentation. The pH ranges from 5.0 to 7.5 in the colon, with high interpersonal variation, while the pH is generally stable in individuals [139, 140]. High pH does for example promote the production of propionate and proteolytic fermentation products, while a low pH is correlated to increased levels of total SCFA, butyrate and lactate [139]. A high pH is needed for proteolytic fermentation to occur successfully, where a too low pH has been shown to inhibit protein fermentation [124, 129]. For example, the production of the proteolytic metabolite indole is inhibited at pH 5.5 [141].

4.2.1 Pea hull fibre

The fibre samples all had similar pH values after 24 hours of fermentation, but the FP-I sample had lower pH at zero hours (Figure 4.1).



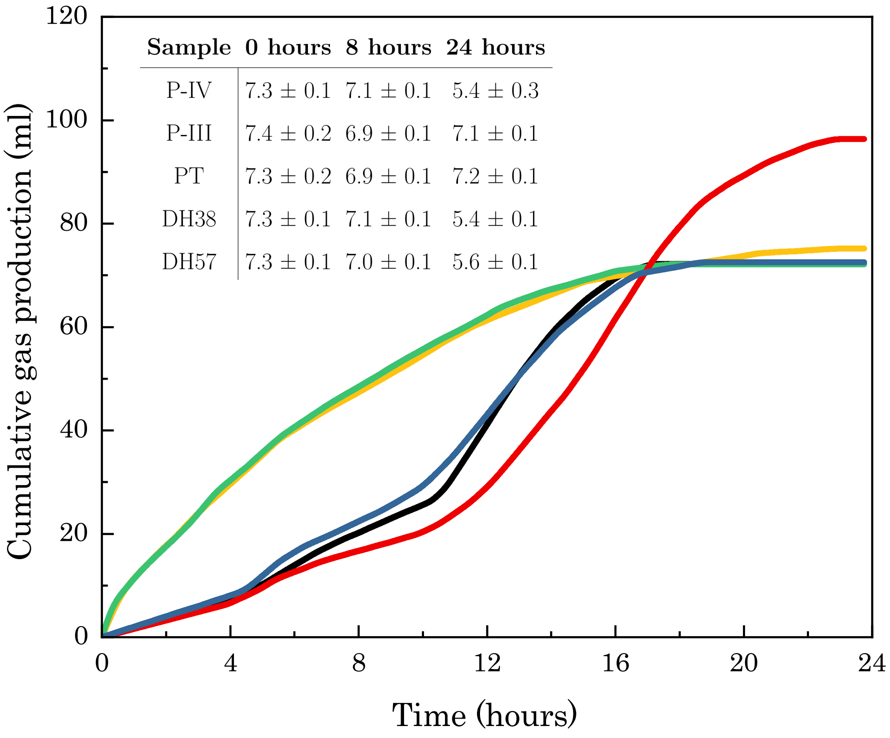
Sample	0 hours	8 hours	24 hours
F-IV	7.3 ± 0.1	6.6 ± 0.1	5.9 ± 0.1
F-III	7.4 ± 0.1	6.6 ± 0.1	6.1 ± 0.5
FT	7.3 ± 0.1	6.4 ± 0.1	5.8 ± 0.2
FP-I	6.4 ± 0.3	6.5 ± 0.1	6.1 ± 0.1

Figure 4.1: Cumulative gas production of the fibre sample over 24 hours. The samples are F-IV (black), F-III (green), FT (red) and FP-I (blue). The table show the measured pH values for each sample at timepoint 0, 8 and 24 hours.

As expected, the pH is reduced over a 24 hour period due to the increased concentration of SCFA, except for the FP-I sample. Furthermore, the gas production over time was similar for all the samples over a 24 hours period. The gas production is a measurement of total gas produced during the fermentation, but cannot distinguish between the most prominent metabolites (CO_2, H_2 and CH_4). Gas production also gives an indication if fermentation is progressing, with a higher production implying higher metabolic activity. A pea hull fibre that has been shown to swell and have an increased water uptake could possibly be easier for the bacteria to ferment, but this is not observed in the modified fibre samples. Nonetheless, the methodology to obtain the more porous fibre included removing parts of the accessible pectin, which is generally considered as a good fermentable polysaccharide [142], and thus imply that the pea hull fibre now has less fermentable polysaccharides present.

4.2.2 Pea protein

The pH values for the protein samples differed over a 24 hour period, where the hydrolysed samples and P-IV had a markedly lower pH at 24 hours compared to P-III and PT, see Figure 4.2.



Sample	0 hours	8 hours	24 hours
P-IV	7.3 ± 0.1	7.1 ± 0.1	5.4 ± 0.3
P-III	7.4 ± 0.2	6.9 ± 0.1	7.1 ± 0.1
PT	7.3 ± 0.2	6.9 ± 0.1	7.2 ± 0.1
DH38	7.3 ± 0.1	7.1 ± 0.1	5.4 ± 0.1
DH57	7.3 ± 0.1	7.0 ± 0.1	5.6 ± 0.1

Figure 4.2: Cumulative gas production of the protein sample over 24 hours. The samples are P-IV (black), P-III (yellow), PT (green), DH38 (red) and DH57 (blue). The table show the measured pH values for each sample at timepoint 0, 8 and 24 hours.

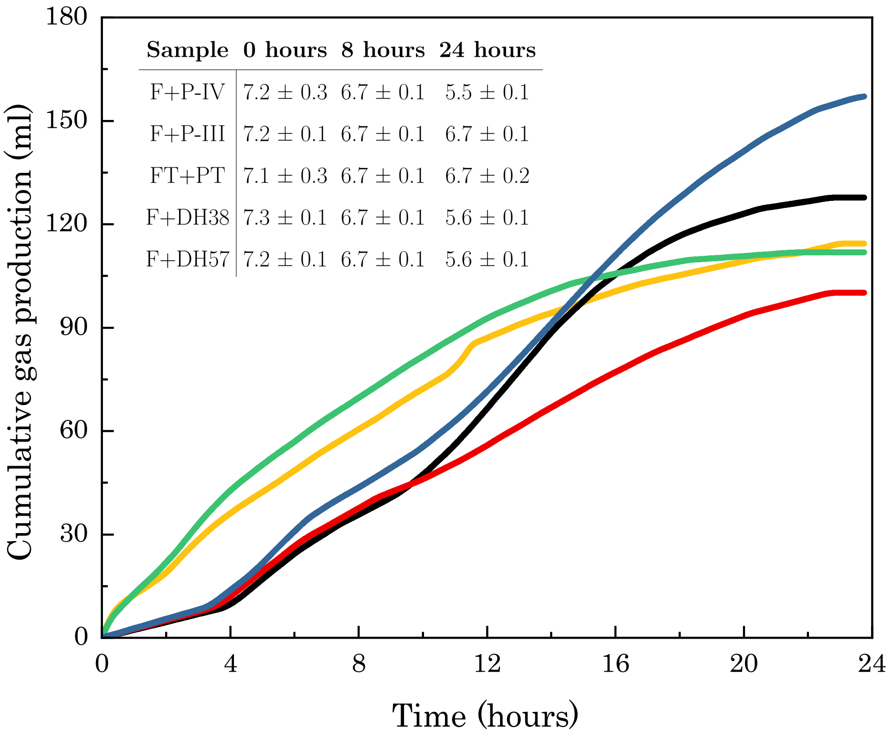
Within the first 8 hours, the pH is only slightly reduced for all samples, which indicated a simultaneous production of metabolites that lower the pH (SCFA and BCFA) and increases the pH (e.g. ammonia). However, at 24 hours there is a difference, where the samples containing P and PT respectively have increased their pH to above 7 while P-IV, DH38 and DH57 is about pH 5.5. Such low pH, especially lower than pure fibre samples, is unexpected as the additional metabolites produced by the protein fermentation is expected to increase the pH [139]. Here, the amount of protein that can be utilised by the bacteria seems to impact the pH but not the gas production. When a lower amount of protein is used, pH reduces at 24 hours, suggesting that there is an elevated production of SCFA and BCFA, but a suppressed production of ammonia. The DH38 sample generates more gas at 24 hours, but the difference is small for all samples. The gas production stagnates around 16 hours for all samples, indicating lower metabolic activity, possibly due to substrate depletion, which is independent of total sample added. An expectation could be that the hydrolysed protein samples would be more quickly fermented as they contain smaller peptides and amino acids that is easier for the bacteria to use, but there is no difference in pH and cumulative gas production is lower for the hydrolysed samples within the first eight hours. However, the gas production for the DH38 and DH57 compared to P-IV are similar, indicating that the difference in gas and pH is due to the microbial community and not the samples.

4.2.3 Protein and fibre blends

A similar trend to the protein samples was observed in the protein and fibre blends (Figure 4.3).

F+P-III and FT+PT did not drastically change pH over 24 hours, only a small reduction compared to zero hours. The F+P-IV, F+DH38 and F+DH57 samples had a steady pH over eight hours, but reduced over 24 hours down to 5.6. The same motivation as for the pure protein samples work for the blended samples, where F+P-IV, F+DH38 and F+DH57 have indications of producing higher levels of SCFA and BCFA, but not as much ammonia. The ratio of fibre to protein is higher in F+P-IV, F+DH38 and F+DH57, which could be an explanation for the reduced pH as a lower pH is expected due to lower protein fraction. It is noticeable that the pure protein samples and the blends when 0.5 grams of protein is used have lower pH than the pure fibre samples (F-IV and FP-I), even when the sample only consists out of 25% protein which is the case for the blends (F+P-IV, F+DH38 and F+DH57). A heightened production of metabolites that lower the pH is therefore produced when protein is present.

The gas production shows low variance between the fibre and protein blend samples, see Figure 4.3. F+DH57 produces the most gas over a 24 hour period, while F+DH38 produces the least of the blends, which is the opposite of what was observed for the pure protein where DH38 produced the most. For the protein and fibre blends, there is also no clear plateau during the 24 hours that was visible in the pure protein sample.



Sample	**0 hours**	**8 hours**	**24 hours**
F+P-IV	7.2 ± 0.3	6.7 ± 0.1	5.5 ± 0.1
F+P-III	7.2 ± 0.1	6.7 ± 0.1	6.7 ± 0.1
FT+PT	7.1 ± 0.3	6.7 ± 0.1	6.7 ± 0.2
F+DH38	7.3 ± 0.1	6.7 ± 0.1	5.6 ± 0.1
F+DH57	7.2 ± 0.1	6.7 ± 0.1	5.6 ± 0.1

Figure 4.3: Cumulative gas production of the blended samples over 24 hours. The samples are F+P-IV (black), F+P-III (yellow), FT+PT (green), F+DH38 (red) and F+DH57 (blue). The table show the measured pH values for each sample at timepoint 0, 8 and 24 hours.

4.3 Metabolite production

The analysed metabolites were the main SCFA (acetate, propionate, butyrate), BCFA (isobutyrate and isovalerate), ammonia, valerate and lactate. The SCFA, BCFA, valerate and lactate were analysed using UHPLC-MS [143, 144] and the ammonia was analysed using a colorimetric method [145].

The fibre samples (F-IV, F-III, FT and FP-I) all had similar metabolic profiles, with acetate, propionate and butyrate contributing to the majority of the SCFA produced, see Table 4.2. Acetate was the most produced SCFA, with 60-65% of total SCFA, while propionate and butyrate produced around 15% of total SCFA each. The amount of acetate and butyrate produced during fermentation of dietary fibre agree well with expectation, while production of propionate is generally higher, but is heavily dependent on substrate [110, 146, 147]. The total amount of SCFA in the F-IV and FP-I sample is about twice the amount compared to the F-III and FT sample, probably due to a different faecal inoculum. The higher levels of SCFA in Control IV compared to Control III can be the explanation for elevated SCFA produced in F-IV and FP-I. There is also a small reduction in total SCFA produced for the FP-I sample comapred to F-IV, but no significant difference. Modifying the pea hull fibre with pectate lyase degrades the pectin and solubilises it, and thus removes it from the insoluble fraction in the modified sample (FP-I). Therefore, a more porous structure and increased viscosity visible in the physico-chemical properties does no increase metabolite production, but might reduce it, most probable due to the removal of pectin. Valerate is a SCFA that is produced to a lesser extent during fermentation, and as with the other SCFA, dependent on the substrate, environmental factors and microbiota. The function of valerate is not as well understood as with acetate, propionate and butyrate, but has been shown to have health effects, such as reduced proinflammatory cytokine production and the ability to regulate barrier functions in epithelial cells [148, 149]. Noticeably, the valerate levels in F-III and FT are 10 to 20 times higher than F-IV and FP-I, which can be explained by difference in microbiota. Consequently, the same trend is observed in Control III, which produces significantly more valerate compared to Control IV at 24 hours. That is a further indication that the difference in valerate stems from variation in microbial community, and not from the samples. It is worth mentioning that the standard deviation for the valerate production is large due to one of the donors producing significantly more than the other donors, *i.e.* due to interpersonal variation (paper III). The BCFA isovalerate also had higher levels in F-III and FT compared to F-IV and FP-I, mainly due to the difference in microbiota. Overall for the fibre samples, there is a trend that metabolic production might be reduced when pea hull fibre is treated with pectate lyase, probably due to the reduction of fermentable pectin.

The metabolites produced when protein was fermented by itself differed, especially between P-IV, DH38 and DH57 compared to P-III and PT, see Table 4.2. The former samples which used a lower total substrate mass of protein (0.5 grams), had lower butyrate levels. The fraction of butyrate produced was also lower in Control-IV compared to Control-III, indicating that the

Table 4.2: Produced SCFA, BCFA and ammonia for all the samples at 24 hours of fermentation. All samples are stated in mmol L^{-1}. Different superscript letters indicate statistically significant differences between the samples at $p<0.05$. n.d - not determined. tr. - < l.o.d.

Sample name	**Acetate**	**Propionate**	**Butyrate**	**Total SCFA**	**Isobutyrate (10^{-3})**	**Isovalerate (10^{-3})**	**Ammonia**	**Valerate (10^{-3})**	**Lactate**
F-IV	29.1 ± 4.7^{ab}	6.3 ± 0.9^{abcd}	9.2 ± 2.4^{ab}	44.6 ± 7.9^{abc}	82.5 ± 14.9^{abc}	170 ± 65.6^{a}	1.4 ± 0.1^{f}	109 ± 53.3^{c}	1.4 ± 0.2^{de}
F-III	11.4 ± 3.2^{bc}	2.9 ± 1.5^{de}	2.4 ± 1.7^{b}	16.6 ± 7.6^{de}	n.d	365 ± 226^{a}	2.9 ± 0.7^{f}	890 ± 592^{c}	tr.
FT	13.0 ± 1.4^{bc}	3.5 ± 0.2^{cde}	3.1 ± 0.5^{b}	19.6 ± 2.4^{cde}	n.d	586 ± 219^{a}	4.9 ± 2.7^{f}	1480 ± 219^{bc}	tr.
FP-I	24.2 ± 1.1^{abc}	5.5 ± 0.4^{abcde}	6.0 ± 0.8^{ab}	35.7 ± 2.3^{abcd}	48.4 ± 14.6^{abc}	85.6 ± 9.6^{a}	n.d	59.9 ± 3.1^{c}	3.1 ± 1.2^{cde}
P-IV	36.1 ± 12.6^{a}	3.3 ± 1.2^{cde}	2.1 ± 0.7^{b}	41.5 ± 12.8^{abcd}	39.0 ± 14.8^{bc}	86.1 ± 26.6^{a}	15.0 ± 4.6^{def}	44.4 ± 29.1^{c}	8.2 ± 2.5^{ab}
P-III	40.2 ± 6.7^{a}	5.3 ± 0.5^{abcde}	9.0 ± 0.9^{ab}	54.5 ± 8.3^{ab}	n.d	1019 ± 769^{a}	58.8 ± 7.1^{ab}	2023 ± 769^{bc}	tr.
PT	28.3 ± 4.4^{ab}	6.6 ± 0.5^{abcd}	9.5 ± 0.5^{ab}	44.4 ± 12.9^{abc}	n.d	896 ± 230^{a}	86.8 ± 25.1^{a}	2066 ± 673^{bc}	tr.
DH38	37.8 ± 7.3^{a}	4.0 ± 0.7^{bcde}	3.1 ± 2.2^{b}	44.9 ± 8.1^{abc}	29.7 ± 6.1^{c}	76.5 ± 14.8^{a}	25.6 ± 8.2^{cdef}	43.2 ± 38.1^{c}	6.5 ± 0.1^{abcd}
DH57	36.1 ± 3.9^{a}	5.2 ± 0.8^{abcde}	2.1 ± 0.6^{b}	43.3 ± 4.1^{abc}	58.1 ± 13.6^{abc}	107 ± 34.8^{a}	24.6 ± 3.9^{cdef}	35.1 ± 9.3^{c}	9.6 ± 2.6^{a}
F+P-IV	36.7 ± 6.3^{a}	7.2 ± 0.4^{abc}	8.7 ± 6.0^{ab}	52.6 ± 2.9^{ab}	51.5 ± 16.4^{abc}	211 ± 25.0^{a}	26.5 ± 4.8^{cdef}	76.3 ± 29.8^{c}	7.0 ± 4.9^{abc}
F+P-III	30.6 ± 6.4^{ab}	7.6 ± 1.1^{ab}	9.0 ± 0.8^{ab}	47.2 ± 9.8^{ab}	n.d	7569 ± 2633^{a}	48.7 ± 23.3^{bc}	9181 ± 2153^{ab}	tr.
FT+PT	30.9 ± 6.6^{ab}	8.7 ± 1.9^{a}	8.6 ± 0.6^{ab}	48.2 ± 9.1^{ab}	n.d	7901 ± 3131^{a}	41.9 ± 34.8^{bcd}	10001 ± 2517^{a}	tr.
F+DH38	37.0 ± 8.7^{a}	8.1 ± 1.5^{a}	9.3 ± 6.6^{ab}	54.4 ± 9.9^{ab}	93.2 ± 43.1^{ab}	226 ± 47.9^{a}	27.4 ± 4.1^{cdef}	111 ± 78.7^{c}	6.9 ± 3.5^{abcd}
F+DH57	42.1 ± 7.2^{a}	8.6 ± 1.9^{a}	13.3 ± 4.1^{a}	64.0 ± 13.1^{a}	99.4 ± 24.9^{a}	209 ± 58.3^{a}	37.1 ± 7.0^{bcde}	141 ± 56.6^{c}	4.2 ± 1.7^{abcde}
Control-III	6.7 ± 0.1^{c}	1.5 ± 0.2^{e}	1.9 ± 0.3^{b}	10.2 ± 1.4^{e}	n.d	1227 ± 89^{a}	4.5 ± 1.0^{ef}	2199 ± 90^{abc}	tr.
Control-IV	26.3 ± 4.4^{abc}	3.5 ± 0.6^{cde}	4.6 ± 0.1^{ab}	34.4 ± 5.0^{bcde}	48.5 ± 14.6^{abc}	85.6 ± 9.6^{a}	1.7 ± 0.0^{ef}	59.9 ± 3.1^{c}	3.1 ± 1.2^{bcde}

lower butyrate concentration is due to microbiota variation. There was also a trend in PT, where the fraction of propionate and butyrate increased after thermal treatment, and less acetate was produced compared to P-III. Thermally treating pea protein alter their viscoelastic properties by swelling, and could thereby be more accessible to the microbiota to ferment. Higher degree of hydrolysis also indicated a larger propionate generation compared to P-IV. A possible explanation for higher propionate levels could be that amino acids and peptides with higher solubility are more easily fermentable compared to unhydrolysed insoluble protein. The rate of peptide hydrolysis is known to be the rate limiting step in protein fermentation, and not the utilisation of amino acids [16]. The free amino acid concentration in DH57 is higher than P-IV, and would thus be fermented quicker. Nonetheless, that trend is not observed in any of the other SCFA.

BCFA produced from protein fermentation was determined, with P-III and PT producing more isovalerate compared to the other protein samples. The elevated isovalerate levels can be connected to the valerate levels in the same samples, and is also visible in Control III versus Control IV. The isobutyrate concentration was not measured for the P-III and PT due to inadequate separation from butyrate, but could be measured for P-IV, DH38 and DH57. There, DH57 produced the highest amount of isobutyrate, followed by P-IV and then DH38. However, there was no statistically significant difference in the isobutyrate concentration between the protein samples.

Differences in ammonia production between the protein samples indicated that modified samples (PT, DH38 and DH57) produced more ammonia than unmodified samples (P-IV and P-III). Once again, a higher solubility could be explanation for a higher ammonia production, but it has also been shown that shorter peptides produces higher ammonia levels than undigested protein [126]. Interestingly, the PT sample is not necessarily expected to have more peptides present, but has been shown to have increased swelling compared to the unmodified protein. A swollen particle could possibly be more accessible for the proteolytic enzymes used by the bacteria. Similar to other metabolites, the ammonia production is dependent on the microbiota, with P-III and PT producing more ammonia than P-IV, DH38 and DH57. Higer concentration in P-III and PT can be correlated to the higher ammonia generation in Control-III compared to Control-IV.

The P-IV, DH38 and DH57 samples also had significantly larger production of lactate, which was not observed in the P-III and PT samples. Different amino acids have been shown to specifically ferment into different SCFA, where the amino acids that are most abundant in pea protein (leucine, lysine, arginine, glutamate and aspartate [128]), mainly produces acetate [127]. Elevated levels of lactate in P-IV, DH38 and DH57 are most probable because of the microbiota, which is shown in Control IV to produce significantly more lactate than Control III. Interestingly, lactate levels of the F-IV and FP-I samples were low, which gives a clear indication that purely dietary fibre does not promote the production of lactate. Increased levels of lactate has been observed in rats fed with a high protein diet [150]. The reduced pH levels that was observed in the P-IV, DH38 and DH57 samples are also promoting lactate accumulation [107]. The valerate levels were higher in the P-III and PT samples, which is expected as the same was observed for the fibres samples, and is mainly attributed to the microbiota used for that study (paper III).

Overall, the protein samples produces more metabolites than the fibre samples, when 1.5 grams of protein is used and similar amount when 0.5 grams is used. Thereby, the microbiota produces more metabolites from pea protein d than the fibre when available. This does not stem from the exceptional fermentative properties of the pea protein, but rather the low fermentability of the pea hull fibre [109, 110]. The pea hull fibre has comparatively low fermentability than other polysaccharides (similar to that of pure cellulose), which gives the perception that pea protein produces a lot of fermentation metabolites. Comparing only the protein samples, trends in the metabolite production after thermal and enzymatic modification are observed. Thermal treatment indicates higher propionate and butyrate fractions, while enzymatic hydrolysis increases propionate production. There is also a trend that the ammonia concentration is higher after either thermal or enzymatic modification. A differences in the metabolite production is also dependent on the microbial community, displaying the importance of interpersonal variation.

In the blended samples, slightly higher levels of acetate was produced in the samples F+P-IV, F+DH38 and F+DH57 compared to F+P-III and FT+PT, but equal amounts of propionate. Butyrate generation was increased for the F+H57 sample, indicating that a higher degree of hydrolysis promotes pathways that metabolises butyrate. The fibre ratio used seemed to have little effect on the metabolites production, where a higher fibre ratio actually produced more total SCFA (F+P-IV, F+DH38 and F+DH57), which does not align when only observing the individual fibre and protein samples. However, the increased SCFA production can be attributed to the difference in microbiota used, and not the fibre to protein ratio, once again highlighting the importance of interpersonal variation. Moreover, the valerate and isovalerate concentrations were significantly higher in F+P-III and FT+PT compared to the other blended samples, but can be connected to the difference in microbiota used for those fermented samples. The presence of fibre increased the fraction of propionate and butyrate in most of the samples, generating a SCFA profile that is more desirable [133], with especially butyrate being correlated to health benefits.

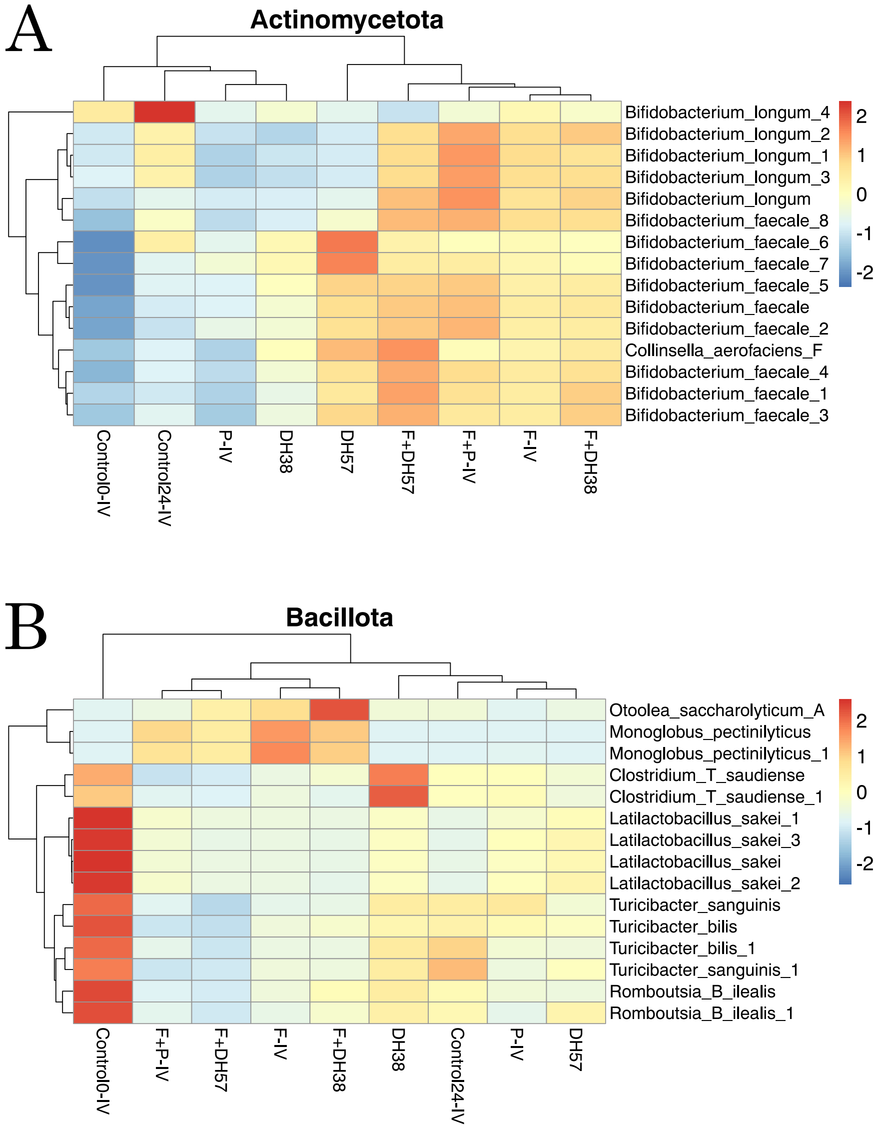
Looking at the BCFA, there is a higher production in all of the blended samples compared to the protein samples, showing that the presence of fibre during proteolytic fermentation matter. The addition of fibre to protein activates cross-feeding pathways that promotes the formation of BCFA. The presence of starch has a small effect on BCFA formation during proteolytic fermentation, but can in some cases increase production [129]. Importantly, the BCFA formation are heavily reduced by decreasing pH, which can play a part in our case where the pH in some of the protein samples are slightly lower than the blended samples. Specifically, the isobutyrate production was higher in the blended samples with hydrolysed protein, compared the unhydrolysed protein, which aligns with was observed for the protein samples. The F+P-IV, F+DH38 and F+DH57 samples also had a production of lactate, however not as prominent as the protein samples. The presence of fibre do suppress the accumulation of lactate, possibly via cross-feeding pathways that generates other metabolites.

The ammonia formation of the blended samples was elevated for the F+P-IV and F+DH57, while it was reduced for the FT+PT sample compared to the protein samples. Ammonia production is expected to be reduced in the presence of carbohydrates, but also by lower pH (similar to BCFA formation), which can give the explanation for the reduction in FT+PT [129]. The increased ammonia production, especially in the F+DH57 sample which produced statistically significant more ammonia than one pure protein samples, is harder to understand. One possible reason could be that microbiota that thrive in the presence of pea hull fibre also has the ability to produce ammonia, while the microbiota present in the pure protein samples instead produce other metabolites, *i.e.* activation of cross-feeding pathways. Another explanation could be that in the presence of dietary fibres that are not that well fermented, accumulation of ammonia can occur. The bacteria want to utilise the ammonia as a primary nitrogen source to grow, but the ammonia is not needed to a large extent because of the low fermentability of the fibre, resulting in higher ammonia concentrations [151, 152]. Ammonia is mainly produced due to deamination of amino acids, either via direct pathways or Sticklad reaction [127], and DH57 and F+DH57 are expected to have the highest amount of free amino acids.

Conclusively, the pea hull fibre does not produce high concentrations of metabolites compared to other dietary fibres, but do have effects when fermented together with pea protein. The the presence of fibre generates higher fractions of propionate and butyrate, but also increases the production of proteolytic fermentation products. Enzymatic modification of pea hull fibre by pectate lyase indicate reduced fermentability. There was a trend that thermally treated pea protein increased the propionate and butyrate fraction compared to untreated protein, while hydrolysis of pea protein increased production of proteolytic metabolites. The SCFA profile was affected by degree of protein hydrolysis in the presence of fibre, highlighting that the modification might have a larger impact when a more complex substrate is used.

4.4 Microbiota

Analysis of the microbiota was performed for the samples F-IV, P-IV, DH38, DH57, F+P-IV, F+DH38, F+DH57 and control after 24 hours (Control24-IV) of fermentation. Control at time 0 (Control0-IV) was also analysed. Sequencing was done by targeting the 16S rRNA gene variable regions 1-8 and then mapped to the Greengenes2 2022.2 database [153]. The four phyla *Actinomycetota*, *Bacillota*, *Bacteroidota* and *Pseudomonadota* represented 99% of all read counts (Figure 4.4). The microbiota composition changes drastically over 24 hours of fermentation for all the samples, even the control sample. The control at zero hours had high abundance from the phylum *Bacillota* and the bacteria *Enterobacter hormaechei*, which both reduced drastically after 24 hours (Figure 4.4.B and D). The samples containing fibre had the highest abundance in the phyla *Actinomycetota* and *Bacteroidota*, revealing that the fibre presence promotes the growth of those bacteria independent of protein (Figure 4.4.A and C). Bacteria from the phylum *Bacteroidota* are diverse and can take part in both carbohydrate



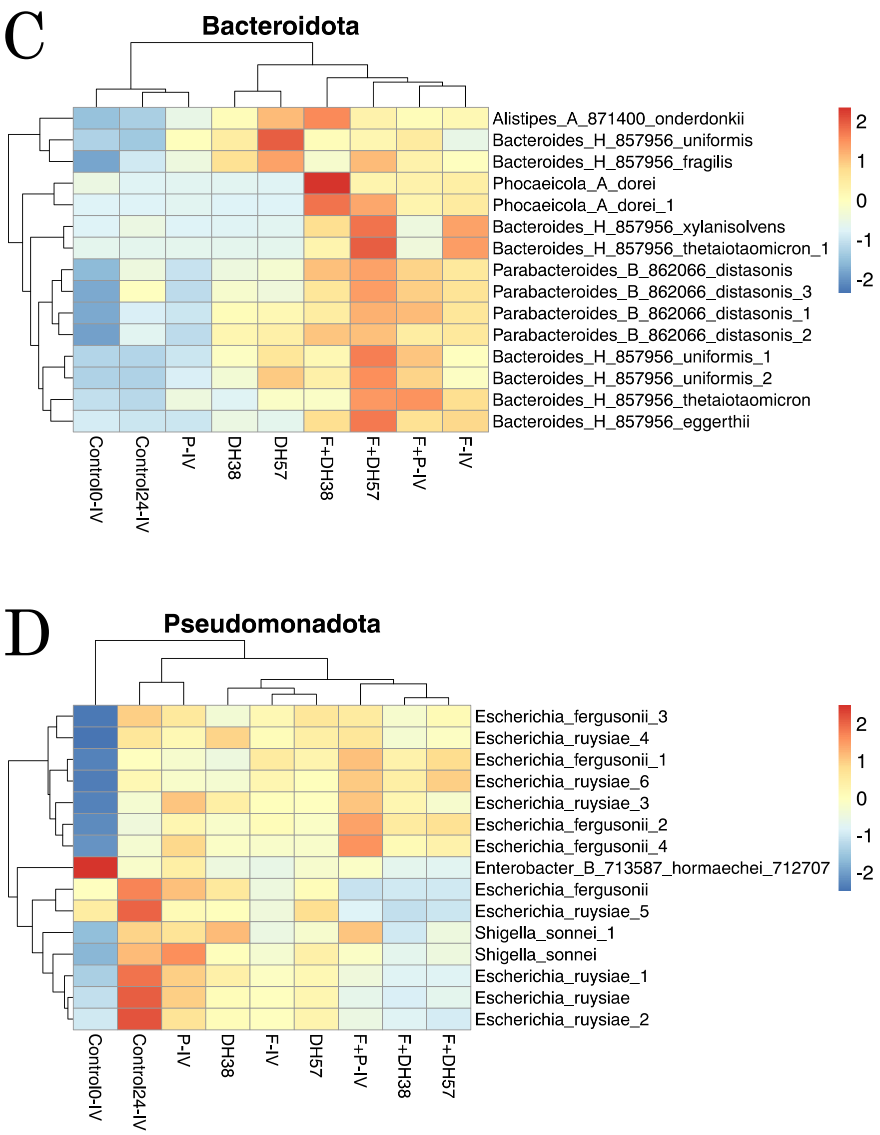


Figure 4.4: Heatmap of the phyla A: *Actionmycetota*, B: *Bacillota*, C: *Bacteroidota* and D: *Pseudomonadota*. The microbial composition for all samples is at 24 hours of fermentation and the control is at time 0 and 24. The top 15 most abundant OTUs for each phyla is shown.

and protein fermentation, but is generally seen as a saccharolytic bacteria [154, 155]. *Bifidobacterium* is the main genus found from the *Actinomycetota* phylum, and is also mainly utilising carbohydrates as an energy source [156]. The protein degree of hydrolysis also affected the mentioned phyla, where a higher DH resulted in a higher abundance. That means that the bacteria in *Bacteroidota* and *Actinomycetota* have the possibility to utilise protein as an energy source, but it depends on the protein structure. A more hydrolysed protein with higher amounts of small peptides and free amino acids is easier for the bacteria to utilise, compared to the unhydrolysed protein. Another trend was observed in the *Bacillota* phylum where the highest abundance were found in the P, DH38 and DH57 samples. Bacteria from the genera *Clostridium*, *Latilactobacillus*, *Turicibacter* and *Romboutsia* were the most numerous, where *Clostridium* and

Romboutsia have been correlated to protein fermentation [157]. However, the fibre containing samples also had high abundance of *Monoglobus pectinlyticus* and *Otoolea saccharolyticum*, which can degrade pectin and hemicelluloses [158, 159]. The pea hull fibre has high amounts of both pectin and hemicelluloses, promoting the growth of those bacteria. The last phylum *Pseudomonadota* had no clear trends but had the highest abundance in control at 24 hours and samples with unhydrolysed protein (P and F+P), see Figure 4.4.D. The two main genera found were *Escherichia* and *Shigella* which are pathogens and acts as pro-inflammatory in the gut [157]. These two genera can ferment protein, but their abundance seems to be reduced with increasing DH. Overall, a protein sample consisting of more free amino acids and small peptides generates a microbial community structure more correlated to fibre fermentation, compared to unhydrolysed protein fermentation. However, the same trend is not observed when analysing the metabolite production of the samples, where the protein samples are all similar independent of protein DH.

The alpha- and beta-diversity of the samples were analysed to determine the microbiota diversity within and between the samples. The Shannon index (alpha-diversity) did not differ significantly between the samples, and ranged between 5.2 to 5.7. The beta-diversity plot shows similarities in microbial community between the samples, see Figure 4.5.A. Samples that contain fibre cluster in the bottom left corner, revealing that if fibre is present, it will impact the community structure. The most interesting message is that differences can be seen in the protein samples depending on protein DH. The same trend was observed in Figure 4.4, showing that a higher DH produces a microbiota more similar to the fibre samples. This gives us insight that the bacteria that utilise fibre as their carbon source also prefers to use free amino acids and smaller peptides, and are different from bacteria that utilises unhydrolysed protein. The unhydrolysed protein sample (and DH38) are more closely related to the control at 24 hours than any other sample, suggesting that the microbiota that grows on less hydrolysed protein are similar to the bacteria during substrate deficiency.

To see if specific bacterial species had any significant effect on individual metabolites production, correlations where investigated using a Spearman's correlation heatmap (Figure 4.5.B) The metabolite that was effected the most by specific bacteria was the ammonia, where species from the genus *Alitiscatomonas* together with *Bacteroides eggerthii*, *Parabacteroides distasonis* and *Phascolarctobacterium faecium* had a significant positive correlation to ammonia production. *Alitiscatomonas* is a known ammonia utiliser but has previously not been correlated to ammonia production [160]. *Bacteroides eggerthii* and *Parabacteroides distasonis* are both part of the *Bacteroidota* phylum and have high abundance in the F+DH57 sample, which also had the largest ammonia concentration. The two bacterial species could possibly utilise the protein to produce ammonia, as some bacteria from the *Bacteroidota* can undergo proteolytic fermentation. The asaccharolytic species *Phascolarctobacterium faecium* uses mainly succinate as carbon source, and is not linked to increased ammonia production [161]. However, the pH in the protein containing samples were low (<5.5), and production of

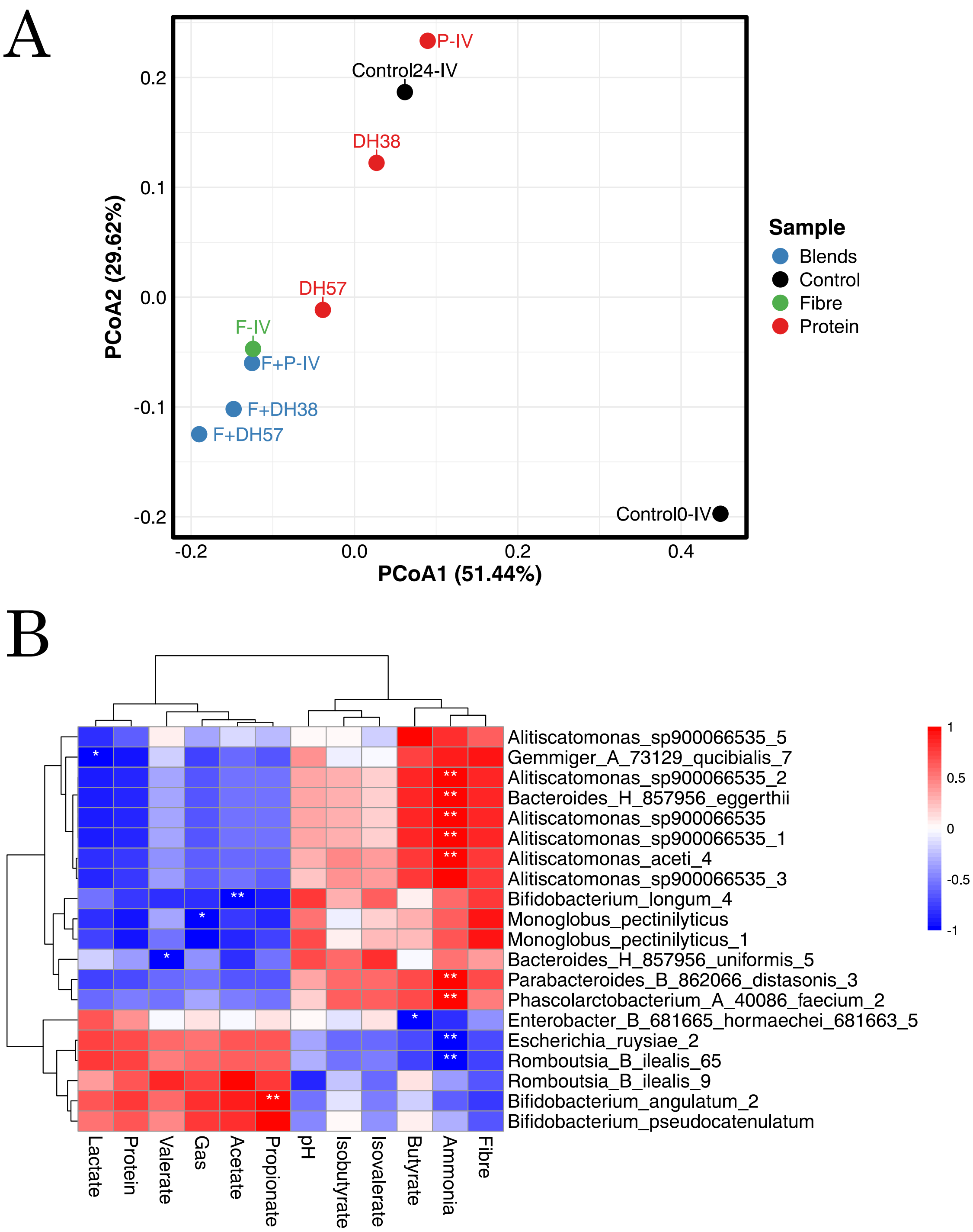


Figure 4.5: A: Beta-diversity among the microbial communities visualised as a PCoA plot using Bray-Curtis distance with the percentage of total variation on each axis. B: Top 20 OTUs with the lowest q-value and metabolites correlated using a Spearmans's correlation heatmap. The stars shows levels of significance where * is q<0.015 and ** is q<0.001. q-values were calculated using Benjamini-Hochberg FDR correction to account for false positives. Correlation is demonstrated by Spearman's rho value from -1 to 1, where -1 is negatively correlated (blue) and 1 is positively correlated (red).

ammonia during acidic conditions can be used to survive the environment by increasing the pH, as observed for *Escherichia coli* [162]. The two bacteria *Escherichia ruysiae* and *Rombousia ilealis* have a strong negative correlation with ammonia, most probably due to their low abundance in the F+DH57 sample. Positive correlation to propionate production was found with *Bifidobacterium angulatum*, which genus has been observed to generate acetate and lactate [133]. Interestingly, a bacteria from the same genus, *Bifidobacterium longum* display strong negative correlation with acetate accumulation. Two bacterial species from the same genus exhibiting different attributes suggest that utilisation of carbohydrates and protein are species dependent, and complicates making conclusions on a phylum or genus level. *Monoglobus pectinilyticus* had a negative correlation with gas production, mainly due to the fibre samples generating small amounts of gas. High abundance of the species *Enterobacter hormaechei* resulted in low amounts of butyrate. *Enterobacter hormaechei* is a known protein fermenter, and were numerous in the protein samples, which produced similar or less butyrate than when the fibre was present. Lastly, the two bacteria *Gemmiger qucibialis* and *Bacteroides uniformis* was negatively correlated to lactate and valerate production, respectively.

There were clear trends in the microbial community structure based on protein DH, however the same trends could not be observed in the metabolite production. This reveals that even if the microbiota is different, similar metabolites in terms of SCFA, BCFA and ammonia can be produced during proteolytic fermentation. Furthermore, the microbiota was affected by fibre presence during fermentation, but proteolytic fermentation products (BCFA and ammonia) were not suppressed. The inclusion of carbohydrates is therefore not the full explanation for protein fermentation suppression. However, other toxic metabolites such as indoles, phenols and *p*-cresol coming from proteolytic fermentation was not analysed, and it is possible that the production of those metabolites could be affected by both protein DH and the presence of fibre. Furthermore, accumulation of metabolites occur because batch cultures were used, where there is no influx of substrate or efflux of produced metabolites. Using batch cultures generates an artificial environment where substrate depletion happens over time, which also activates specific cross-feeding pathways that might not occur *in vivo*.

Chapter 5

Concluding remarks

The overall aim of this thesis was to evaluate the effect of modifications to pea hull fibre and pea protein on their physico-chemical properties and *in vitro* colonic fermentation. The first objective focused on thermal and enzymatic treatment of pea hull fibres, and how modifications in physico-chemical properties could be related to possible changes in *in vitro* colonic fermentation. Physical and compositional properties was altered using thermal and enzymatic treatment. Those treatments induced changes in the insoluble and solubilised fraction compositions, specifically solubilising the pectic fraction after treatment. Enzymatic modification with pectate lyase had the largest effect on composition, which also resulted in differences in physical properties and cell wall structure. Degradation and swelling of the particles caused by enzymatic degradation using pectate lyase slightly improved the viscosity of pea hull fibre suspensions. Consequently, these particle suspensions exhibited soft particle behaviour and the experimental data could be fit using models by Mendoza and Leverrier to predict rheological behaviour [83, 84]. Nonetheless, the pea hull fibres still displayed low fermentatbility, and induced changes in physico-chemical properties by treatment did not effect *in vitro* colonic fermentation metabolite production.

The second objective was to investigate the effects of pH and heating on pea protein physico-chemical properties. It was observed that swelling and protein-protein interaction could be adjusted by changes in pH, but resulted in similar patterns during thermal gelation. The outcome of heating and degree of hydrolysis on pea protein was also explored, showing that reduction in molar mass and increased solubility of the protein was successfully achieved by proteases. Protein hydrolysis caused minor shifts in the metabolite production and differences in the microbiota during *in vitro* colonic fermentation. When utilising protein with a higher degree of hydrolysis, resemblance of the microbial community was closer to that of fibre than to unhydrolysed protein.

The third objective was to explore how addition of pea hull fibre to pea protein affects physico-chemical properties of the blend. The results revealed limited physical interactions between the fibre and the protein. However, rheological properties are affected by the addition of fibre, by an additive effect.

That is, the viscosity of the blend is similar to the sum of the viscosity of the individual pea fibre and pea protein. The outcome in *in vitro* colonic fermentation showed when fibre is present during fermentation, it generally acts as the preferred substrate. The effect on fermentation varied depending on modification, where some proteolytic fermentation products increased in the presence of fibre. The microbiota was dominated by the fibre if present, independent of protein modifications. However, the same could not be observed in metabolite production, showing that the metabolic profile is not fully controlled by the microbial community.

Conclusively, modifications of pea hull fibres by thermal and enzymatic treatment resulted in changed viscosity, but no difference in the *in vitro* colonic fermentation as evaluated by metabolite production. Physico-chemcical properties of pea protein could be modified by heating and enzymatic treatment, which can affect the metabolic and microbial response during *in vitro* colonic fermentation.

5.1 Outlook

Rheological changes in pea hull fibre show that modifications are able to affect physico-chemical properties. However, larger differences in composition, particle size and rheological properties could be obtained by other treatments or combinations of them. Sequential treatment by heating and then enzymatic degradation could be an option, but also the use of steam explosion.

Fermentation of individually fractionated peptides and protein subunits could reveal microbiota preference, and consecutively the health effect in terms of metabolite production. Expanding the current thesis to other protein sources or analysing biomarkers during clinical studies would also be of interest. The combined fermentation of dietary protein and fibre showed that the microbiota and metabolite production is complex. Varying the fibre type to include a more fermentable fibre could induce other trends in the fermentation outcome.

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