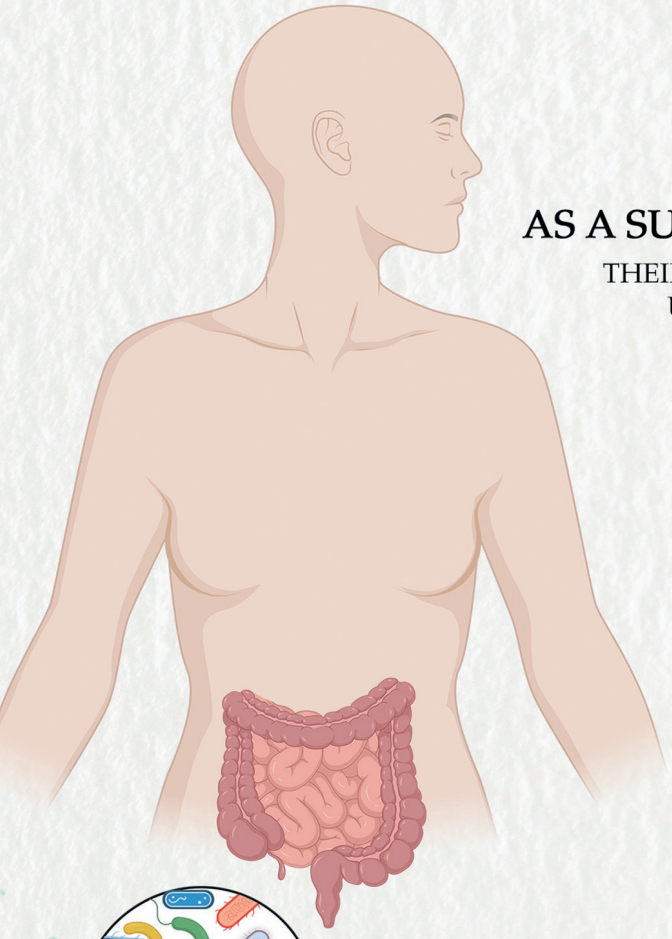
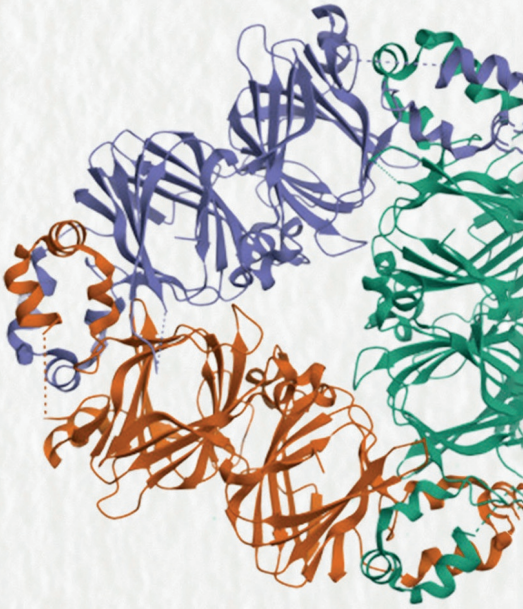


PULSE PROTEINS AS A SUSTAINABLE ALTERNATIVE:

THEIR DIGESTIBILITY AND THE ROLE OF THE UNDIGESTED FRACTION IN THE COLON



RUTH TAKYIWAAH BOACHIE

Propositions

1. Protein structure at the point of gastrointestinal hydrolysis is the main determinant of protein nutritional quality.
   (this thesis)

2. Undigested proteins in the colon are as relevant for health as the absorbed fraction.
   (this thesis)

3. Fundamental science is more suitable at the advanced educational level than the basic level.

4. Rules of scientific writing confines creative interpretation of the scientist.

5. Sustainability is viewed differently in developed and developing countries.

6. Adaptability is more important than determination.

Propositions belonging to the thesis, entitled

Pulse proteins as a sustainable alternative: their digestibility and the role of the undigested fraction in the colon

Ruth Takyiwaah Boachie
Wageningen, 12 September 2023

Pulse proteins as a sustainable alternative: their digestibility and the role of the undigested fraction in the colon

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Pulse proteins as a sustainable alternative: their digestibility and the role of the undigested fraction in the colon

Ruth Takyiwaah Boachie

Thesis

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by the authority of the Rector Magnificus,
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
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Chapter 1

General Introduction

1.1 Shift from animal proteins to sustainable alternatives

The global population is increasing rapidly, and this rapid growth has created a demand for an increase in global food production. Thus, global food production has increased significantly to meet the increasing demand, and the environmental impact has been detrimental [1]. About 40% of the world's land constitutes farmlands and pasture and has become a major source of depletion of freshwater resources [2]. Additionally, a quarter of greenhouse emissions are products of agricultural activities [3]. Therefore, while increasing food production, sustainable food production methods are needed to reduce the detrimental consequences on the environment. Plant-based diets have been recommended due to the minimal impact and their beneficial role in enhancing biodiversity.

Additionally, the enormous global population growth has instigated an increase in global protein demand to meet nutrition security needs. Changes in dietary patterns, driven globally by urbanization have also contributed to the increased protein demand [4,5]. The emerging changes in protein consumption patterns in most developing countries are due to 'westernization' and improving economic conditions. Current consumption trends in developed countries show a gradual transition towards plant proteins mainly due to ongoing awareness of the environmental impact of animal proteins, animal welfare concerns [6], and the perception that they are healthy [7].

Current dietary patterns involve consumption of high caloric, fat, and sugary foods, which are typically highly processed. As a result, an increase in prevalence of micronutrient deficiency, obesity, and related metabolic conditions has been recorded [8]. Transformation to more healthy choices that are nutrient dense is needed. According to the FAO, over 50% of the global supply of food proteins are plant-based, followed by meat, dairy, and fish and seafood (Figure 1.1) [8,9]. However, regionally, plant proteins, such as pulses, are more commonly consumed in Sub-Saharan Africa, because they are less expensive and more accessible. In western countries, cereal proteins from wheat are commonly consumed as bread, whereas rice and millet are commonly consumed in developing countries [4].

1.2 Sustainable protein alternatives

Diversifying the sources of proteins to meet the current high demand for proteins to feed the global population is more sustainable than expanding animal protein

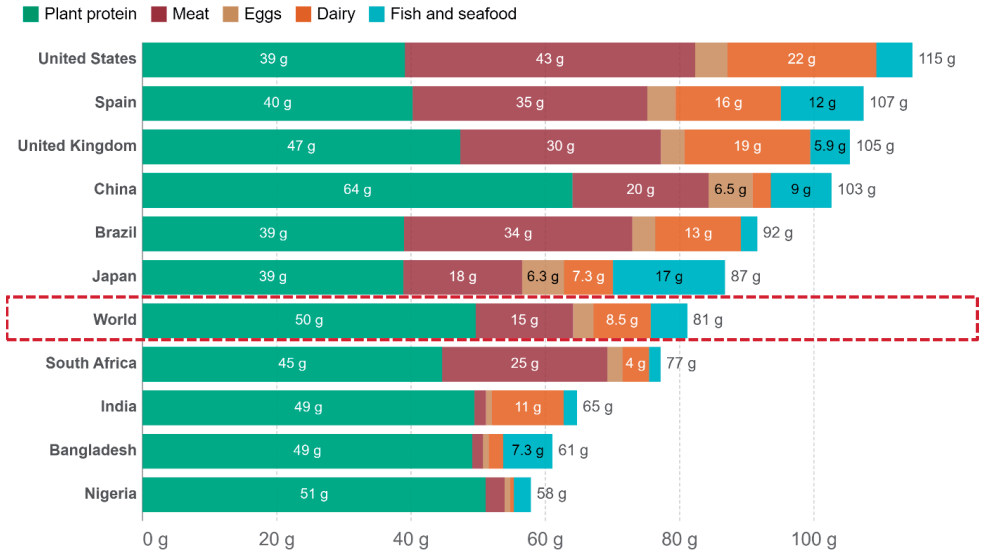

Figure 1.1: Per capita protein sources in 2019, as reported by the FAO [9]

production [1]. Current accepted alternative sources of food proteins include insects, algae, legumes and pulses, cereals and pseudo-cereals, oilseeds, and tubers [6]. These alternative proteins can exist as storage proteins, as in pulses or metabolic proteins as in algae. However, protein contents of most plant-based alternatives are lower than those of the traditional animal source. For instance, the protein content of legumes and pulses is between 16 – 30% [10], and even less for cereals and potatoes. Production of these alternatives entirely for their proteins rather aggravates the ongoing environmental crisis. Therefore, considering the low protein yield, recovery of proteins from side streams of product processing of these alternatives or further use of protein extraction by-products is recommended [6].

In considering the sustainability of a protein alternative, the trade-off between the environmental impact and the benefit of these alternatives must be considered [11]. The relatively low quality of plant proteins coupled with their low protein yield means that high production levels are needed to meet protein demands. Increased production and the related environmental impact can outweigh the benefit of these alternatives. Therefore, protein quality and yield should be considered as significant factors in determining sustainability of protein alternatives. Weindl et al suggests that a variety of low-quality protein alternatives can be used complementarily in diets [11]. The alternatives with low

digestibility can be used complementarily to high quality protein alternatives or to moderate amounts from animal sources.

1.2.1 Pulse proteins as a more sustainable food protein

Pulses comprise the edible dry seeds of the Leguminosae family, which include *Cicer arietinum* (chickpeas), *Vigna subterranea* (Bambara beans), *Vicia faba* (Faba or broad beans), *Phaseolus vulgaris* (common beans), *Vigna unguiculata* (cowpeas), *Pisum sativum* (peas*), Cajanus cajan* (pigeon peas), *Lens culinaris* (lentils), *Lupinus sp.* (lupins), and *Vicia sativa* (vetches) [12]. Among these, common beans, cowpeas, chickpeas, lentils, and peas are commonly consumed globally [12,13]. Pulses are the second most consumed food group globally [14].

Pulses contain about 40-70% starch, 20-29% protein, 12-14% dietary fiber, and 1-2% fat [15]. Their protein fractions, however, differ. For example, lentil proteins consist of 70% globulins, 16% albumins, 11% glutelin, and 3% prolamins [16], whereas pea proteins consist of 55-80% globulins and 18-25% albumin with minute levels of glutelin and prolamins [17]. Globulins, being the predominant proteins, contain substantial amounts of arginine, aspartic acid, glutamine, and lysine hence the reported high lysine content of pulses [18]. Therefore, pulses are largely consumed as staples along with cereals to meet protein needs, mostly in developing countries where access to animal proteins is limited. High lysine content in pulses complement the high methionine, tryptophan, and cystine content of cereals to enhance the amino acid composition of the diet [18].

Agricultural activities generally have detrimental effects on the environment; however, growing pulses is beneficial because they produce less greenhouse gases and require less water and agrochemical use. Pulses also sequester carbon and thus reduce atmospheric carbon dioxide whereas animal production rather increases it [19]. Additionally, several pulses are resilient to climate change. For instance, pigeon peas are drought resistant. Beyond that, pulses contribute to biodiversity due to their ability to fix nitrogen into the soil with symbiotic bacteria such as Rhizobium. They can also release phosphorus that is bound to the soil. These activities collectively improve soil fertility, enhance soil microbial biomass, promote soil nutrient availability, and enhance soil biodiversity [20].

1.2.2 Techno-functionality of sustainable protein alternatives

As we explore more sustainable sources of food proteins, it is important to also consider the techno-functionality of these alternative proteins in food product applications, either as ingredients or additives. Fundamental structural differences between animal and plant proteins limit expected techno-functional

properties. Food proteins have foaming, gelling, emulsifying, and stabilizing abilities; therefore, they can be used to either create or stabilize the structure of food products [6,21]. The dense globular structure of the principal proteins in pulses and their high molecular weight pose a challenge to these functional properties [22,23]. For example, their low solubility makes their use in food products challenging. Solubility of globulins depends on the hydrophobicity of the protein surface, pH, ionic strength, and molecular weight [10]. The solubility of proteins is key in several functional properties, such as emulsification, foaming, water absorption, and gelling capacity [22,24]. The gelling ability of proteins also influences their water retention, fat binding, emulsifying, and foaming abilities and determines their viscosity and adhesiveness [6,21].

Plant proteins have intense cross-linkages and tend to aggregate either in the native state or after extraction [25], hence physical, chemical, and/or biochemical modifications are needed to improve their functionality. Globulins and albumins can form gels after modification processes that enhance structural unfolding needed in the three-dimensional network of protein-solvent and protein-protein interactions in gels [26]. Although challenging, globulins can form fibrous structures to mimic tissues in food applications [6].

Physical treatments, such as heating, can denature the proteins and expose the hydrophobic residues that are within the core of the protein structure. Pulse proteins can be made more hydrophilic by changing the pH and net charge or forming conjugates with hydrophilic compounds, such as sugars and polysaccharides. Glycation of proteins during Maillard reaction has proven effective in improving the functional properties of plant proteins, since the amino group side chains of the proteins bind to the reactive sides of the sugars to produce hydrophilic products [24,27,28]. However, advanced glycation involves covalent interactions which can cause protein cross-linkages and reduce digestibility of the protein [29].

1.3 Fate of proteins along the gastrointestinal tract (GIT)

The GIT is exposed to exogenous and endogenous proteins daily. The exogenous sources are dietary proteins whereas the endogenous sources include cell turnover from mucosal epithelium, gastrointestinal secretions, or even plasma proteins [30,31]. About 100 g and 35 g of dietary and endogenous proteins are presented in the GIT daily [31,32]. Digestion of proteins begins in the stomach where parietal cells in the gastric lining and the gastric chief cells secrete hydro-

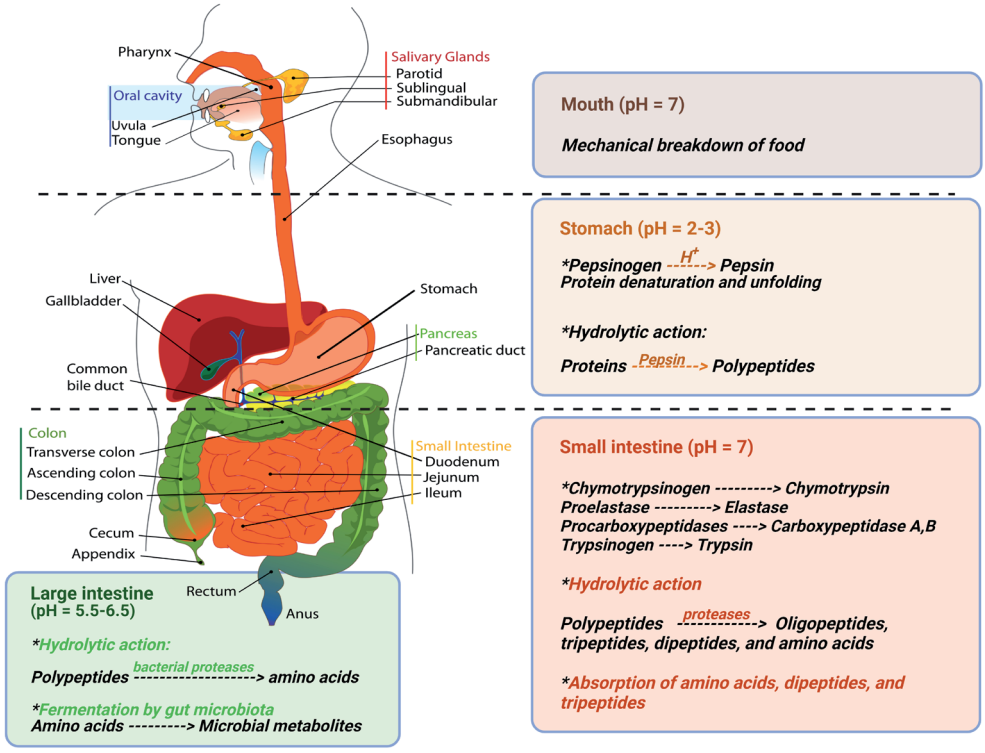

Figure 1.2: Fate of dietary proteins along the gut [39]

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chloric acid and pepsinogen, respectively (Figure 1.2). The resulting acidic condition (pH 2-3) activates pepsinogen into pepsin through autocatalysis [33]. The low pH also disrupts the hydrogen bonds and electrostatic forces holding the native structure of the protein, alters their structure, and facilitates unfolding of intact proteins or aggregates. Pepsin is an endopeptidase and preferentially cleaves the carboxylic group of aromatic residues to produce a mixture of polypeptides, oligopeptides, and rarely, a few amino acids. With the help of mechanical contractions and peristaltic movement, pepsin mixes with the proteins and cleaves about 15% of the peptide bonds [34].

The chyme moves to the duodenum where pepsin is deactivated due to the neutral pH of the small intestine (Figure 1.2). The polypeptides are extensively hydrolyzed by elastase, chymotrypsin, trypsin, and carboxypeptidases A and B [35]. These enzymes are secreted in the inactive form by the acinar cells of the pancreas. Their activation is triggered by enterokinase which is secreted by the intestinal cells in duodenum and jejunum [30]. Elastase, chymotrypsin, and trypsin

are endopeptidases. Specifically, trypsin cleaves the carboxyl end of basic amino acids, chymotrypsin cleaves carbonyl groups of aromatic residues, and elastase cleaves carboxyl groups on small hydrophobic amino acids. Carboxypeptidases A and B hydrolyze single amino acids residues from the carboxyl terminal [35]. These enzymes act on the chyme within the lumen to produce oligopeptides, tripeptides, dipeptides, and amino acids. The peptides and amino acids produced are actively transported from the brush border into the enterocytes. The brush border membrane has several peptidases that further hydrolyze peptide bonds. The amino acids, dipeptides, and tripeptides are transported passively into the portal blood through the basolateral membrane or actively transported with the help of carrier-mediated transport systems [31]. Partially digested hydrolysates and undigested proteins (endogenous and exogenous) move to the colon (Figure 1.2).

About 12-18 g of partially digested dietary protein hydrolysates and endogenous proteins are transported to the colon daily depending on protein intake and protein digestibility [36]. The digestibility of dietary proteins can be modified by structural change in the native protein. These structural changes can be caused by extrinsic factors such as processing temperature and pH [37]. Residual proteins reaching the colon can be hydrolyzed by the microbiota to produce metabolites to support their growth requirements and influence host's metabolism [38].

1.4 Factors influencing protein digestibility

Protein digestibility is a measure of the protein’s susceptibility to hydrolysis and the absorbable fraction released [40]. Intrinsic factors including primary sequence, structural folding, and cross-linkages along with extrinsic factors such as temperature, pH, ionic conditions, and presence of other molecules in the food can influence protein digestibility [41,42]. Good sources of plant proteins require processing to reduce endogenous protease inhibitors and antinutritional factors, such as phytic acid tannins, to make them safe for consumption. Given that healthy dietary choices should include diverse nutrients, proteins can co-exist with other food components, including dietary fiber, phenolic compounds, and reducing sugars. These food components can interact with the proteins because proteins possess amino acid residues with reactive functional groups in their side chains. These include α-amino, α-carbonyl, hydroxyl, imidazole, and sulfhydryl groups [43]. The processing conditions can also facilitate interactions among components within the food matrix.

Their interaction can result in complexes that alter the protein's susceptibility to the human digestive enzymes and either enhance their bioactivity or hinder them [44]. Moreover, proteins can interact with other endogenous biomolecules as they move along the GIT. These factors evoke interest in the following: (1) How do food processing methods alter the native structure and consequently affect the digestibility and bioactivity of the proteins? (2) How do proteins interact with other food biomolecules in the food matrix and how does this interaction impact their digestibility? The effect of food processing and the presence of other food components are further discussed in the following sections.

1.4.1 Effect of food processing on protein digestibility

Various processing methods may be applied to enhance shelf life or safety of food for consumption. These processing methods include concentration/extraction, enzyme modifications, thermal, and pressure processing. Thermal processing is commonly used and known to alter the native structure of proteins and alter their susceptibility to proteases (Figure 1.3). During thermal processing, intermolecular collision increases and disrupts intermolecular forces that are maintaining the secondary and tertiary structure of the protein [45,46]. Hydro-

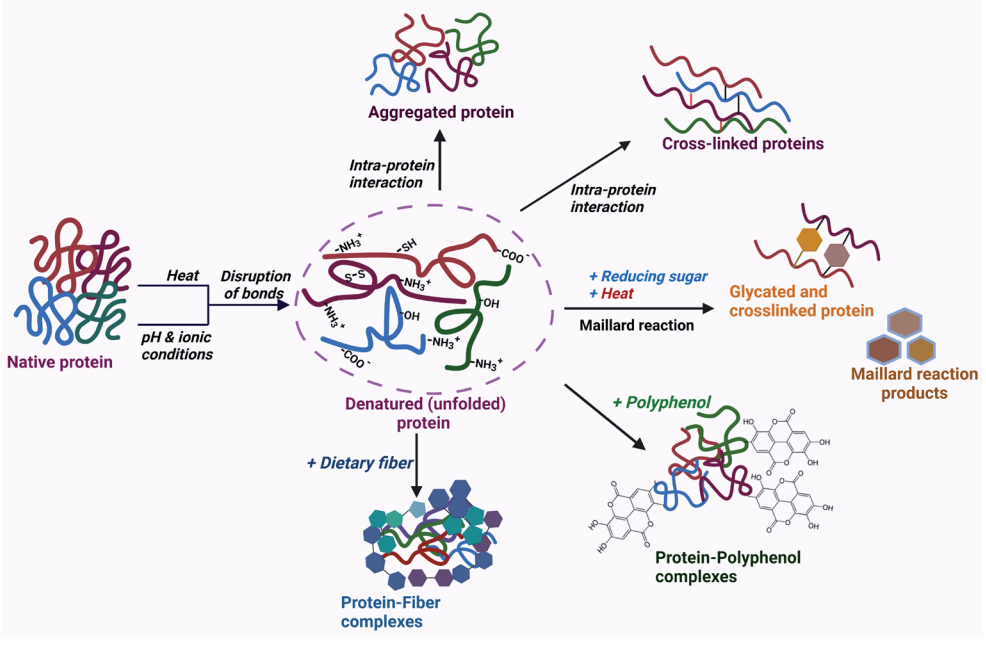

Figure 1.3: Possible changes in tertiary structure of native proteins and the products formed during food processing.

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gen bonds, ionic bonds, and electrostatic interactions can be broken when proteins are heated above their denaturation temperature and cause the protein to unfold and expose the reactive groups that were in the core of protein structure. As shown in Figure 1.3, the unfolded protein can aggregate, form cross-linkages, or the exposed reactive groups can interact with other components in the food matrix as observed in Maillard reaction [45].

Maillard reaction commonly occurs in foods that are thermally processed. It involves covalent bonding between the carbonyl group of a reducing sugar and α-amino group at the N-terminal of residue amino acids, that is ε-NH_2 of Lys, or the guanidino-NH_2 of Arg. Reversible condensation between the carbonyl group from the reducing sugar and free amino group, which is the Schiff base reaction, initiates the early stage and produce N-substituted glycosylamine as intermediates. Amadori rearrangement occurs from the N-substituted glycosylamine to produce N-substituted 1-amino-1-deoxyketoses due to their susceptibility to heat in the advanced stage. The final stage includes several structural modifications, including cyclization, dehydration, and elimination [22,47]. This change may influence organoleptic properties, shelf life, physicochemical, and textural properties, but more importantly alter digestibility. Digestibility can be reduced when structural modifications like cross-linkages cause steric hindrance and limit access of the digestive proteases to cleavage sites. The cleavage sites can also be modified during the condensation reaction and become less susceptible to the digestive enzymes [48–50].

The effect of temperature is dependent on the native structure of the proteins and as such the susceptibility of different proteins to temperature changes differ. Proteins with high amounts of Ile, Leu, Val, and Phe are less susceptible to thermal denaturation than hydrophilic amino acids [51]. Additionally, processing time and water activity are also relevant in protein denaturation and subsequent crosslinking interactions, yet temperature-time factor is more significant [46].

1.4.2 Effect of Interactions with other food components on protein digestibility

The food matrix comprises the nutritive and non-nutritive components of the food, the physical organization of these components, and their intermolecular interaction [52,53]. The major intermolecular forces include hydrogen bonds, electrostatic interactions, hydrophobic forces, and covalent disulfide bonds [54]. The structure and physical state of the food matrix affect digestion and determine the amino acids released for absorption [53,55]. In addition, the food matrix structure influences the gastric emptying and transit time. For instance,

when food matrix structure is solid and contains firm tissues, they take longer to move along the GIT due to a slower digestion rate. Processing methods used can cause structural modification to the food matrix and increase the ability of proteins to interact with other proteins, carbohydrates, and secondary plant metabolites due to their reactive side chains and functional groups (Figure 1.3). The interactions between food proteins and other components in a food matrix depends on the molecular size, pH, hydrophobicity, amino acid residues, and structure of the protein [56].

Proteins interact with other proteins through aggregation or even phase separation, depending on the net forces of the proteins involved [54,57]. Electrostatic attraction among proteins due to the opposite surface charge can result in aggregation of these proteins. When the concentration of the more reactive protein is low, phase separation can occur. pH is key in these interactions. Proteins aggregate at the isoelectric point, however, at other pH, the side chains form both intramolecular and intermolecular complexes [58].

In addition to proteins, carbohydrates and phenolics commonly exist in plant-based foods. These components also have functional groups that can interact with proteins. The nature of their interactions is discussed in the following subsections.

Protein-Polysaccharide Interaction

Proteins and polysaccharides can interact through covalent bonds, or non-covalent bonds including hydrogen bonding, hydrophobic forces, Van der Waals forces, and electrostatic bonds. The interaction is dependent on pH, ionic strength, surface charges, concentration, and biopolymer ratio [59]. Protein-polysaccharide interactions are mostly electrostatic due to the charges on their backbones, except in the case of Maillard reaction where a reducing sugar residue of a polysaccharide can covalently bond to α-amino group of the protein as discussed in the previous section. Protein-polysaccharide complexes can coacervate and form soluble mixtures [60]. The complexes can also be insoluble and evenly dispersed or form separate phases of the biopolymers [61].

Dietary fibers are frequently used in several food products for their gelling, viscosifying, and bulking properties or are added to fortify food products [62]. β-glucans have recently received considerable attention due to their solubility in water and high viscosity which imparts them with hypocholesterolemic and hypoglycemic abilities [63,64]. Barley, oats, bacteria, algae, yeast, and mushrooms are good sources of β-glucans [64]. The structure and molecular weight

of β-glucan vary depending on the source [65]. β-glucans from cereals are linear homopolysaccharides, consisting of β-(1→4)-linked D-gluco-pyranosyl units (oligomeric cellulose segments). The β-(1→4)-linked units are separated by β-(1→3)-linked cellotriosyl and cellotetraosyl units [66]. The molar ratio of these units determines the molecule's functional properties, such as water solubility, viscosity, and gelation ability.

Proteins contain charged groups that can easily react with the negatively charged groups of β-glucans through electrostatic, hydrogen, or hydrophobic interactions, yet electrostatic forces dominate. The interaction is dependent on pH, ionic strength, surface charges, concentration, and biopolymer ratio [59]. Due to their gelling ability, β-glucan can increase the viscosity of the food and form viscous solutions in the gut [67]. In the gut, increase in viscosity delays gastric emptying and increases transit time in the intestinal tract. The increased viscosity also limits access of digestive enzymes due to inadequate mixing [68]. Therefore, the interaction of β-glucan with proteins can limit protease action and protein digestibility, yet studies on the nature of interaction between pulse proteins and dietary fibers are limited.

Protein-Polyphenol Interactions

Secondary plant metabolites, such as phenolic compounds, occur commonly in plant food sources. They are widely consumed due to diverse associated health benefits including their antioxidant activities [69]. Protein-phenolic compound interactions can occur through covalent bonds or non-covalent bonds, including hydrogen, ionic, and hydrophobic bonds [70].

The hydroxyl group of the phenolic compounds is the main hydrogen donor to the protein carbonyl groups but oxidized phenolic compounds can also be involved [62,70]. They form hydrogen bonds with the carbonyl, hydroxyl, or amine group of the protein but hydrophobic interactions among the non-polar groups can also occur. The nature of the interaction depends on the molecular weight and levels of hydroxylation, methylation, hydrogenation and glycosylation of the phenolic compounds and the molecular weight, net charge, and conformation of the protein [70,71].

Physical conditions, such as pH and temperature, affect the interaction between phenolics and proteins. For example, high temperatures cause protein unfolding to expose hydrophobic groups that might be buried in the core of the protein structure therefore favoring hydrophobic interactions with phenolics [72]. Simi-

larly, at low pH, protein binding sites are exposed because ionic forces holding the native structure of the protein are disrupted, causing the protein to unfold.

Protein-polyphenol complexes can be soluble or insoluble [69]. Formation of these complexes can enhance functionality of the proteins, such as the gelling ability [73]. On the other hand, it can cause stearic hindrance and limit the digestive enzymes' access to the protein or modify the cleavage site and consequently reduce the protein's susceptibility to the digestive enzymes, and thus reduce their digestibility. Residual and unbound polyphenols can also bind the enzymes and inhibit their hydrolytic ability [74]. The unbound residues can also stabilize the enzyme structure, thus, cause better fitting of the protein on the enzyme activation site [75]. However, the inhibitory effect of protein-polyphenol complexes on protein digestion is more common [74,76,77]. Based on the factors discussed, it is relevant to understand structural modifications that occur upon pulse proteins interaction with polyphenolic compounds and how these changes influence protein digestibility.

1.5 Role of food proteins/peptides beyond their nutritional role

Food proteins have bioactive properties that go beyond their role as macronutrients involved in energy production and body building. Previous studies have shown that specific peptide sequences exhibit bioactivity when released from native proteins. Although native proteins show some level of bioactivity, these activities are higher in their hydrolysates [78]. These bioactivities are mostly observed after exogenous or endogenous protease hydrolysis or from microbial fermentation [79]. Diverse types of peptides can be released from proteins, depending on the conditions used for hydrolysis. These conditions include enzyme specificity, pH used for hydrolysis, enzyme-substrate ratio, temperature, and time. Therefore, bioactivity of the hydrolysates depends primarily on the amino acid sequence, which in turn determines molecular weight, structure and spatial conformation, hydrophobicity, and surface charge [80,81].

Reported bioactivities include cholesterol-lowering, antioxidant, hypotensive, antimicrobial, and hypoglycemic abilities. These properties imply that bioactive peptides can be used in the management of chronic diseases, hence providing an alternative to synthetic pharmaceutical products. Bioactive peptides can reduce risk of metabolic disorders by regulating digestive enzymes, nutrient absorption in the GIT, and gut hormones [82–84]. The bioactivity of peptides in the gut relat-

ing to metabolism of the gut microbiota and gut hormones are discussed in the sub-sections below.

1.5.1 Modulation of gut microbiota and their metabolites by peptides

The gut microbiota is ubiquitously distributed along the intestinal tract but highly colonizes the large intestine and is mainly inhabited by Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, and Verrucomicrobia [85]. In the large intestine, they ferment undigested or partially digested food components that are transported to the colon. When undigested proteins reach the colon, they are further hydrolyzed by the mix of endogenous microbial proteases available from several members of the gut microbiota including *Bacteroides, Fusobacterium, Clostridium, and Lactobacillus* [86]. Additionally, residual proteases from the small intestine might contribute to proteolysis in the colon. The amino acids produced can either be incorporated in the bacterial cell for protein biosynthesis or catabolized into metabolites such as short chain fatty acids (SCFAs), branched chain fatty acids, ammonia, hydrogen sulfide, carbon dioxide,

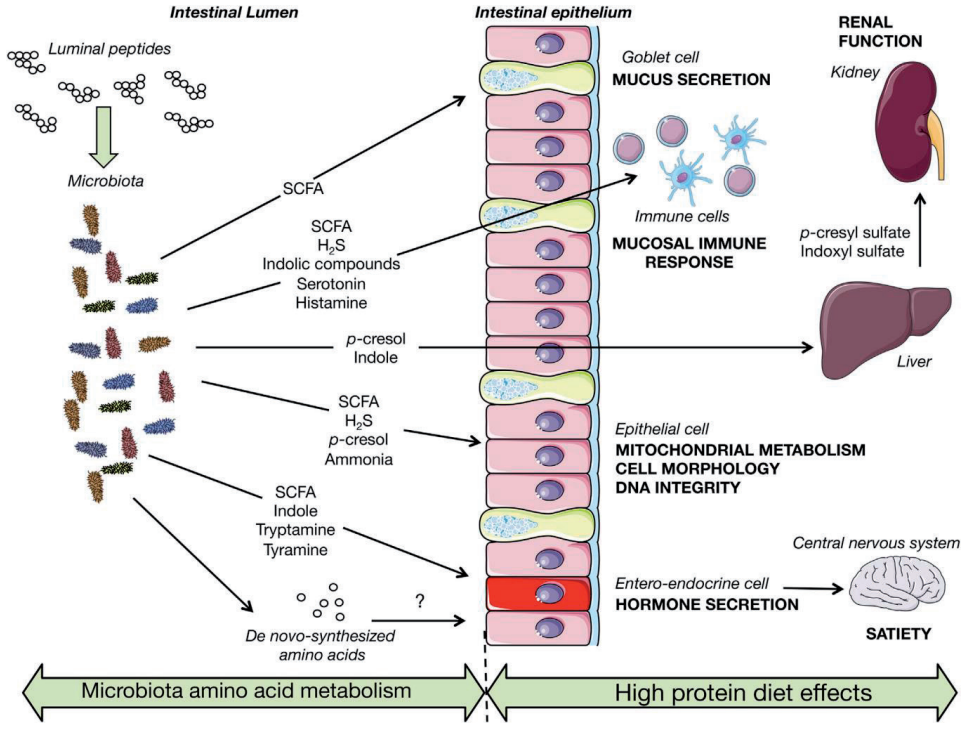

Figure 1.4: Role of gut microbiota fermentation of peptide fractions in the colon on host's health.

Figure was adapted from Portune et al [38]

phenols, and organic acids via deamination, decarboxylation, and transamination (Figure 1.4) [38]. The gut microbiota uses different catabolic pathways, and different bacterial species use different amino acid substrates [87]. Microbial fermentation of proteins depends on the proportion of available carbohydrate present concurrently with proteins [88]. Beyond the type of substrate present in the diet, transit time, and resulting pH condition due to substrate fermentation might influence the gut microbiota [38]. Several studies have reported the ability of large peptides to modulate the microbiota metabolism depending on the source and concentration [89–92].

Once absorbed by the colon cells, the metabolites can also be distributed through the systemic circulation to organs and tissues far from the gut and influence host's health by producing neuroactive compounds like neurotransmitters [38], improving mucosal barrier functions, controlling inflammatory parameters [93], or influencing energy metabolism of the host, depending on the growth phase of the microbiota (Figure 1.4) [38,94]. For instance, SCFAs act as ligands for G protein-coupled receptors in L-cells located in the jejunum, ileum, and colon [95].

Findings concerning the role of gut microbiota in host energy metabolism suggest an interplay between gut-brain signaling and microbiota-host interactions [38]. This sparks interest in understanding the interaction between the gut microbiota and protein hydrolysates in the colon, and the influence of this interaction on microbial metabolism.

1.5.2 Satiating effect through the gut hormones

Gut hormones, such as Cholecystokinin (CCK) and GLP-1 are secreted in the small intestine where they can regulate production of digestive secretions in the stomach, pancreas, and gallbladder; hence, their ability to regulate digestion [96,97]. CCK delays gastric emptying to halt further food intake or functions indirectly through luminal CCK-releasing factor. GLP-1 enhances satiety, and thus reduces food intake. Additionally, GLP-1 stimulates insulin release from the β-cells of the pancreas when blood glucose levels increase [98].

The enterocytes in the small intestine secrete these gut hormones when secretory cells are exposed to nutrients released from digestion. Hence, they peak postprandially, and their secretions are differentially stimulated by the type of nutrients produced [99]. The secretion of these gut hormones is determined by location of secretory cells and rate of exposure to the nutrients post-digestion. CCK is mostly secreted by enteroendocrine I cells in the duodenum, whereas GLP-1 is secreted by L-cells in the jejunum and ileum upon amino acid and oligopeptide

stimuli [99]. CCK and GLP-1 secretion are mainly stimulated by amino acids and oligopeptides through proton-coupled peptide transporters and Na^+-coupled amino acid transporters in the apical membrane of entero-endocrinal cells [99]. Secretion of gut hormones in the distal gut is reported to be stimulated by SCFA produced by the gut microbiota rather than nutrient-sensing as observed in the upper gut [100].

Several animal proteins have been extensively studied and have shown the ability to stimulate secretion of CCK and GLP-1 and inhibit dipeptidyl peptidase-IV (DPP-IV) activity [82,84,101]. DPP-IV inhibition prevents the cleavage and inactivation of GLP-1, which reduces the risk of hyperglycemia and consequently Type 2 Diabetes [102–104]. Additionally, animal proteins are involved in glucose metabolism by enhancing insulin secretion and consequently reducing postprandial blood glucose levels [105,106]. Although pea proteins and their hydrolysates have been reported to reduce risk of metabolic disorders by stimulating production of gut hormones such as CCK in the small intestines [107,108], data on other pulse proteins is scarce. Additionally, considering the low digestibility of these pulse proteins, significant amount of partially digested pulse proteins is transported to the colon, yet little is known about the ability of large peptides reaching the distal gut to directly stimulate the production of these gut hormones.

1.6 Aim of thesis

Healthy dietary choices include consumption of plant proteins due to their bioactivity beyond their role as a macronutrient. However, these proteins usually exist in complex food matrices where other components like dietary fiber, phenolic compounds, and reducing sugars can interact with them. The interaction between plant proteins such as pulse proteins and other biomolecules including dietary fiber and phenolic compounds can result in structural modifications that reduce their susceptibility to human digestive enzymes. Therefore, this thesis aimed to investigate how these interactions impact digestibility of the proteins (Figure 1.5). In **Chapter 2**, we investigated the influence of β-glucan interaction on the particle characteristics and *in vitro* digestibility of proteins isolated from lentil and yellow pea seeds. In **Chapter 3**, we evaluated the effect of tannic acid interaction on particle characteristics, structure, thermal stability, peptide profile, and in vitro peptic digestibility of lentil proteins. Lentil and yellow pea proteins were selected as model plant proteins because these pulses are commonly consumed, and their proteins are gaining attention for use in food applications.

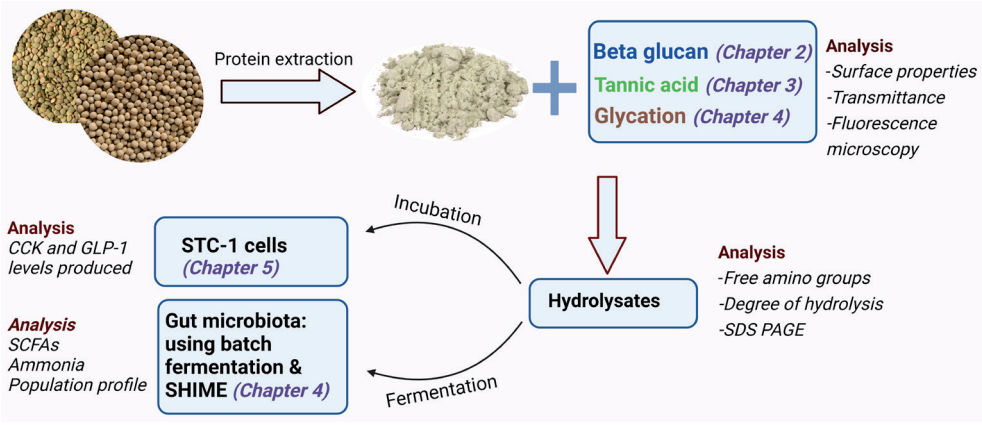

Figure 1.5: General overview of the thesis

(Hydrolysates used in Chapter 4 and 5 were separately prepared from in vitro digestion of protein concentrates).
Figure was created in BioRender.com

After digestion, residual proteins and partially hydrolyzed polypeptides move to the colon. In the colon, these hydrolysates can interact with the enteroendocrine cells and the microbiota. The digestibility of proteins can be affected by irreversible structural modifications that occur in commonly occurring processes such as Maillard reaction. To investigate this, in **Chapter 4** we assessed how glycated lentil protein hydrolysates that reach the colon affect the metabolites and population of the gut microbiota using batch fermentation and the Simulator of Human Intestinal Microbiome Ecosystem (SHIME®). Several studies have reported that presence of food proteins and peptides in the small intestines stimulate secretion of satiety hormones such as CCK and GLP-1. However, it is unclear if the presence of hydrolysates in the colon leads to a similar outcome. Hence, **Chapter 5** explored the effect lentil protein hydrolysates have on CCK and GLP-1 secretion in intestinal endocrine cells, using Secretin Tumor Cell line (STC-1). **Chapter 6** summarizes the findings and discusses the relevance of the above-mentioned studies. The limitations and future directions are also discussed.

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2

Chapter 2

β-Glucan Interaction with Lentil (*Lens culinaris*) and Yellow Pea (*Pisum sativum*) Proteins suppresses their *In vitro* Digestibility

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Abstract

Interaction between food proteins and food matrix biomolecules can affect protein digestibility. In this study, barley β-glucan interaction with lentil and yellow pea proteins, and the effect on the *in vitro* digestibility of the proteins under simulated static gastrointestinal conditions was investigated. The proteins were mixed with β-glucan at mass ratios of 1:0.5, 1:1, and 1:2, and subjected to simulated *in vitro* digestion. The interaction between β-glucan and the isolated proteins was demonstrated by the decrease in transmittance, surface charge and increase in particle size of the complexes. Brightfield microscopy showed the formation of aggregates between the biopolymers, although increased molecular size was not observed by discontinuous native polyacrylamide gel electrophoresis. Fluorescence microscopy of the mixtures indicated that β-glucan formed aggregates with lentil proteins while the interaction with yellow pea proteins appeared as distinct phases of protein within the β-glucan network. The *in vitro* protein digestibility of lentil and pea protein decreased by 27.3% and 34.5% in presence of β-glucan mass ratio 1:2, respectively. The findings confirm the possibility to modulate protein digestibility by changing the physical characteristics of a food matrix.

Keywords: yellow pea protein, lentil protein, β-glucan, protein-fiber interaction, protein digestibility, simulated gastrointestinal digestion, fluorescence microscopy

2.1 Introduction

There is enormous evidence to support health benefits of food proteins beyond nutrition, yet animal source proteins have traditionally been used for these studies. Also, considering the need for more sustainable food protein sources, legume proteins have emerged as an acceptable alternative. Legumes contain 18-32% protein, making them a good and sustainable source of plant proteins. Specifically, protein contents of yellow pea (*Pisum sativum)* and lentil (*Lens culinaris*) seeds range from 20-30% and 20.6-31.4%, respectively.[1,2] Lentil protein consists of 70% globulins, 16% albumins, 11% glutenins and 3% prolamins.[3] Pea proteins mainly consists of 55-80% globulins and 18-25% albumin with minute levels of glutenins and prolamins.[2,4] Despite the substantial protein content, the digestibility of legume proteins is hindered by naturally occurring cell walls, protease inhibitors and the interaction between the proteins and other biomolecules in the food matrix.[5] Proteins and polysaccharides are commonly used in food formulations due to their functional properties. Hence, there is a high possibility of polysaccharides such as β-glucans to co-exist with legume proteins in a food matrix. Interaction between these biopolymers either during processing or along the gastrointestinal tract can influence their physicochemical properties and in turn modify their functional properties, and bioactivity.[6] The structural conformation, charge, and ionic strength are important factors that determine the nature of interpolymer interaction. For example, an electrostatic interaction may occur between a negatively charged group of the polysaccharide and the protein's cationic groups. Thus, making pH, temperature, biopolymer ratio and concentrations relevant conditions in the type of complexes formed whether soluble, insoluble, or existing as distinct phases in solution[7,8]

β-Glucans from cereals are linear homopolysaccharides that largely consist of β-(1→4)-linked D-gluco-pyranosyl units separated by β-(1→3)-linked cellotriosyl and cellotetraosyl units.[9] The molar ratio of these units determines the physical properties of β-glucans, such as water solubility, viscosity, and gelation ability. The presence of β-Glucan like other soluble dietary fibres can cause phase separation of digesta, as well as increase the viscosity of the digesta due to its gel-forming capacity.[10] This phenomenon can physically hinder distribution of digestive enzymes due to inadequate mixing with substrates.[11,12] Consequently, co-existence of β-Glucan and legume proteins in a food matrix can influence protein digestion. Moreover, the varying pH and ionic conditions of the gastrointestinal tract can produce a modulatory effect on the structure and behaviour of β-glucans, legume proteins, and the possible interaction that can occur between both biomolecules.[12] Zielke et al. reported that at low pH, ß-glucan can form

aggregates with gliadin and whey proteins possibly through electrostatic interactions since the proteins are positively charged at pH below their isoelectric point.[13] In addition, the principal proteins in legumes, globulins can dissociate reversibly or irreversibly with changes in pH and ionic conditions. Increase in ionic strength can also result in aggregation of globulins via disulphide bonds, consequently reducing solubility.[4]

Thus, it is important to understand the structural behaviour of legume proteins in a matrix containing other food biopolymers. Therefore, the objective of this study was to investigate the influence of β-glucan interaction on the particle characteristics and *in vitro* digestibility of proteins isolated from lentil and yellow pea seeds using the INFOGEST static digestion method. This study approach elucidates interaction between β-glucan and legume proteins, and the effect of gastrointestinal conditions on the *in vitro* protein digestibility.

2.2 Materials and Methods

2.2.1 Materials:

Dry lentil and yellow pea seeds were donated by Pulse Canada (Winnipeg, Manitoba, Canada). β-Glucan (94% purity, high viscosity, molecular weight 251 kDa) derived from barley was purchased from Megazyme (Bray, Ireland). α-Amylase (from porcine pancreas, type VI-B, ≥5 units/mg solid), pancreatin (from porcine pancreas, 8xUSP specification), pepsin (from porcine gastric mucosa, ≥250 units/mg solid), and bile extract (from porcine) were purchased from Millipore Sigma (Burlington, MA, USA). NativeMark™ protein standard and GelCode™ blue safe protein stain were obtained from Fisher Scientific (Toronto, ON, Canada). Fast Green FCF (dye content ≥85%) and Calcofluor White (Calcofluor White M2R, 1 g/L and Evans Blue, 0.5 g/L) were purchased from Millipore Sigma (St. Louis, MO, USA).

2.2.2 Protein isolation:

Protein was extracted from green lentil and yellow pea seeds. First, seeds were soaked overnight in water (seed to water, 1:3 w/v) at room temperature. Thereafter, the seeds were crushed with a food processor (Waring commercial blender, Torrington, USA) and freeze-dried. The freeze-dried seeds were milled into a flour with a food processor. The flour was suspended in 0.05 M NaOH (10%, w/v) and pH was adjusted to 10. The suspension was stirred for 4 h and then centrifuged for 30 min at 7000 g and 4 °C to separate the insoluble components. The pH of the resulting supernatant was adjusted to 4.0 using 3 M HCl and further stirred

for 2 h to precipitate the proteins. The residue was recovered by centrifugation at 7000 g and 4 °C for 30 min and resuspended in deionized water afterwards. The pellet was neutralised by adjusting the pH to 7.0 using 3 M NaOH solution. Finally, the extracted proteins from yellow pea seeds (PPI) and lentil seeds (LPI) were freeze-dried to obtain the protein powders, which were stored at -20 °C for further analysis.

2.2.3 Preparation of protein-β-glucan mixtures

Protein and β-glucan stock solutions were prepared separately. The isolated lentil and yellow pea proteins were dissolved in Milli-Q water at a mass concentration of 1% (w/v), and the resulting solution was stirred. Afterwards, the protein solutions were centrifuged at 7000 g and 25 °C for 5 min to remove insoluble particles. A solution of the β-glucan in Milli-Q water was prepared at a mass concentration of 2% (w/v) and heated at 85 °C for 1 h under continuous stirring as previously reported.[14] Mixtures were prepared using protein:β-glucan mass concentration ratios of 1:0.5, 1:1, and 1:2.[13] Protein (1:0) solution was used as control. The resulting solutions contained 5 mg/mL of either lentil or yellow pea protein isolates, and 2.5, 5, or 10 mg/mL β-glucan depending on the mass ratio. The mixtures were stirred for 2 h at room temperature prior to further analysis.

2.2.4 Determination of protein-β-glucan interaction:

The interaction between LPI or PPI and β-glucan was determined by measuring the absorbance as previously reported by Zielke et al.[13] The absorbance of the protein-β-glucan mixtures and the control at time 0 min and 120 min of incubation at room temperature was measured in a multimode microplate reader (Tecan, Switzerland) at 590 nm. The transmittance of the mixtures was compared to the control to identify changes resulting from addition of β-glucan.

2.2.3 Particle characterization by dynamic light scattering:

The average particle size, ζ-potential, and polydispersity index (PDI) of the protein-β-glucan mixtures and the controls were determined by inserting 1 mL of the mixtures in a capillary cell. Measurements were performed in Milli-Q water (pH 7, refractive index 1.330, viscosity 0.8872 cP 192 and dielectric constant 78.5) at 25 °C after equilibrating for 120 s, using the Zetasizer Nano ZS with non-invasive backscatter optics (Malvern Instruments Ltd., UK).

2.2.4 Simulated in vitro gastrointestinal digestion.

The simulated *in vitro* gastrointestinal digestion was performed by modifying the COST INFOGEST three-phase static method.[15] Briefly, 0.5 mL of the protein and β-glucan aqueous solutions were mixed with 350 µL of simulated salivary

fluid (SSF), 0.25 µL of α-amylase (1500 U/mL), 2.5 µL of $CaCl_2$, and 97.5 µL water. The 950.5 µL solution was incubated for 2 min at 37 °C. The oral bolus was mixed with 750 µL of simulated gastric fluid (SGF), 0.8 µL porcine pepsin (25000 U/mL) and 0.5 µL of $CaCl_2$ (0.3 M). The pH of the 1.79 mL digesta was adjusted to 3.0 using 1 M HCl and the digesta subsequently incubated for 2 h with constant shaking (90 rpm). The gastric chyme was mixed with 1.1 mL of simulated intestinal fluid (SIF), 2.5 µL of pancreatin (800 U/mL based on trypsin activity), 12.5 µL of bile extract (10 mM), and 4 µL of $CaCl_2$. Next, 131 µL of water was added to reach a final volume of 3.06 mL. The pH was readjusted to 7.0 using 1M NaOH. The intestinal phase digestion was conducted for 2 h with continuous shaking (90 rpm). Afterwards, enzymatic activities were halted by vortex mixing and transfer of the samples into a freezer (-20 °C).

2.2.5 Degree of hydrolysis determination:

The degree of hydrolysis were determined after the intestinal phase by measuring the amount of free amino groups by the o-phthaldialdehyde (OPA) method.[16] In a microplate, 30 µL of serine standard, blank (Milli-Q water) and sample solutions were mixed with 225 µL of the OPA reagent. The microplate was incubated while shaking for 2 min at room temperature, before reading the absorbance at 340 nm using a microplate reader (Tecan, Switzerland). The degree of hydrolysis were calculated as reported by Nielsen et al.[16]

2.2.6 Discontinuous native polyacrylamide gel electrophoresis (PAGE):

The discontinuous native PAGE (Ornstein-Davis) was performed according to the Mini-PROTEAN® Tetra cell instruction manual (Bio-Rad, USA). A gel with 6%, 9%, 12%, 15%, and 18% polyacrylamide gradients was cast with a 3% (w/v) stacking gel. Two hundred µL of the digesta and undigested mixtures were mixed with 400 µL of sample buffer (containing 1.25 mL of 0.5 M Tris-HCl pH 6.8, 3mL of glycerol, and 0.2 mL of 0.5% bromophenol blue in 5.55 mL deionized water). Thereafter, 15 µL of the sample solutions or 5 µL of the NativeMark™ protein standard was loaded onto separate wells of the gel. Electrophoresis was conducted at 120 V for 90 min in a BioRad Mini-PROTEAN Tetra Cell electrophoresis unit (Bio-Rad, USA). The gel was stained overnight in 100 mL GelCode™ blue safe protein and rinsed three times with Milli-Q water. The gel was then de-stained and imaged with a ChemiDoc MP Imaging System (Bio-Rad Inc., Canada).

2.2.7 Bright field and fluorescence microscopy:

The interaction between the proteins and β-glucan was observed using bright-field and fluorescence microscopy. Samples were observed with bright field microscopy (Axio Vert A1, Carl Zeiss, Germany) as previously reported.[17] For fluo-

rescence microscopy, proteins and β-glucan controls were stained separately. The proteins were stained with Fast Green FCF whereas β-glucan was stained with Calcofluor White. The protein-β-glucan mixtures were then stained with a mixture of both dyes, which consisted of 0.1% w/v Fast Green FCF solution and 0.05% v/v Calcofluor White at a ratio of 1:1 (v/v). The sample mixture (500 µL) was gently mixed with 50 µL of the mixed dye as previously reported.[18] The stained samples were observed on a slide under UV light using the DAPI channel with excitation/emission wavelengths of 353/465 nm for Calcofluor White and the Cy5 channel at 650/673 nm for Fast Green FCF, using the Axio Imager 2 microscope equipped with an Axiocam 506 camera (Carl Zeiss, Germany). The Zen 2.3 pro software (Carl Zeiss, Germany) was used to process the images. Relative fluorescence intensities of β-glucan and protein were quantified from aggregates present in the stained mixtures using ImageJ software (NIH, Bethesda, MD).

2.2.8 Statistical analysis:

Results were reported as mean ± standard deviation of triplicate experiments. One-way analysis of variance with the post-hoc Tukey test was used to compare significant differences between means at a confidence interval of 95% ($P < 0.05$). Unpaired two-tailed t-test was used for analysis of the fluorescence image quantification data.

2.3 Results and Discussion

2.3.1 Protein-β-glucan interaction:

In this study, a protein-β-glucan model system was set up to study how the interaction of β-glucan with lentil or yellow pea protein can affect particle characteristics of the protein, and the *in vitro* digestibility. Protein/β-glucan was mixed at mass ratios of 1:0.5, 1:1, and 1:2 and stirred for 2 h. While β-glucan exists in lower concentrations in foods, the selected ratios are relevant in understanding the mechanism of protein/β-glucan interaction in food product formulations and along the gastrointestinal tract. Lentil or yellow pea protein-β-glucan interaction was evaluated by measuring the amount of light transmitted by the mixtures. Absorption in the visible spectrum has been used to evaluate biopolymer interactions that cause agglomeration or aggregation.[11,13] Insoluble complexes formed from biopolymer interaction can increase the amount of light absorbed by the mixture hence reducing the amount of light transmitted.

Although a dose-dependent change was not observed in the current study, percent transmittance significantly reduced over 2 h in mixtures with the highest

β-glucan concentration. For lentil protein/β-glucan, there was a 14.78% *($p < 0.05$)* absolute decrease at the highest β-glucan concentration. The most significant absolute decrease of 22.72% (*$p < 0.05$)* was observed for PPBG 1:2 mixture after 2 h, as shown in Figure 2.1A&B. Decrease in transmittance indicates the formation of aggregates or agglomerates between the biopolymers. Thus, there is no apparent agglomeration or aggregation occurring in LPBG 1:0.5, 1:1, and PPBG 1:0.5 mixtures based on transmittance. At pH greater than the isoelectric point, the net charge of the protein moves towards a negative net charge. Therefore, the near neutral pH of the mixtures in this study favored electrostatic repulsion between the proteins and the β-glucan.

Zielke et al.[13] observed a decrease in transmittance upon the formation of insoluble complexes from whey protein/β-glucan ratios of 1:0.5, 1:1, and 1:2. Although pH 3-9 was used in the study, the observed change occurred at pH 3-4. No changes were observed beyond pH 4. However, in the gliadin/β-glucan mixtures, a decrease in transmittance was only observed in the 1:2 mixture at pH 3. This finding suggests that protein/β-glucan aggregation goes beyond pH and isoelectric point of the protein but also depends on the type of protein involved. Furthermore, a time-dependent change in turbidity previously reported for whey protein/β-glucan mixtures was attributed to phase separation of the biopolymers. The increase in turbidity was observed in mixtures with high molecular weight and concentrations of β-glucan.[11]

2.3.2 Particle characteristics of the protein/β-glucan mixtures:

Increase in the amount of β-glucan resulted in larger particle sizes, with a more substantial increase observed for the 1:2 mixtures (Figure 2.1C&D). There was a six-fold increase in the mean particle size for LPI/β-glucan 1:2 and 1.5-fold increase for PPI/β-glucan 1:2 when compared to the mean particle size of the respective proteins. The LPI control had a mean particle size of 281.93 ± 2.37 nm while PPI control had 267.20 ± 5.67 nm. Particle sizes of the LPI and PPI controls were similar to the values previously reported for pea proteins.[19,20] Dynamic light scattering technique measures the biomolecular size as the average hydrodynamic size of aggregates.[19] Therefore, increase in particle size in the mixtures as the concentration of β-glucan increased could be attributed to the aggregation between the proteins and β-glucan. It was also observed that although transmittance suggested less aggregation in LPI/β-glucan 1:2 compared to PPI/β-glucan 1:2, the size distribution suggested otherwise. This could be because of the relatively higher sensitivity of DLS in detecting the aggregated species in LPI/β-glucan.

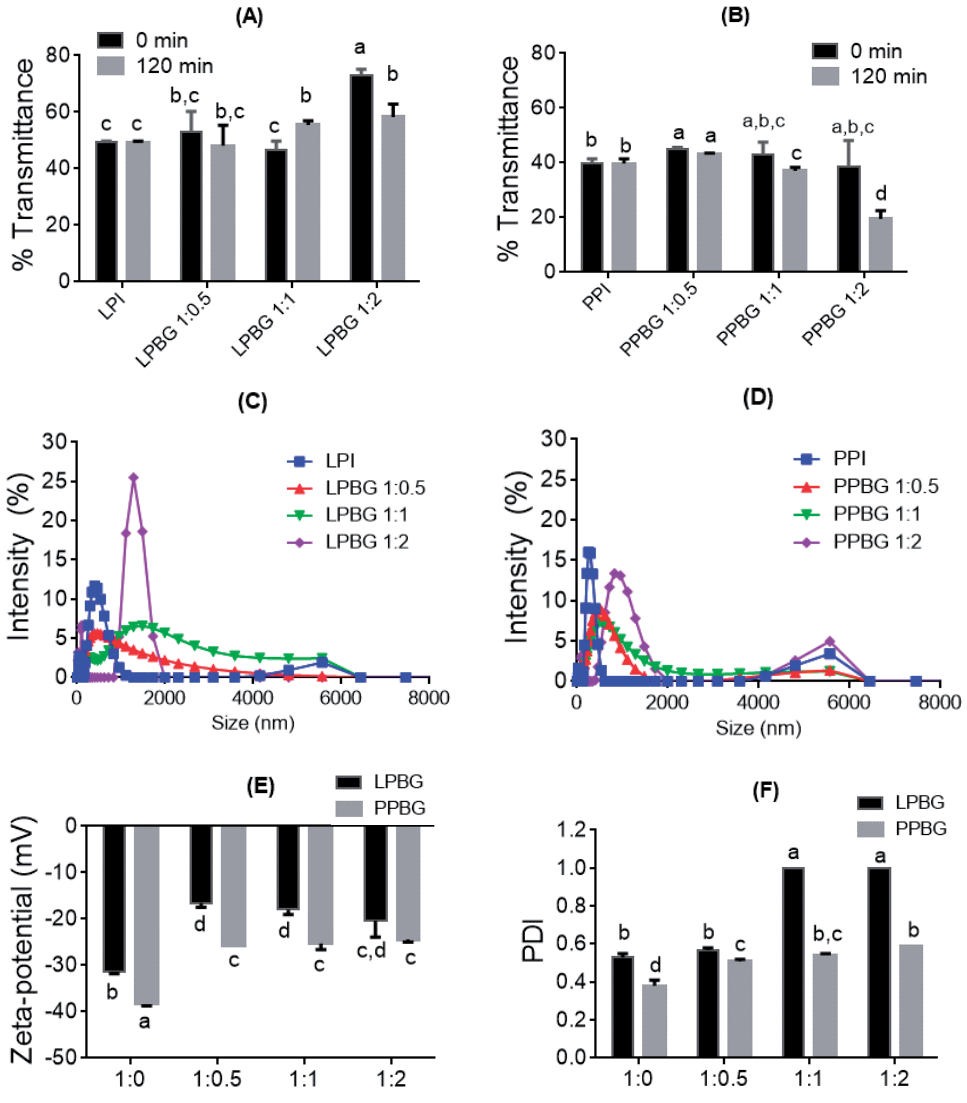

Figure 2.1: Transmittance at 590 nm at 0 and 120 min of protein/β-glucan interaction for (A) lentil protein (LPI), lentil protein/β-glucan mixtures (LPBG), (B) pea protein (PPI), and pea protein/β-glucan mixtures (PPBG). Particle size distribution of (C) lentil protein/β-glucan mixtures and (D) pea protein/β-glucan mixtures. Particle characteristics of the proteins and their β-glucan mixtures: (E) zeta potential (surface charge) and (F) polydispersity index (PDI).

Experiments were conducted in triplicate prior to the simulated static digestion and bars with different letters in each chart indicate significantly different mean values ($P < 0.05$).

The PPI and LPI with β-glucan mixtures recorded significantly lower ζ-potential than the proteins (less negative), irrespective of the β-glucan amount (Figure 2.1E). The ζ-potential indicates the electrical charge present on the surface of the biomolecules. This suggests some form of interaction of β-glucan that shielded the charged residues of LPI and PPI, thus reducing the ζ-potential of the mixtures. The reduced charge results in lower repulsion between the biopolymers and thus lower stability of the particles, and agglomeration or aggregation. Before the simulated *in vitro* digestion, the protein and protein/β-glucan samples were at a neutral pH, above the isoelectric point of the proteins (pI 4.4-4.6).[21,22] Also, at low concentrations, the phosphate group of β-glucan are thought to be responsible for the negative charge.[23] Thus, it is expected that the negative charge of both the protein and β-glucan would result in electrostatic repulsion between the polymers.

Increasing β-glucan concentration resulted in an increase in PDI for both LPI/ β-glucan and PPI/ β-glucan mixtures. The increase in PDI was however higher in the lentil mixtures. The LPI control recorded a higher PDI than the PPI control (Figure 2.1F) and this could be associated with the differences in their surface charges. A PDI of 0.39 for pea proteins has also been reported by Wu *et al.*[24] PDI is a measure of the broadness of molecular weight distribution of a formulation. A PDI close to 1 indicates a polydisperse formulation whereas values less than 0.3 represent monodispersed formulations. Therefore, this suggests that the control samples have particles that are monodispersed or moderately vary in sizes and changes to be more disperse upon addition of β-glucan. Comparable increase in PDI has been observed on pea protein interaction with other biopolymers.[25]

2.3.3 Effect of β-glucan on *in vitro* digestibility and profile of the proteins:

In this study, β-glucan decreased the *in vitro* digestibility of LPI and PPI. The DH obtained for the LPI and PPI control (1:0) was higher than that of the mixtures, irrespective of the β-glucan concentration (Figure 2.2A). PPI/β-glucan however recorded the most pronounced effect on digestibility with a relative decrease of 34.5% at 1:2 compared to protein alone. Protein interaction with β-glucan could produce complexes that are resistant to hydrolysis by gastric and intestinal proteases. β-Glucan can also form viscous gels that hinder the digestive enzymes from accessing the proteins.[5] However, it is unlikely that the β-glucan can significantly contribute to the viscosity of digesta at the concentrations used in the simulated digestion. Existing evidence show that the digestive enzymes do not directly affect the β-glucan structure. [26,27] Therefore, the differential ef-

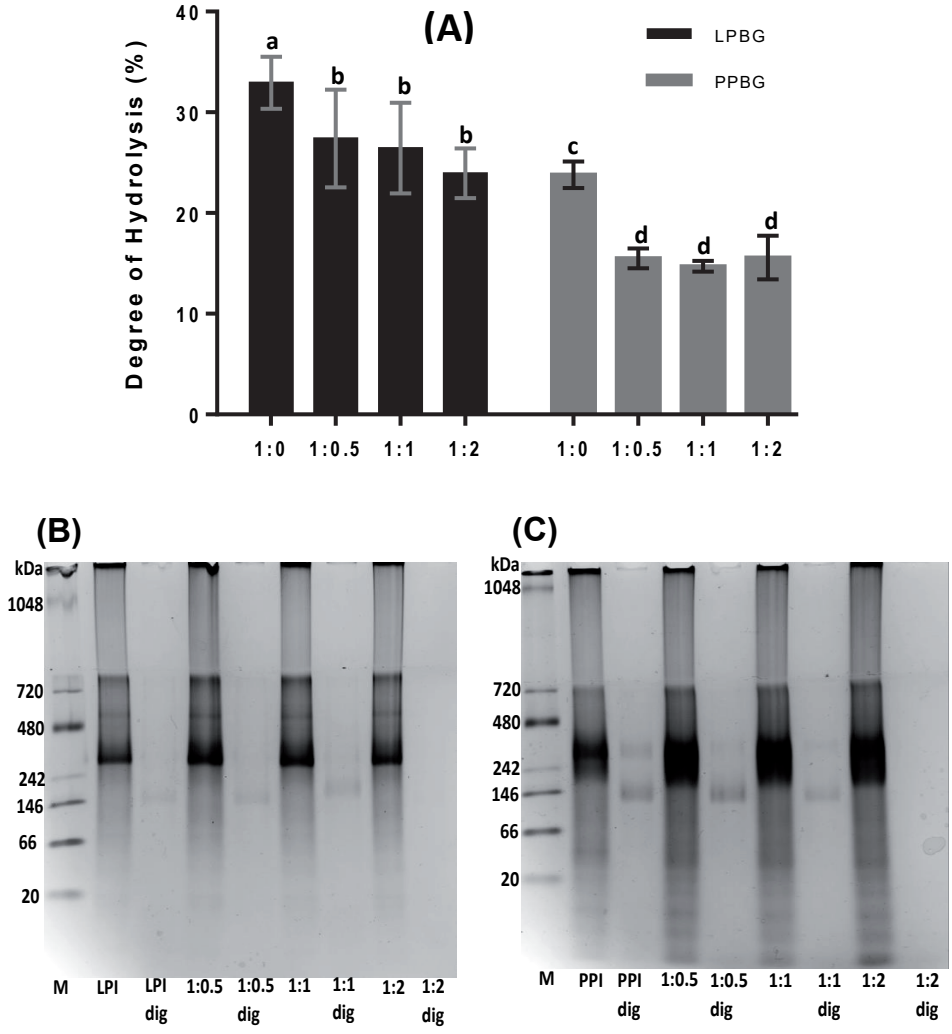

Figure 2.2: (A) ***In vitro*** protein digestibility (%) of lentil protein and pea protein in the absence and presence of different concentrations of β-glucan after simulated static digestion; bars with different letters in each chart indicate significantly different mean values (***P*** < 0.05). Native-PAGE profiles of (C) lentil protein/β-glucan mixtures and (D) pea protein/β-glucan mixtures before and after simulated static digestion. [lentil protein control (LPI), lentil protein/β-glucan mixtures (LPBG), pea protein control (PPI), and pea protein/β-glucan mixtures (PPBG)]

fect on the proteins could be attributed to difference in protein profile, which determines protein behaviour and interaction with the other biomolecules. Pea protein isolates obtained under the experimental conditions mainly consist of globulins (55-80%) and albumins (18-25%) with negligible amounts of gluten-

ins and prolamins.[2,4] Conversely, lentil protein isolates are composed of 11% glutenins and 3% prolamins. Differences in the protein type, composition and structure from the two sources were also confirmed by the difference in degree of hydrolysis of the pea and lentil protein controls themselves.

Discontinuous native PAGE was used to evaluate the profile of the legume proteins without denaturation. From the prominent bands observed in Figure 2.2B&C, the proteins fall within a molecular weight range of 242-480 kDa. Storage proteins including legumin, vicilin and convicilin, have molecular weights of 320-410, 150-200, and 280-290 kDa, respectively.[28–31] Lentil proteins are characterized by legumin-like proteins with molecular weights of 320-380 kDa.[3,32] After simulated digestion, the prominent bands disappeared and a less intense band appeared at 146 kDa. However, this shift does not happen in the 1:2 protein/β-glucan mixture. As observed for LPI (Figure 2.2B), the bands disappeared after digestion of the PPI/β-glucan 1:2 mixture (Figure 2.2C). The decrease in band intensity with increasing β-glucan concentration suggests a possible entrapment of the existing protein molecules. However, this was not supported by the degree of hydrolysis data for LPI (Figure 2.2A).

2.3.4 Brightfield and fluorescence imaging of the protein-β-glucan mixtures:

The brightfield images in Figure 2.3 display the microstructures of the lentil and yellow pea protein solutions (Panel A, D), β-glucan solution (Panel B, E), and protein/β-glucan solution at 1:2 ratio (Panel C, F) after staining. The lentil protein (Panel A) contained small aggregates, indicated with arrows. The β-glucan solution (Panel B, E) displayed a connected structure that contains similarly sized aggregates. The lentil protein/β-glucan structure (Panel C) was largely homogeneous even with large sized aggregates present. The size distribution of the aggregates makes the mixture heterogeneous, which is supported by the high PDI value (Figure 2.1F). On the other hand, the yellow pea protein solution (Panel D) had a loose structure compared to the lentil protein structure (Panel A), in line with its lower PDI value. The yellow pea protein/β-glucan solution (Panel F) showed a compact structure with some smaller aggregates compared to lentil protein/β-glucan. These findings suggest the formation of aggregate between the yellow pea proteins and β-glucan. The larger aggregates observed in Figure 2.3C may be the protein-fibre complexes responsible for reducing the digestibility of lentil proteins at the highest β-glucan concentration.

Fluorescence imaging was used to enhance the visibility of the biopolymers and to confirm the protein-β-glucan interactions in relation to the effect on protein

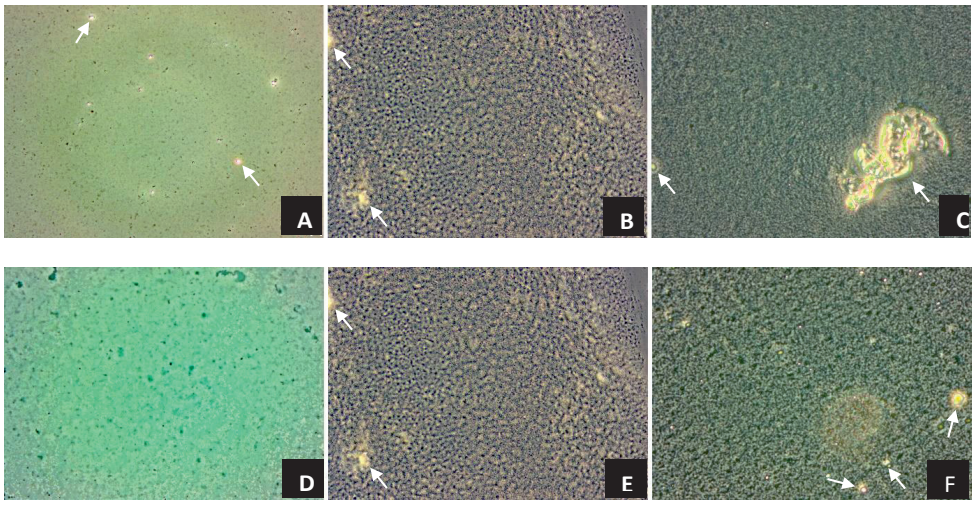

Figure 2.3: Brightfield microscopy images (A-F) at 40× magnification. (A) lentil protein solution, (B) β-glucan solution, (C) lentil protein/β-glucan solution at ratio 1:2, (D) pea protein solution, (E) β-glucan solution, (F) pea protein/β-glucan solution at ratio 1:2.

Arrows indicate presence of aggregates.

digestibility. The protein/β-glucan solutions (1:2) were stained separately and imaged at the same location in the field (Figure 2.4). In the lentil protein/β-glucan mixture (Panel A-C), Panel C shows an overlap of the LPI (purple) and β-glucan (blue) signals suggesting that the lentil protein and β-glucan aggregated. The relative fluorescence intensities of both β-glucan and lentil protein confirm the presence of both species within the aggregates (Figure 2.4G). This supports the result obtained by brightfield imaging (Figure 2.3C). The lentil protein-β-glucan aggregates may have hindered the digestive proteases from accessing cleavage sites of lentil proteins leading to a decrease in protein digestibility. In contrast, the yellow pea protein/β-glucan mixture (Panel F) indicated that signals for both protein and polysaccharides are visible and mostly do not overlap but rather the pea protein and β-glucan separated into distinct phases in water (Figure 2.4H). Quantification of the relative fluorescence intensities of both biopolymers within the aggregates indicate a significantly lower amount of protein within the β-glucan aggregates. Rather, the protein is mostly localized in the open structure of the β-glucan network, except for a few interactions occurring at the interface of the biopolymers. Consequently, the yellow pea proteins were entrapped within the β-glucan network, thus physically obstructing the diffusion of the digestive enzymes. This significantly reduced yellow pea protein digestibility and supports the stronger effect of β-glucan on degree of hydrolysis of yellow pea protein. At mass ratio of 1:2, the relative decrease in *in vitro* digestibility of lentil

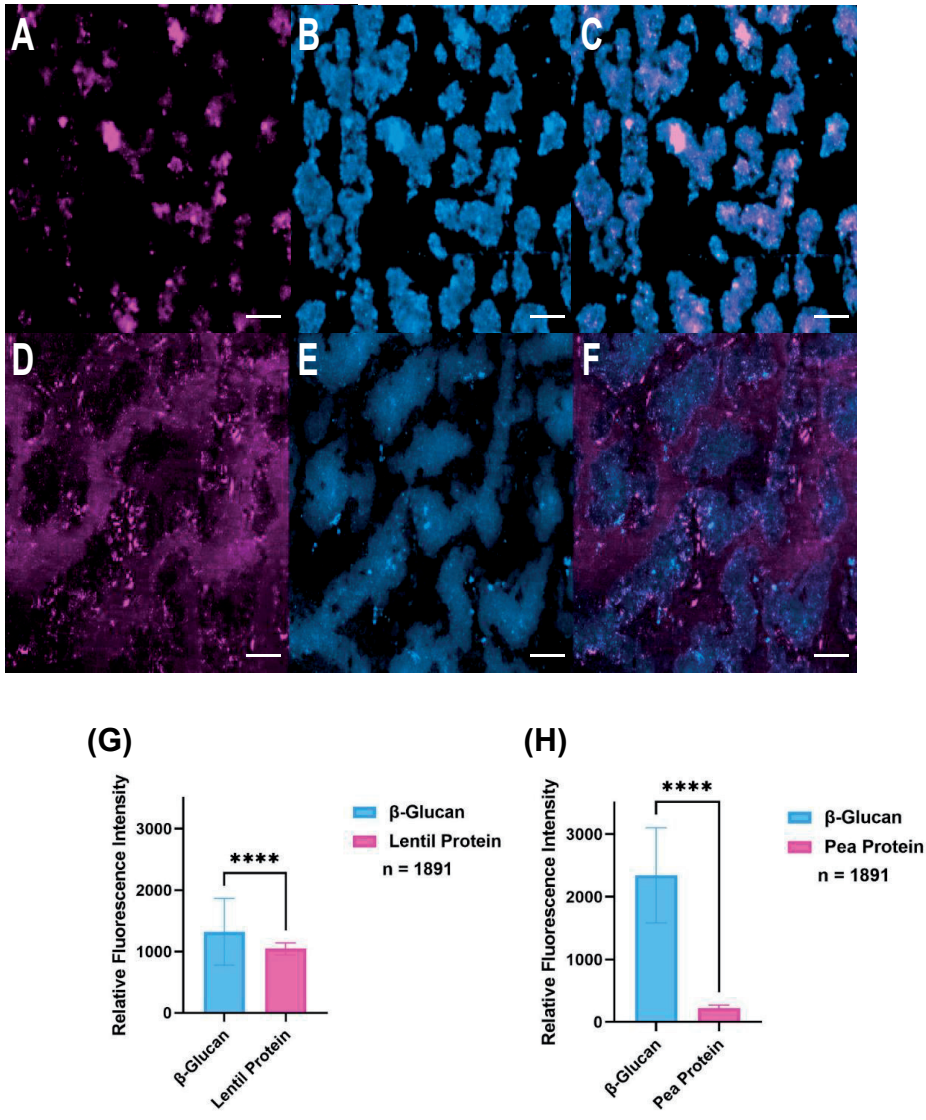

Figure 2.4: Fluorescence microscopy images at 20× magnification. (A) Protein visualised with Fast Green FCF in lentil protein/β-glucan solution at ratio 1:2. (B) β-glucan visualised with Calcofluor White in lentil protein/β-glucan solution at ratio 1:2. (C) Both protein and β-glucan visualised with dye mixture in lentil protein/β-glucan solution at ratio 1:2. (D) Protein visualised with Fast Green FCF in pea protein/β-glucan solution at ratio 1:2. (E) β-glucan visualised with Calcofluor White in pea protein/β-glucan solution at ratio 1:2. (F) Both protein and β-glucan visualised with dye mixture in pea protein/β-glucan solution at ratio 1:2.

Scale bars indicate 50 µm. Relative fluorescence intensities of β-glucan and protein quantified from aggregates present (n=1891) for (G) lentil protein-β-glucan and (H) pea protein- β-glucan mixtures. **** indicates that mean values are significantly different at $P < 0.0001$.

protein was 27.3% while that of yellow pea protein was 34.5%. Based on the ζ-potential (Figure 2.1E), the pea protein and β-glucan are negatively charged at a near-neutral pH, resulting in electrostatic repulsion among the molecules. The electrostatic stabilization explains the distinct phases formed where the pea protein occupied the pockets created by the β-glucan in water. In addition, all protein fractions in lentils have been reported to be glycosylated, thus causing a significant difference in lentil and yellow pea protein interaction behaviour.[1,33]

2.4 Conclusion

In conclusion, lentil and yellow pea protein can interact with β-glucan in solution and this resulted in lower *in vitro* protein digestibility in the lentil protein/β-glucan mixtures and the yellow pea protein/β-glucan mixtures compared to the proteins only. However, the extent of decrease in *in vitro* protein digestibility was higher in yellow pea protein/β-glucan mixtures. Based on fluorescence imaging of the aqueous mixtures, lentil protein and β-glucan formed aggregates whereas yellow pea protein was trapped within the β-glucan network forming distinct phases. Although lentil and yellow pea have similar protein types, the relative amounts of the fraction with different molecular features are different. Lentil proteins contain more glycosylated fractions, glutenins, and prolamins than yellow pea proteins, hence the different interaction behaviours with the β-glucan. These findings provide insight into the protein/fibre interactions that can occur in a food matrix, and confirms the possibility to modulate protein digestibility by changing the physical characteristics of the food matrix.

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Notes

The authors declare no competing financial interest.

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3

Chapter 3

Lentil Protein and Tannic Acid Interaction Limits *In Vitro* Peptic Hydrolysis and Alters Peptidomic Profiles of the Proteins

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Abstract

In this study, the nature of lentil protein-tannic acid (LPTA) interaction and its effect on *in vitro* pepsin digestion were investigated. LPTA mixtures containing 1% w/v LP and 0.001-0.5% TA were prepared and characterized in terms of particle size, thermal properties, and secondary and tertiary structures. A twenty-fold increase in particle size was observed in LPTA0.5% compared to LP control (without TA), indicating aggregation. Static quenching of tryptophan residues within the protein hydrophobic folds was observed. Increasing TA levels also enhanced protein thermal stability. Over 50% reduction in free amino groups of LPTA0.5%, relative to LP, was observed after pepsin digestion. Cleavage specificity of pepsin and peptidomic profile of LP were modified by the presence of TA in LPTA0.5%. This study showed that 0.5% w/v TA induced protein aggregation and reduced LP digestibility by hindering accessibility of pepsin to the protein network, thus modifying the profile of released peptides.

Keywords: lentil protein, tannic acid, *in vitro* protein digestibility, peptic digestion, food matrix interaction, biomolecular interactions, peptidomics

3.1 Introduction

Legume proteins, including lentil proteins, are gaining increasing attention due to their associated functional and bioactive properties. Their bioactivities include blood glucose regulatory, antifungal, antioxidative, and antihypertensive properties.[1–3] Protein content of lentils varies from 20% to 30%, and comprises 70% globulins, 16% albumin, 11% glutelins, and 3% prolamin fractions.[4,5] Lentil seed proteins mainly exist as storage proteins, serving as a primary source of energy during seed development.

Plant proteins, particularly legume proteins, are currently being considered as a more sustainable alternative for food applications, and functional food formulations. However, in complex food matrices, protein interaction with food components can alter their structure and functionality. Secondary plant metabolites, such as tannic acids, are abundant in specific food or added to functional food formulations due to their health benefits. To obtain these health benefits, they need to reach the absorptive site intact by escaping enzymatic hydrolysis or oxidative breakdown. Therefore, legume protein-polyphenol complexes can be used as a delivery option in functional food formulations.[6] The interaction between legume proteins and tannic acid can modify protein structure and behavior, affecting their susceptibility to digestive enzymes and nature or amount of bioactive fractions of protein released after hydrolysis.[7]

A combination of hydrogen bonding and hydrophobic interactions has been suggested to occur between the hydrophobic and hydrophilic regions of the globular fractions of food proteins and tannins.[8,9] The interaction begins with hydrogen bonds between the hydroxyl moiety of the tannin phenol group and the carbonyl group of the peptide, serving as an acceptor. It can advance to a hydrophobic bonding to yield complexes that can be soluble or insoluble. At a favorable protein-tannic acid ratio, and pH (around isoelectric point of the protein), insoluble complexes are formed due to reduced electrostatic interactions.[10–13] Although tannic acid binding might be non-specific, net charge, conformation, and size influence protein binding. Conditions, such as temperature, ionic strength, pH, and concentration, are also essential.[14–16] The pH can affect the stability of the hydrophilic bond and be reversed. The effect of tannic acid on protein digestibility is influenced by the concentration of tannic acid present.[10] However, the native sequence and structure of proteins dictate the mechanism of protein-tannic acid interaction.[9,17]

The evaluation of possible modifications that occur after lentil protein and tannic acid interaction, and the resulting effect on their digestibility are relevant to understand the effect of the interaction on the protein's functionality and bioactivity. Hence, this study sought to find the effect of tannic acid interaction on particle characteristics, structure, thermal stability, peptide profile, and *in vitro* peptic digestibility of lentil proteins. Although *in vitro* peptic digestibility does not reflect overall digestibility, the physical state in which complexes are presented for digestion is relevant since the structure determines their susceptibility to digestive enzymes, and the type of peptides released.

3.2 Materials and Methods

3.2.1 Materials:

Dry green lentils were donated by Pulse Canada (Winnipeg, Manitoba, Canada). Tannic acid (gallotannin, tannin, molecular weight 1701.20 g/mol) from Chinese natural gallnuts and pepsin (≥250 units/mg solid) from porcine gastric mucosa were purchased from Millipore Sigma (Burlington, MA, USA). NativeMark™ protein standard and GelCode™ blue safe protein stain were purchased from Fisher Scientific (Toronto, ON, Canada). Fast Green FCF (≥85%) was also acquired from Millipore Sigma (St. Louis, MO, USA).

3.2.2 Lentil protein extraction:

Dry green lentil seeds were soaked overnight in a 1:3 (w/v) seed-to-water ratio at 20 °C. The soaked seeds were then freeze-dried after partial grind in a food processor (Waring commercial blender, Torrington, USA). Afterwards, the dried seeds were pulverised with a food processor into fine powder. Solution of the flour (10% w/v) was prepared in NaOH (0.05 M) and stirred constantly for 4 h with pH maintained at 10.0. The suspension was then centrifuged at 7000 × g, 4 °C for 30 min. The supernatant was recovered, pH adjusted to 4.0 using 3 M HCl and stirred for 2 h to precipitate the lentil proteins. Thereafter, the resulting suspension was centrifuged at 7000 × g, 4 °C for 30 min. The pellet fraction was then resuspended in deionized water and pH adjusted to 7.0 with 3 M NaOH and freeze-dried. The lyophilized lentil protein powder (LPI) was stored at -20 °C. The LPI had 69.59 ± 5.77% protein content as determined by Lowry assay.

3.2.3 Preparation of lentil protein-tannic acid (LPTA) complexes:

Stock solutions of LP powder (2% w/v) and TA (1% w/v) were prepared in deionized water. Thereafter, the two solutions were mixed to obtain 1% w/v LP and 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, or 0.5% TA after diluting stock solutions

with deionized water.[18] These samples are hereafter referred to as LPTA0.001%, LPTA0.005%, LPTA0.01%, LPTA0.05%, LPTA0.1%, and LPTA0.5%. For the control (LPI), the extracted lentil protein was mixed with deionized water in place of TA.

3.2.4 LPTA interaction study:

A 100 µL of LP stock and 100 µL of TA dilutions or water (as control) were added into a clear 96-well microplate. The plate was instantly shaken in a microplate reader (Tecan, Switzerland) for 20 s. The absorbance of the mixture was measured at 590 nm every 3 min for 162 min at 20 °C. Interaction between LP and TA was reported as percent transmittance using the relation:

3.2.5 Surface properties of the protein-TA complexes determined by dynamic light scattering (DLS):

The average particle size, ζ-potential, and polydispersity index (PDI) of LP-TA mixture or control (1 mL) were analysed with Zetasizer Nano ZS (Malvern Instruments Ltd., UK) after equilibrating the suspension for 120 s at 25 °C. The measurement was carried out at a refractive index of 1.450, absorption of 0.001, in Milli-Q water (pH 7.0, refractive index 1.330, viscosity 0.8872 cP and dielectric constant 78.5). Results of triplicate analysis measured using the Smoluchowski model at F(ka) 1.50 and backscattered angle of 173° were reported.

3.2.6 Fluorescence imaging:

Fluorescence microscopy was used to visualize the relative distribution of LP and TA after their interaction. LP was stained by mixing 50 µL of Fast Green FCF (0.1% w/v) and 500 µL of LPTA0.5%, and the mixture loaded on to a slide. The samples were visualized under UV light using the Axio Imager 2 microscope equipped with an Axiocam 506 camera (Carl Zeiss, Germany). The DAPI channel with excitation/emission wavelengths of 353/465 nm was used for TA and the Cy5 channel at 650/673 nm for Fast Green FCF. The image processing was conducted with Zen 2.3 pro software (Carl Zeiss, Germany).

3.2.7 Thermal stability determination using Differential Scanning Calorimetry (DSC):

Differential scanning calorimeter (DSC Q2000, TA instruments, USA) was used to investigate the effect of LPTA interaction on the thermal stability of LP. About 1.65–8.25 mg freeze dried LPTA mixtures were weighed into clean aluminum pans. Afterwards, pans were purged with pure nitrogen gas at a flow rate of 20 mL/min. Samples were then heated to 25–250 °C at a scan rate of 10 °C/min as previously reported.[19] With an empty pan as reference, the corresponding thermogram was recorded and Origin 9 software (OriginLab Corporation, USA)

was used to obtain the transition enthalpies from the integrated peak or dip area of the plot of

$$\frac{heat\ flow\ (\frac{mJ}{s})}{weight\ of\ sample\ (mg)}\ vs\ time\ (s).$$

3.2.8 Interaction parameters determined from fluorescence quenching:

Fluorescence quenching technique was used to evaluate the nature and strength of LPTA interaction using a Varian Cary Eclipse (Agilent, USA). Stock solutions of LP (0.25 mg/ml) and TA (0.294 mM) were prepared in MilliQ water. Further dilutions of TA were prepared to obtain 0.03–58.8 μM (0.0005 – 0.01% w/v) TA when mixed with equal volume of LP. Final LP concentration in the mixtures was maintained at 0.125 mg/mL (0.0125% w/v). The fluorescence intensity of the mixture at different TA concentrations were measured at 25 °C, using an excitation wavelength of 280 nm for tryptophan.[19] Binding parameters were calculated using the Stern-Volmer equations (Eq. 1-3) by assuming that fluorescence quenching is as result of LPTA binding.

$F_0/F_0\text{-}F = 1/fK\,[Q] + 1/f$ (1)

$F_0/F = 1 + K_Q t_0[Q] = K_D[Q]$ (2)

$\log\,(F_0\text{-}F)/F = \log K_A + n\log[Q]$ (3)

F and F_0 are the fluorescence intensities in the presence and absence of TA respectively; f is the accessible fluorophore fraction; K is the Stern-Volmer quenching constant; $[Q]$ is TA concentration; K_Q is the bimolecular quenching rate constant; K_D is the Stern-Volmer quenching constant; and t_o is the lifetime of the protein fluorophore in the absence of the quencher, usually in the order of 10^{-9} seconds.[19] K_A is the binding constant and n is the substantive number of binding sites for TA. These parameters were determined from the plots of Eq. 1-3.

3.2.9 Circular dichroism (CD) spectroscopy:

The effect of LPTA interaction on the secondary structure of the proteins was evaluated by measuring the circular dichroism spectra with Jasco J-715 (Jasco International Corporation, Japan). A stock solution of LP (0.25 mg/ml) and TA (0.294 mM) were prepared in MilliQ water. The TA stock solution was diluted to obtain selected TA concentrations (0.001%, 0.005% and 0.01%). Equal volumes of LPI and the TA dilutions were then mixed. The resulting solutions contained 0.0125 w/v% LPI, and 0.0005, 0.0025, 0.005 %w/v TA. MilliQ water was used as control. LPTA complexes containing 0.125 mg/mL (0.0125%w/v) LP was added to a quartz cuvette (path length of 0.01 cm). The sample was then subjected to a spectrum scan at 50 nm/min from 185 nm to 260 nm, and a bandwidth of 1 nm at

25 °C in nitrogen gas. CD Pro software was then used to estimate the composition of the protein secondary structures.[19]

3.2.10 *In vitro* peptic hydrolysis of proteins:

LPTA solutions containing 1% w/v LP and 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, or 0.5% w/v TA were constantly stirred for 30 min. Thereafter, pepsin stock solution (25000 U/mL) was prepared in Milli Q water. A 30-µL pepsin stock solution was added to 5 ml solutions of the complexes after adjusting pH to 3.0 with 1 M HCl. Samples were incubated at 37 °C for 2 h with constant shaking at 90 rpm. Afterwards, pH of digesta was adjusted to 7.0, vortex-mixed and frozen to halt enzymatic activities. *In vitro* peptic protein digestibility was determined by the *o*-phthaldialdehyde (OPA) method. Briefly, OPA reagent (225 µL) was added to 30 µL of serine standard, blank (Milli-Q water), or sample solutions in a microplate. The microplate was subjected to 2 min incubation with shaking at room temperature. The absorbance was read at 340 nm using a microplate reader (Tecan, Switzerland). The level of free amino groups was determined as previously reported and expressed as serine-equivalent.[20]

3.2.11 Determination of protein profile from discontinuous native polyacrylamide gel electrophoresis.

Native PAGE was conducted following the Mini-PROTEAN® Tetra cell instruction manual (Bio-Rad, USA). Polyacrylamide gradient gel (6%, 9%, 12%, 15%, and 18% w/v) and a 3% (w/v) stacking gel were cast. A sample buffer, comprising 1.25 mL Tris-HCl (0.5 M) with pH 6.8, 3 mL of glycerol, and 0.2 mL of bromophenol blue (0.5%) in 5.55 mL deionized water, was prepared and mixed with samples at a ratio of 1:2 v/v. The NativeMark™ protein standard (10 µL) was also loaded into the first well of the gel. A 15-µL aliquot of the sample buffer-sample solution was then loaded into separate wells. Afterwards, the loaded gel was mounted into a BioRad Mini-PROTEAN Tetra Cell electrophoresis unit (Bio-Rad, USA) and run at 120 V for 90 min until the bands had migrated sufficiently. The gel was rinsed with deionized water and stained with 100 mL Coomassie Brilliant Blue R-250 staining solution for 2 h and de-stained overnight. Image was acquired with a ChemiDoc MP Imaging System (Bio-Rad Inc., Canada).

3.2.12 Liquid chromatography with tandem mass spectrometry (LC-MS-MS) of high molecular weight hydrolysates

Sample preparation

The LPI and LPTA0.5% digesta was adjusted to pH 4.0 to precipitate the high molecular weight hydrolysates. The samples were centrifuged for 30 min at 7000

× g, and 20 °C. The precipitates were then freeze-dried for characterization of the peptides. About 5 mg were solubilized in 1000 µL of the buffer consisting of 20 mM HEPES, 5% glycerol, and 0.1% *n*-dodecyl β-D-maltoside (DDM). The portion of 100 µL was then reduced using 4 µL TCEP solution (100 mM) and incubated at 25 °C for 55 min on a shaking plate at 450 rpm. Samples were then alkylated with 4 µL iodoacetamide solution (500 mM) and incubated at 25 °C for 55 min on a plate shaking at 450 rpm. The resulting solutions were then acidified with formic acid (2 µL, 100%), vortexed, and centrifuged at 10,000 ×*g* for 30 s. Samples were desalted using C18 TopTips (Glygen) columns, as per the manufacturer's instructions, then vacuum dried.

Nano-LC–MS/MS:
Protein samples (~5 µg of protein) were analyzed by an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific) coupled to an UltiMate 3000 nanoRSLC (Dionex, Thermo Fisher Scientific) as previously reported.[21]

MS Data Analysis.
MS raw files were analyzed with MaxQuant (version 2.0.3.1) and the Andromeda search engine.[22,23] Peptides were searched against a UniProt FASTA file for the tribe *Fabeae* (163743), encompassing 509 protein sequences (25.11.2020) and a contaminants database. Default parameters were used if not mentioned otherwise. Peptides from unspecific cleavage within the size of 6 to 25 amino acids were searched. N-terminal acetylation and methionine oxidation were set as variable modifications, and cysteine carbamidomethylation as fixed modification. Peptides were identified with an initial precursor mass deviation of up to 10 ppm and a fragment mass deviation of 0.5 A false discovery rate (FDR) of 0.01 was applied at both peptide and protein level, determined by searching against a reverse sequence database. 'Match between runs' and 'de-novo sequencing' were enabled with default settings to increase the number of identified peptides within and among samples.

Bioinformatic Analysis.
Proteins and peptides matching to the reverse database were discarded. The MaxQuant output tables were then loaded in the R programming environment where all subsequent analyses were conducted. Peptide properties were obtained from the *Peptides* R package, cleavage site sequence logos were made with *ggseqlogo* and all other plots were produced with *ggplot2*.

3.2.13 Statistical analysis.

Mean ± standard deviation of triplicate experiments was reported, and one-way analysis of variance with the post-hoc Tukey test was used to determine significant differences between mean values at $P < 0.05$.

3.3 Results and Discussion

3.3.1 Effect of LPTA interaction on the complex characteristics

Transmittance:

Percent transmittance was used to quantify the interaction between LP and TA that affects transmission of light. Transmittance (%) of LPTA0.001%, 0.005% and 0.01% were comparable to that of LPI control (0% TA), while LPTA0.05% and 0.1% produced a slightly lower transmittance. There was an absolute decrease from 45.6% (for LPI control) to 4.6% for LPTA0.5%. Unlike other complexes, LPTA0.5% further reduced over the 162 min (Figure 3.1A). This shows that increasing concentration of TA resulted in the formation of complexes that hinder the transmission of light. In addition, the slight increase observed in LPTA 0.05% and 0.1% could be due to disintegration of instant unstable aggregates formed initially while there was further aggregation in LPTA 0.5% over time. Kaspachak *et al.* (2020) observed an increase in turbidity of TA/BSA mixtures as the concentration of TA increased.[10]

The initial step of the complexation process involves hydrophilic bonding of TA hydroxyl groups to polar groups of the protein to produce possibly soluble particles, thus leaving a less hydrophilic covering on the protein surface. Therefore, a subsequent interaction among complexes yields aggregates and precipitates. In addition, TA can also initiate protein cross-linkages to form precipitates.[10,24,25] The current study shows that this phenomenon depends on the concentration of TA present. From the vast decrease (36.3%) observed from LPTA0.1% to LPTA0.5%, high concentration of TA seems to facilitate this process. The pH of the LPTA mixtures was neutral, which is above the isoelectric point of LP. The pH of LPI and LPTA0.5% were 7 and 6.4, respectively. Thus, the aggregation can be attributed to the formation of protein-tannic acid complexes.

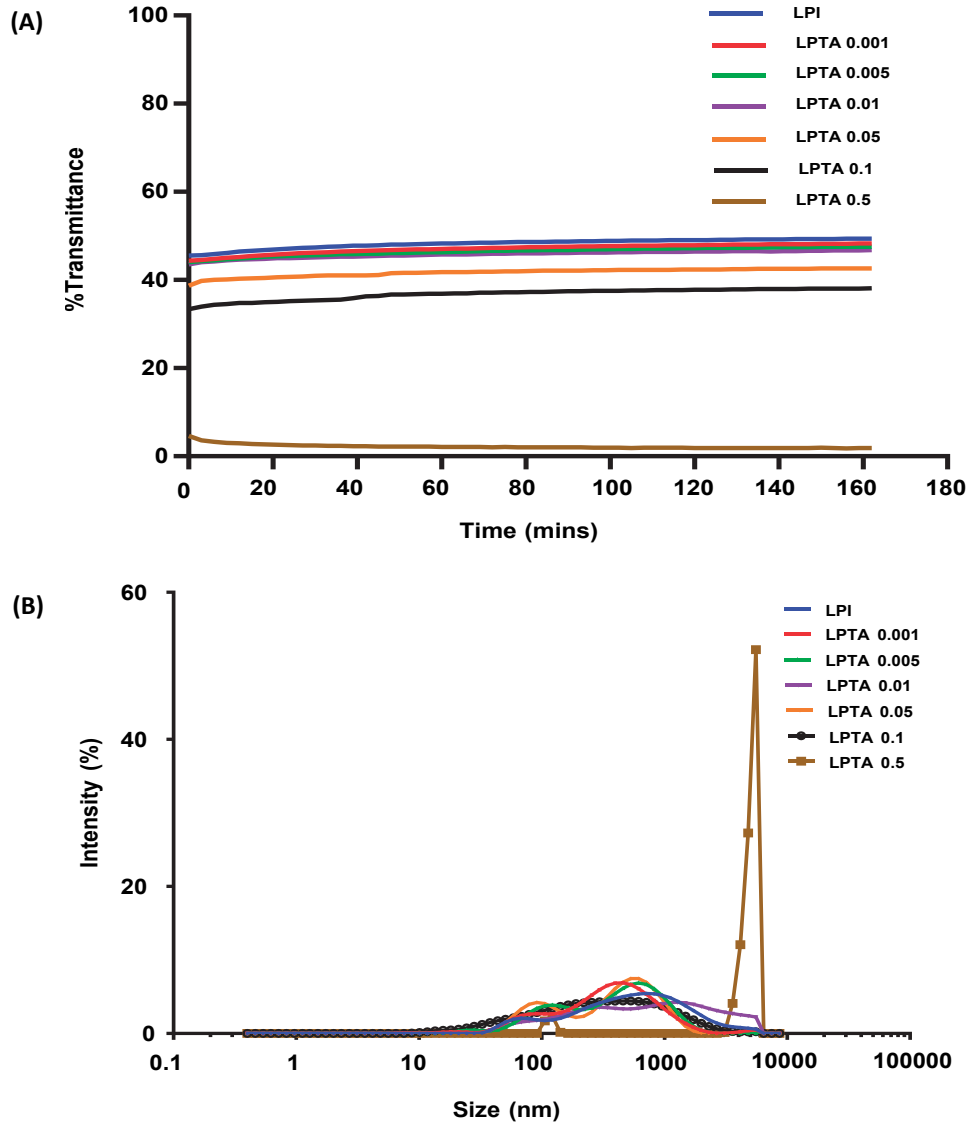

Figure 3.1. Particle characteristics of mixtures. **(A)** Transmittance measured at 590 nm over 162 min and **(B)** particle size distribution of lentil protein and lentil protein-tannic acid mixtures.

Surface properties of the LPTA complexes:

Formation of complexes from LPTA interaction can be evaluated by assessing the particle characteristics of these complexes. The particle size distribution curve shows that about 70% of LPI particles were evenly distributed between 200 nm and 2000 nm (Figure 3.1B). Similar distributions were observed in LPTA0.001%,

0.005%, and 0.01%. Meanwhile about 96% of LPTA0.5% was within 3000-6000 nm. Again, increasing TA concentration to 0.5% resulted in a significant increase ($P < 0.05$) in average particle size in LPTA0.5%. This twenty-fold increase in size suggests the formation of aggregates at high TA levels. In sodium caseinate/TA complexes, average particle size increased when TA levels were increased to 0.8% due to agglomerates formed from protein cross-linkages enhanced by high TA levels.[26] Apart from LPTA0.001% and LPTA0.5%, all samples recorded a PDI of 0.6, indicating moderate heterogeneity in sample size distribution (Table 3.1). Similar observation was reported for sodium caseinate/TA complexes where, as TA levels increased, PDI approached 0.5.[26] LPTA0.01% produced the most heterogeneous mixture while LPTA0.5% produced the least, with PDI of 1.00 and 0.21, respectively. This confirms the particle size distribution observed, where over 90% of LPTA0.5% fall within a narrow range (3000-6000 nm). A strong surface charge suggests a potential to partake in strong electrostatic interaction. Thus, a decrease in zeta potential indicates a lower stability of the biomolecule in solution, leading to aggregation. In this study, all mixtures recorded similar zeta potential (-40 mV) as the LPI control. This therefore suggests that the form of interaction happening does not affect the surface of the protein but rather involves the hydrophobic core of the proteins. Pea protein-TA complexes have

Table 3.1. Particle characteristics, transition temperatures, and enthalpies of lentil/tannic acid mixtures.

Sample	Z-ave (nm)	PDI	ZP (mV)	Transition Temperature (°C)	Transition enthalpies (J/g)
LPI	299.67 ± 5.76^{a}	0.58 ± 0.04^{a}	-41.2 ± 2.08^{ab}	143.2	36.7
LPTA 0.001%	213.50 ± 6.21^{a}	0.57 ± 0.05^{a}	-38.47 ± 1.12^{a}	143.6	67.1
LPTA 0.005%	211.03 ± 3.52^{a}	0.60 ± 0.01^{a}	-40.27 ± 0.31^{ab}	149.2	82.1
LPTA 0.01%	308.50 ± 3.80^{a}	1.00 ± 0.00^{b}	-41.77 ± 1.00^{ab}	113.4	32.7
LPTA 0.05%	193.13 ± 1.07^{a}	0.59 ± 0.00^{a}	-41.67 ± 1.00^{ab}	149.2	31.9
LPTA 0.1%	174.27 ± 1.33^{a}	0.59 ± 0.00^{a}	-42.90 ± 0.79^{b}	147.2	105.5
LPTA 0.5%	6128.00 ± 1214.34^{b}	0.21 ± 0.06^{c}	-39.90 ± 2.71^{ab}	182.3	107.1

Different superscript letters represent statistical difference in means.

been reported to exhibit similar zeta potential values. However, Li *et al.* reported a substantial increase in negative surface charge as concentration of TA increased in the pea protein-TA complexes,[18] indicating that protein-TA interaction could be occurring on the surface rather than within the hydrophobic core of the protein.

Fluorescence imaging of LPTA mixtures.

Visualization of the LPTA0.5% complexes by fluorescence microscopy further elucidated their interaction by showing their relative distributions. From the fluorescence images obtained, the protein and TA signals co-existed in the same location (Figure 3.2A-C). This indicates aggregation between protein and TA at 0.5% w/v TA, hence the presence of large particles in the mixture, confirmed by the average particle size and the size distribution (Table 3.1 & Figure 3.1B). Moreover, the relative fluorescence intensities showed the presence of both protein and TA in the aggregates.

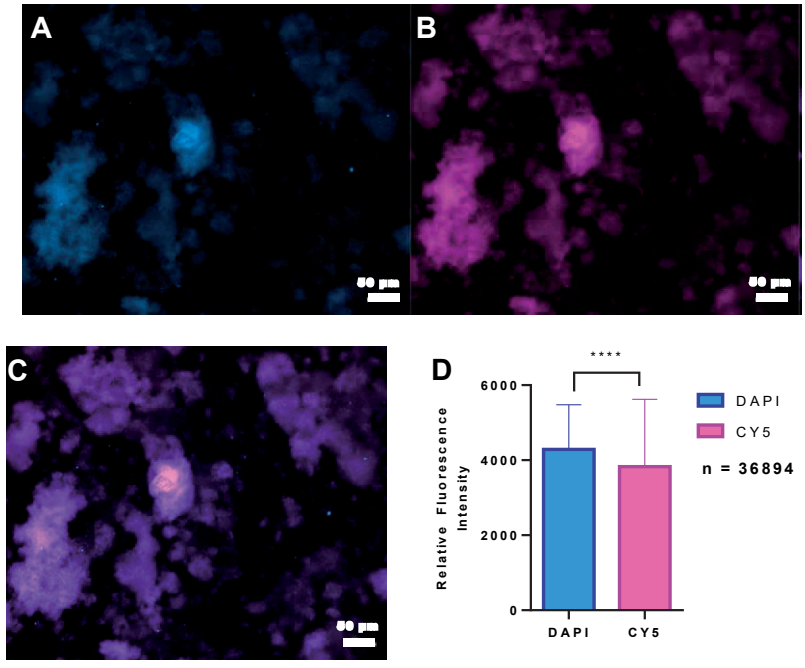

Figure 3.2. Fluorescence microscopy images at 20× magnification. **(A)** Tannic acid in LPTA0.5%, **(B)** lentil protein in LPTA0.5% visualised with Fast Green FCF, and **(C)** merger of both lentil protein and tannic acid signals in LPTA 0.5%. Scale bars represent 50 µm. **(D)** Quantification of relative fluorescence intensities of tannic acid and protein in each aggregates present (n = 36,894).

**** indicates significantly different mean values at $P < 0.0001$.

3.3.2 Nature and strength of LPTA interaction

Intrinsic fluorescence and binding parameters:

Examining the tryptophan fluorescence spectra provides insight into molecular interactions between a protein and other chemical compounds. From Figure 3.3A, it was observed that when a constant concentration of LP was mixed with increasing concentration of TA, the fluorescence intensity decreased. The fluorescence emission of the native LP peaked around 325 nm. The initial excitation scan showed the highest peak at 280 nm for tryptophan. Tryptophan is usually embedded within the hydrophobic core of the protein. Hence, decreasing fluorescence intensity suggests binding of the tryptophan residues, a proximity of the binding sites to the tryptophan residue or binding of the ligand to a distant site that causes conformational changes in tryptophan residue.[27] Therefore, the LPTA interaction occurs within the protein hydrophobic folds. Again, the maximum emission wavelength also increased, depicting a red shift. This suggests

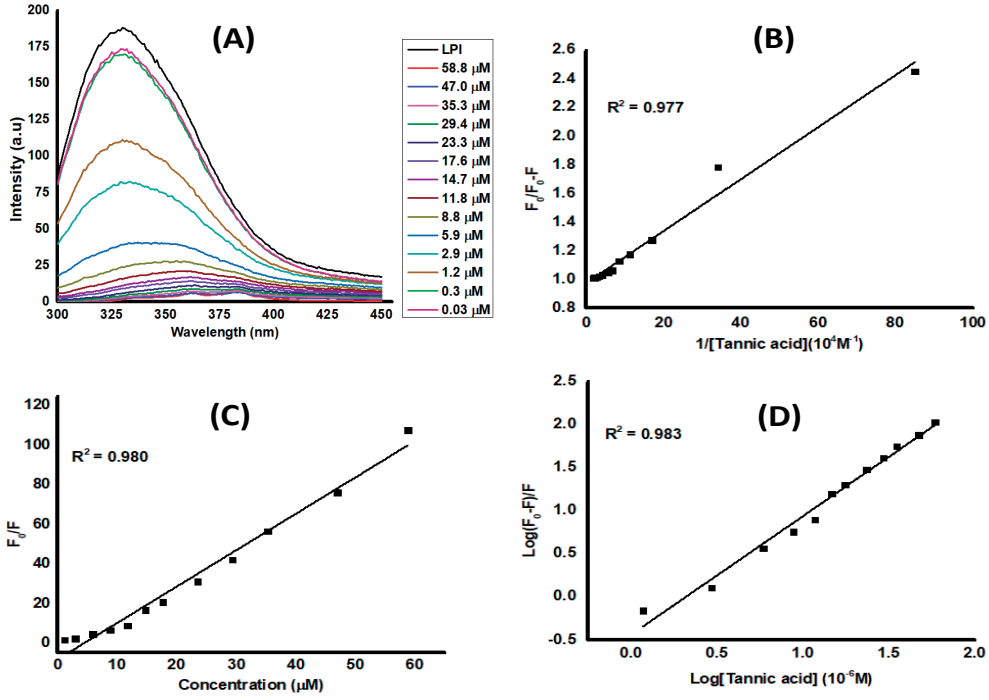

Figure 3.3: (**A**) Fluorescence emission spectra of LPI at various concentrations of tannic acid. (**B**) Plot of $F_0/(F_0-F)$ vs. 1/ [Tannic acid] for binding constant (K) determination. (**C**) Stern-Volmer plot for quenching constant determination. (**D**) Plot of $\log(F_0 - F)/F$ vs. log[Tannic acid] for determination of the number of bound tannic acid (n).

that increasing TA concentration enhanced polarity of the microenvironment of the tryptophan residues. Hydrogen bonding within the complexes change the molecular structural conformation of the protein and expose tryptophan residues. Similar quenching effect of TA on food proteins has also been reported by previous studies [27,28].

To further understand the mechanism of binding between LPI and TA, the Stern-Volmer equation and plots were used to evaluate the binding parameters. From equation 1, a plot of *1/Q* against F_0/F_0-F (Figure 3.3B) was used to determine the binding constant, *K*, and the accessible fluorophore fraction, *f,* assuming that quenching is directly associated with binding. The binding constant *K* was determined to be 5.6±1.44 ×10^5 M^{-1}. This is higher than previous reports on pea protein-curcumin interactions suggesting the formation of stronger hydrophobic interactions.[19] The accessible fluorophore fraction, *f,* was also estimated as 1.02. Equation 2 was used to find the quenching constant, K_D, by plotting *[Q]* against F_0/F, as shown in Figure 3.3C. The quenching constant, K_D (1.8±0.04 ×10^6 M^{-1}), obtained in this study, was higher than those of previous report on food protein and TA complexes [27], suggesting a high binding affinity. Xie *et al.* studied the interaction between TA and bovine serum albumin, egg ovalbumin, and bovine β-lactoglobulin. The highest quenching constant recorded (4.02×10^5 M^{-1}) was reported for bovine β-lactoglobulin, which is lower than K_D recorded in the present study.[27] This shows a higher binding affinity in LPTA complexes.

A fluorophore quenching could be due to collision or complex formation with the quencher. The F_0/F vs. *[Q]* plot was mostly linear, indicating that one quenching mechanism predominated. However, the bimolecular quenching rate constant, K_Q, was found to be 1.8±0.04 ×10^{15} $M^{-1}S^{-1}$. K_Q of 10^{10} $M^{-1}S^{-1}$ is the maximum for a quenching mechanism dependent on the diffusion of the quencher.[29] Therefore, the current result points to a static quenching of tryptophan residues by TA. As calculated from Eq. 3 and shown in Figure 3.3D, approximately one (1.37±0.01) TA molecule was bound to a protein molecule in the complex. The static quenching is as a result of formation of a stable non-fluorescent complex that changes the conformation and secondary structure of LP.[30]

Secondary structure:

CD employs the absorption levels of the optically active and asymmetrical structure of amino acid residues to provide insight into the secondary structures of proteins.[31] The different fractions including α-helix, β-strands, and random coils generally produce varying positive and negative bands during the spectra scan from 185 nm to 260 nm. In this study, CD was conducted for selected concentra-

Table 3.2. Effect of lentil protein-tannic acid interaction on secondary structure.

LPI – tannic acid interaction at various tannic acid concentration						
Tannic acid concentration (%w/v)	Secondary structure fractions (%)					
	Regular α-helix	Distorted α-helix	Regular β-strand	Distorted β-strand	Turn	Unordered
0	2.4	5.5	26.0	12.2	22.7	31.3
0.0005	1.8	5.1	27.3	12.5	22.9	30.5
0.0025	3.3	5.8	26.5	12.6	22.4	29.4
0.005	8.4	6.5	24.5	11.6	21.3	27.6

tions of TA used in the fluorescence quenching spectroscopy to assess changes in the different secondary structure fractions that occurred due to complexation with TA. As shown in Table 3.2, increasing TA content caused an overall increase in the regular and distorted α-helix fractions. On the other hand, regular and distorted β-strand fractions remained constant but reduced marginally at the highest TA concentration. These findings concur with those reported by Zhao *et al.*[28] The change in the secondary structure demonstrates that interaction in LPTA altered the hydrogen bonding in the proteins. These changes were supported by the concentration-dependent decrease in fluorescence intensity of proteins observed at the selected TA concentrations (Figure 3.3A).

Thermal stability:

Thermal stability is determined by disulfide bonds in globular proteins, proportion of acidic/basic residues, and polar uncharged residues present.[32] Thus, structural and conformational changes induced by interaction with TA can influence protein peak temperature and be evaluated by studying the thermal stability of the proteins.

The DSC thermograms (Figure 3.4) show that high concentrations of TA modified the thermal stability of LP. The transition temperature of LPTA0.5% was observed at 182.3 °C, compared to that of the LPI, which was 143.2 °C (Table 3.1). The highest transition enthalpy was observed at 0.1% and 0.5% w/v TA. The transition enthalpy shows the level of structural unfolding of the proteins.[32] These observations indicate that high TA concentrations enhanced the thermal stability of the proteins, since a higher energy was required to modify the peak temperature of the protein. High peak temperatures suggest compact tertiary structure of the proteins, and disruption of hydrogen bonds holding the protein structure. High level of disulphide bonds can also enhance thermal stability.[32] The improved thermal stability of the proteins confirms that hydrogen bonding

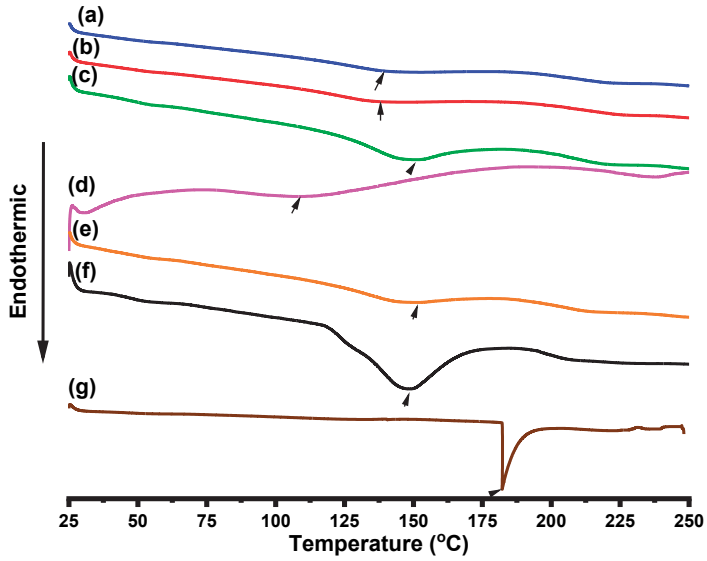

Figure 3.4: Differential scanning calorimetric thermogram of **(a)** LPI **(b)** LPTA 0.001% **(c)** LPTA 0.005% **(d)** LPTA 0.01% **(e)** LPTA 0.05% **(f)** LPTA 0.1% **(g)** LPTA 0.5%

is involved in the maintenance of the LPTA complexes. The increased peak temperature of the LPTA complexes at high TA concentration indicate the additional energy needed for the disruption of the hydrogen bonds holding their compact tertiary structure.[32,33] Thus, lower peak temperatures were observed in the LPI control and complexes with low TA concentrations.

Several peak temperatures of thermal denaturation, including 79.26 °C and 106.5 °C, have been reported for lentil proteins, depending on the method of protein preparation or cultivar of lentils used.[33,34] More important is the effect of high legumin content, since their solid structure makes them tougher to unfold.[35]

3.3.3 Effect of TA on *in vitro* protein digestibility by pepsin.

Insoluble complexes formed from LPTA interaction could hinder enzymatic hydrolysis of proteins, due to the hydrophobic obstruction of protein surface, coupled with a low gastric pH that favors the stability of protein-TA complexes. Particle characteristics of the mixtures demonstrate that LPTA0.5% resulted in the highest aggregation. This reflected in the content of free amino groups recorded after protein digestion by pepsin. As shown in Figure 3.5A, LPTA0.1% and 0.5% had the lowest free amino groups content, with LPTA0.5% showing a value that is 56.8% less than LPI. The insoluble complexes formed in LPTA0.5%

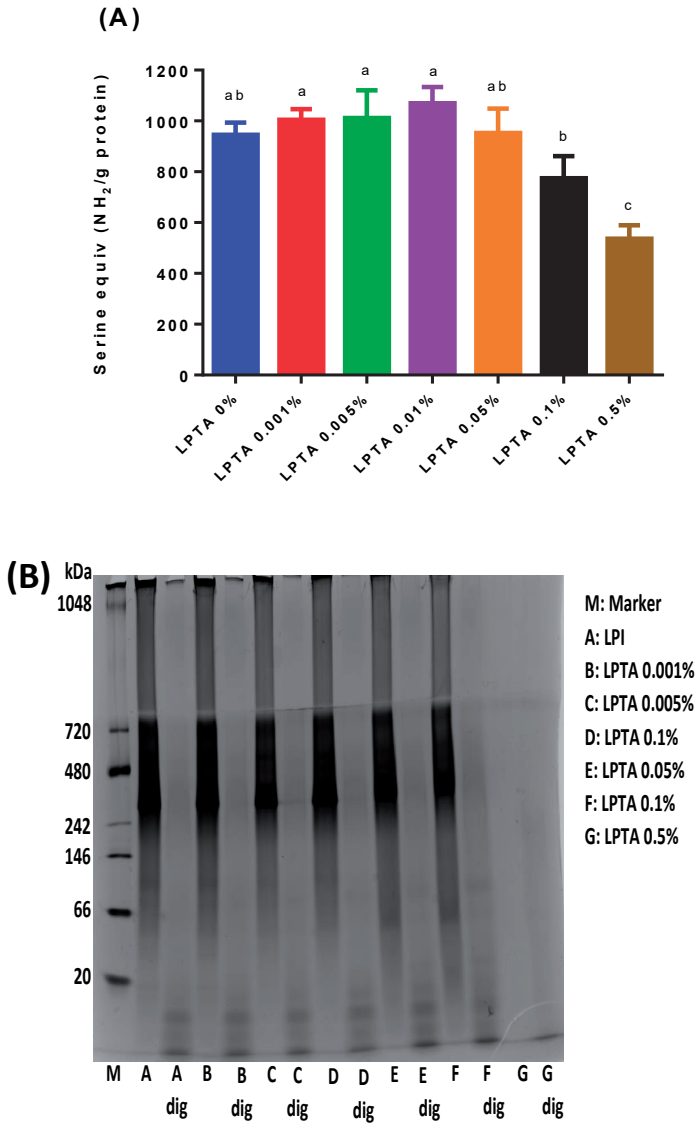

Figure 3.5: (A) Free amino nitrogen content after peptic digestion and **(B)** native polyacrylamide gel electrophoresis profiles of lentil protein/tannic acid mixtures.

evidently played an integral role in hindering hydrolysis of the proteins. In a BSA-TA mixture, *in vitro* protein digestibility of BSA was also hindered significantly.[10]

At 0.1% w/v TA, random hydrophobic stacking of LP and TA could have further obstructed peptic hydrolysis.[9] Gallotannin, which is a hydrolysable tannin, has been reported to effectively inhibit enzymatic activity even at low concentra-

tions, due to their high density and several hydroxyl groups.[7] Moreover, previous reports suggest that the inhibition of peptic digestion by TA is more towards blockage of the protein surface than retarding pepsin activity.[11] At low TA levels, the protein structures are altered by the hydrogen bonds occurring between LPTA, but the free amino groups were comparable to LPI. However, increasing to 0.1% and 0.5% w/v caused a significant decline. These results show that peptic hydrolysis was reduced because TA physically hindered access to cleavage sites. However, it cannot be excluded that some tannic acid may have bound to pepsin and contributed to the overall inhibitory effect. Tannins can bind to the free enzyme or the enzyme-substrate complex, although they bind more tightly to the free enzymes.[7]

From the Native PAGE, most prominent bands in LPI, and LPTA mixtures were observed slightly above 242 kDa. This represents legumin, which has molecular weight of 300–400 kDa. Faint bands observed beneath 146 kDa could be due to vicilin, which has a molecular weight range of 145–190 kDa.[36]. In addition, bands in the digested LPI sample shows the presence of low molecular weight peptides (<20 kDa) after peptic hydrolysis (Figure 3.5B). Prominent bands were also observed in digested samples of LPTA0.001–0.1%. These bands were also mostly below 20 kDa. However, at 0.1% w/v TA, the low molecular weight bands were less prominent, while other faint bands emerged slightly above 66 kDa. The LPTA0.5% wells had no bands, even after digestion. This confirms the formation of large complexes with molecular weights likely beyond 1,048 kDa, or those that exhibit low solubility in the buffer, hence the significantly lower digestibility observed in LPTA0.1% and LPTA0.5% compared to LPI.

3.3.4 Characteristics of high molecular weight hydrolysates:

High molecular weight hydrolysates obtained after pepsin digestion of LPTA0.5% and LPI (control) were characterized with LC-MS-MS. Due to an incomplete protein sequence database on UniProt for *Lens culinaris* subsp. culinaris (Cultivated lentil; Taxonomy identifier 362247), the tribe *Fabeae* (163743) encompassing 509 protein sequences was used as a reference for peptide identification.

Pepsin is described to exhibit a broad cleavage specificity, but preferably cleaves after hydrophobic and bulky residues such as phenylalanine and tyrosine to produce high molecular weight polypeptides.[37] Therefore, the MS spectra were processed with unspecific cleavage and a peptide length ranging from 6-25 residues. A total of 1,146 peptides were identified from the high molecular weight hydrolysates of LPI and LPTA0.5% (Figure 3.6A), with 589 overlapping peptides among the two samples. Therefore, more peptides were released from the high

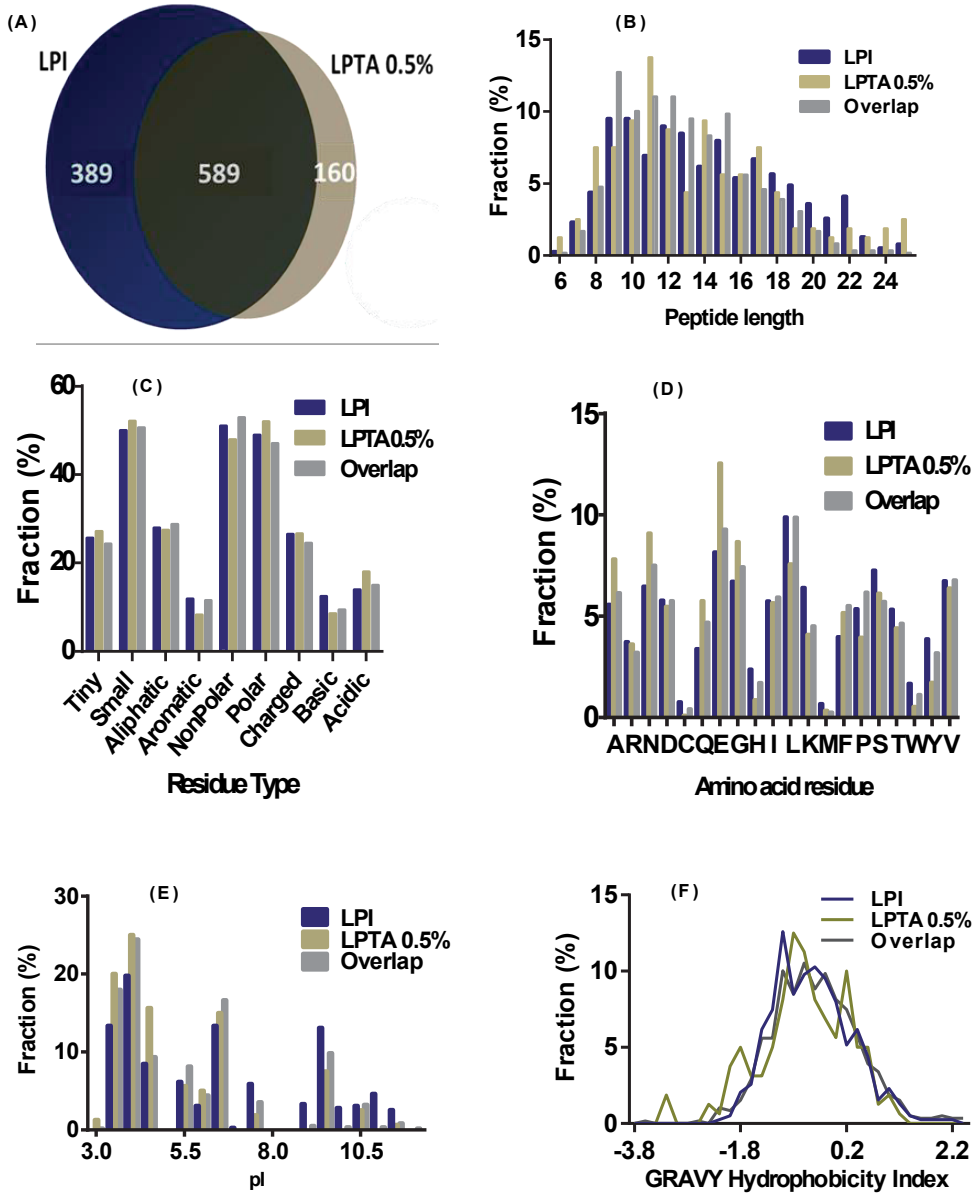

Figure 3.6: Characteristics of high molecular weight hydrolysates obtained after pepsin digestion of LPI (control), and LPTA0.5%. **(A)** Number of peptides released, **(B)** percentage of lengths of identified peptides, **(C)** percentage of residue group types, **(D)** distribution of amino acid residues of peptides, **(E)** distribution of peptide isoelectric point (pI), and **(F)** distribution of peptide hydrophobicity index (Kyte-Doolittle scale).

molecular weight fraction after digesting LPI with pepsin, whereas the complexes in LPTA0.5% reduced the cleavage of the native proteins. The principal proteins in lentils and globulins, comprising legumin and vicilin, have molecular weights of about 300–400 kDa and 145–190 kDa, respectively.[36] Hence, these proteins may not be completely hydrolyzed, thus releasing high molecular weight peptides. A concurring finding indicates that most of the pulse proteins that escape *in vitro* digestion are globulins, 7S vicilin and 11S legumins.[38]

Over 50% of the peptides identified in each sample have 8–14 residues (Figure 3.6B). Only 3.7% of the overlapping peptides had 20–25 residues, whereas 10.6% of the LPTA0.5% peptides were 20–25 long. Hence, over 50% 'small' peptides were produced in all three groups (Fig. 3.6C). Similar polar and non-polar fractions were observed for all the groups. Generally, the amino acid composition of LPI residues were comparable to the overlap peptides, except for a lower amount of Phe and a higher amount of Ser (Fig 3.6D). LPTA0.5% recorded slightly lower amounts of Pro, Tyr, Trp, and a considerably higher amount of Asn and Glu. This shows that LPTA complexes altered the distribution of amino acid residues released.

The physicochemical properties including isoelectric point (pI), and hydrophobicity of peptides were also estimated (Figure 3.6E & F). Majority of the peptides had a pI of 4.0. About 60.63% of LPTA0.5% peptides recorded a pI of 3.5–4.5, whereas 41.65% of LPI peptides were within this range. The grand average of hydropathicity (GRAVY) was estimated with the Kyte-Doolittle scale, where a score below 0 indicates hydrophilicity while values above 0 indicate hydrophobicity.[39] The most occurring score of the overlapping peptides, unique peptides in LPI, and peptides in LPTA0.5% were observed at -0.6, -1, and -0.8, respectively. Thus, peptides produced were mostly hydrophilic.

Finally, the cleavage motifs were compared among the three groups of peptides (Figure 3.7). Leu, Phe, and Glu were mostly involved in the cleavage motif of LPI and the overlapping peptides. On the other hand, Leu, Glu, Phe, Asn, and Asp were involved for LPTA0.5%. It was also observed that in LPTA0.5%, pepsin exhibited less specificity for major residues, Leu, and Phe, whereas Asn and Gln participated significantly in its cleavage window. Considering that pepsin prefers to cleave after Phe and Leu, the modified cleavage specificity observed in LPTA0.5% showed that the cleavage specificity of pepsin was modified by the presence of TA in LPTA0.5%, resulting in the decreased digestibility of LPTA0.5% compared to LPI.

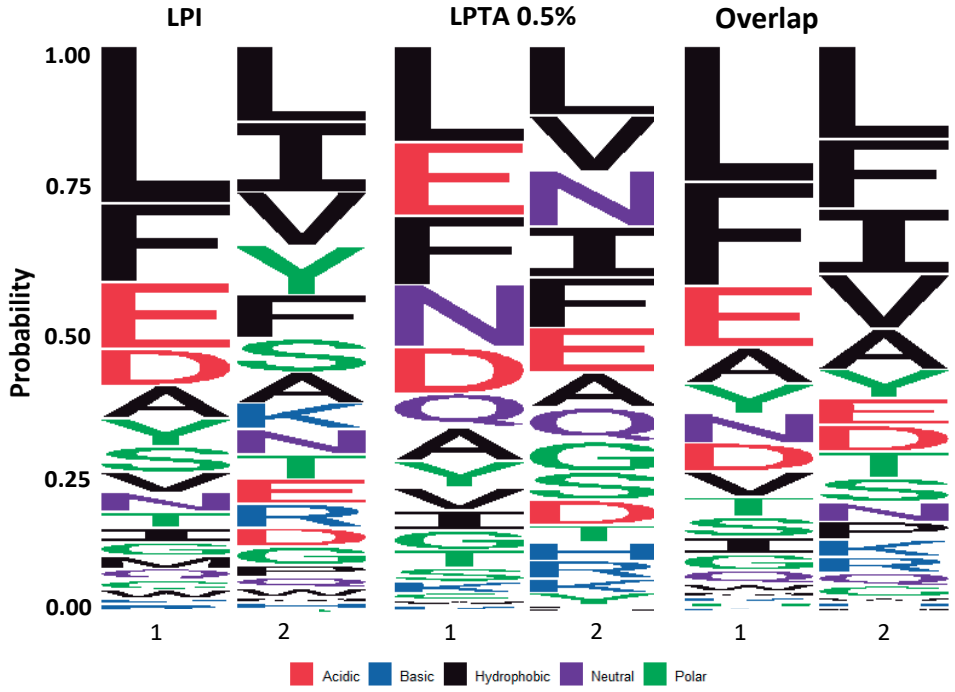

Figure 3.7: Consensus motifs of cleavage sequences displaying probability of residues to be on the N-terminal position or P1 **(1)** and C-terminal position or P1′ **(2)**.

Alphabets represent one-letter amino acid codes. Large alphabets represent higher occurrence of residue in identified peptides.

3.4 Conclusion

In conclusion, LPTA interaction yielded complexes with modified particle size. Based on fluorescence spectroscopy, it was observed that the interaction occurred within the protein hydrophobic folds. At 0.5% w/v TA, the secondary structure of LPI was modified and the thermal stability enhanced. The fluorescence imaging of the complexes showed that both biopolymers aggregated together, hence their signals co-existed in the same location. The decreased *in vitro* peptic digestion suggested that the LPTA complexes obstructed the protein surface and inhibited access of pepsin to the protein. The hydrolysates obtained after the pepsin digestion had mostly 8–14 residues. However, LPTA0.5% hydrolysates contained about three times more of larger peptides than LPI. Therefore, TA modified the cleavage specificity of pepsin, hence altering the type and size of peptide fragments released. Findings from this study confirm the possibility that LPTA interaction and its effect on digestibility are dependent on TA levels

in the matrix. For the first time, the peptides released after pepsin hydrolysis of LPI were characterized. This study provides a framework for further studies on peptides that resist the entire simulated *in vitro* digestion, which will provide in-depth understanding of the possible outcome of LPTA interaction on the distal gut region and gut microbiota.

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Notes

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Supplementary material:

Lentil Protein and Tannic Acid Interaction Limits *In Vitro* Peptic Hydrolysis and Alters Peptidomic Profiles of the Proteins

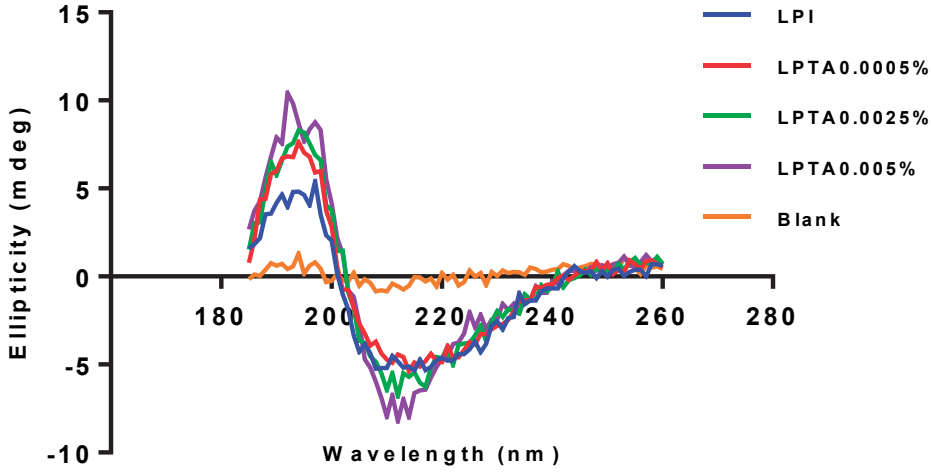

Figure 3.S1: Circular dichroism spectra of lentil protein isolate (LP) in the absence (LPI) and presence of different concentrations of tannic acid (LPTA).

The spectra was obtained with Jasco J-715 spectropolarimeter (Jasco International Corporation, Japan). MilliQ water was used as blank.

4

Chapter 4

Undigested glycated lentil proteins modulate the gut microbiota profile but not their metabolites *in vitro*

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Abstract

Glycation enhances plant protein techno-functionality; however, digestibility and the equilibrium between peptides absorbed and those reaching the colon can be altered. This study evaluated how undigested glycated lentil proteins, potentially reaching the colon affect the gut microbiota using batch fermentation and the Simulator of Human Intestinal Microbiome Ecosystem (SHIME®). Lentil protein-fructose mixtures were incubated at 60°C for 0, 24, or 48h (conjugates labelled LP+Fr0, LP+Fr1, LP+Fr2). Maillard reaction markers increased by over 10-fold and *in vitro* protein digestibility decreased by 23.5% after 48-h incubation. Short- and branched-chain fatty acids produced by 48h-fermentation of the insoluble hydrolysates of conjugates were comparable. LP+Fr2 hydrolysates caused 42% relative increase in Bacteroidetes in the proximal colon of Donor 1 whereas 26% increase was observed with LP+Fr0 hydrolysates. Bacteria population profile in the colon sections was differentially modulated depending on the donor. Our findings show that the extent of glycation does not affect short- and branched-chain fatty acid levels produced in the colon, while the effect on microbiota population is dependent on host and colon section.

Keywords: Maillard reaction, plant protein digestibility, gut microbiota, SHIME, lentil proteins, food protein fermentation, dietary Advanced Glycation end-products (dAGEs)

4.1 Introduction

To meet the protein needs of the increasing global population, the variety of food protein sources must be widened. Hence, plant protein sources, such as pulses, continue to receive attention as sustainable alternatives to traditional animal protein sources. Pulses are widely consumed, even in areas where food security is a challenge. Moreover, previous reports have shown that pulse protein hydrolysates can provide beneficial functions, such as blood glucose modulation by inhibiting the activities of α-amylase, α-glucosidase, and dipeptidyl peptidase IV [1,2]. The health benefits of pulse protein consumption have also prompted an increase in their acceptance in food products.

Beyond their nutritional value, the techno-functional properties of food proteins are relevant in food product applications. Traditional food applications of proteins have been from animal sources; however, due to the need for more sustainable alternatives, pulse proteins are being considered for their suitability in food product applications. The major challenge of pulse proteins is the compact globular structure of their predominant proteins and their high molecular weight which limits their techno-functionality [3,4]. For instance, pulse proteins are mostly seed storage proteins with hydrophobic compact structures, whereas milk and egg proteins are hydrophilic and hence more soluble [4].

The solubility of proteins is key in several functional properties, such as emulsification, foaming, gelling, and water absorption capacity [3,5]. Due to the low solubility of pulse proteins, their use in food products is challenging, hence physical, chemical, and/or biochemical modifications are needed to improve their functionality. Additionally, pulses have undesirable flavours. Harsh pH conditions used during pulse protein extraction can also cause structural changes [6]. Physical treatments, such as heating, can denature the proteins and expose the hydrophobic residues that are usually within the core of the protein structure.

Pulse proteins can be made more hydrophilic by changing the pH and net charge or forming conjugates with hydrophilic compounds, such as sugars and polysaccharides. Glycation has proven effective in improving the functional properties of plant proteins, since the amino group side chains of the proteins bind to the reactive sides of the sugars to produce hydrophilic products [5,7,8]. Glycation of the proteins occurs in the initial stage of Maillard reaction to produce intermediate Amadori products, and advances further to produce melanoidins as end products. Melanoidins are brownish nitrogenous polymers [3,9]. Previous reports show that the effect of glycation on the functional properties of the

proteins depends on the extent or stage of Maillard reaction. The early stage enhances the functional properties of the proteins, whereas advanced glycation yields off-flavours, reduced digestibility, and protein cross-linkages [3,7].

Glycation in foods can progress to the advanced stage of the Maillard reaction, where irreversible structural modification of the protein occurs, hence affecting protein digestibility [10,11]. After protein hydrolysis in the small intestine, partially hydrolysed and unabsorbed polypeptides move to the colon, where they can interact with the microbiota. If the proteins are modified, their digestibility and the nature of hydrolysates that reach the colon can be affected [11]. Additionally, this change can influence the metabolites and population profile of the gut microbiota [10,11]. Dominika *et al.* reported higher abundance of commensal bacteria when the gut microbiota was exposed to glycated pea proteins compared to non-glycated proteins, whereas levels of short- and branched-chain fatty acids produced were not significantly different [12].

Maillard reaction is common in processed foods with high protein content, whether animal sourced or plant-based, yet the effect of glycation on behaviour of proteins along the gastro-intestinal tract has not been extensively studied. This gap in literature is particularly notable for plant proteins. This study therefore aimed to evaluate the impact of glycation on the digestibility of lentil proteins as a model plant protein. Additionally, we studied the effect that the insoluble fraction of glycated lentil protein hydrolysates that potentially reach the colon has on gut microbiota metabolites and population, using batch fermentation and the Simulator of Human Intestinal Microbiome Ecosystem (SHIME®).

4.2 Materials and Methods

4.2.1 Materials

Dry green lentils (*Lens culinaris*) were purchased from a local grocery store in Wageningen, Netherlands. D-Fructose was purchased from Merck Life Science NV, Netherlands. Pepsin (from porcine gastric mucosa, ≥250 units/mg solid), pancreatin (from porcine pancreas, 8× USP specification), and bile extract (from porcine) were purchased from Merck Life Science NV, Netherlands. NuPAGE® LDS Sample buffer (4X), NuPAGE® SDS Running Buffer (20X), and NuPAGE®12% Bis-Tris Gel (1.0 mm, 10 Well) were purchased from ThermoFisher Scientific, Netherlands. Coomassie Brilliant Blue R-250 Staining Solution was purchased from Merck Life Science NV, Netherlands. BlueRay Prestained Protein Marker 10–180

kDa was purchased from Jena Bioscience GmbH, Germany. Ammonia Assay Kit (Rapid) was purchased from Megazyme Ltd, Netherlands.

4.2.2 Lentil protein extraction

Green lentil seeds were soaked in water (1:3 w/v) at 20°C overnight, freeze-dried, and milled into fine powder using a food processor. The flour was suspended in 0.05 M NaOH (10%, w/v), and pH was adjusted to 10 to solubilize the proteins. The suspension was stirred constantly for 4 h and then centrifuged at 7000 g for 30 min at 20 °C. The solubilized proteins in the supernatant were precipitated by adjusting pH of the supernatant to 4 and stirring for 2 h. pH was adjusted using 3 M HCl and 3 M NaOH. Afterwards, the suspension was centrifuged at 7000 g for 30 min at 20°C. The extracted lentil proteins (LP) were recovered as pellets and lyophilized. Prior to lyophilization, the pH was adjusted to 7. The protein content of LP was 67.67±0.20%, as determined by the Dumas method. The nitrogen**:** protein conversion factor of 5.5 was used as suggested by Mossé [13].

4.2.3 Glycation of lentil proteins

LP and D-fructose stock solutions (5% w/v) were prepared in deoinized water. Equal volumes of LPI and fructose stock solutions were mixed and lyophilized. The dry LP-fructose powder samples were incubated at 60°C in a desiccator with a relative humdity of 79% [14]. Relative humdity was achieved by adding saturated potassium bromide solution to the desiccator. The samples were incubated for 0, 24, and 48 h and hereafter referred to as LP+Fr0, LP+Fr1, and LP+Fr2, respectively. After incubation, samples were stored at –20°C until subsequent analysis.

4.2.4 Determination of particle size distribution

The particle size distribution of the glycated and non-glycated proteins were determined by dynamic light scattering using a Malvern Zetasizer. Sample solutions containing 1 mg/mL protein were prepared in Milli-Q water. A disposable cuvette was filled with a 1 mL aliquot, and the particle sizes were measured. Duplicate measurements were performed for each sample.

4.2.5 Evaluation of molecular weight profile of glycated proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular weight profile of the glycated and non-glycated LPs were evaluated by SDS-PAGE according to the NuPAGE® electrophoresis system manufacturer's instruction. First, 2 µL of LP, LP+Fr0, LP+Fr1, and LP+Fr2 (with protein concentration of 0.5–1 mg/mL) were mixed with 5 µL of LDS sample buffer and 15 µL MilliQ water. The mixture was centrifuged for 1 min at 20°C, 2000 rpm.

Thereafter, the samples were incubated at 70°C for 10 min and spinned again at 2000 rpm and 20°C for 1 min. The protein marker (5 µL) and samples (10 µL) were loaded unto the gel wells. The electrophoresis was run at 200 V for 35 min. The gel was then gently rinsed with water and stained with Coomassie Brilliant Blue R-250 Staining Solution for 1 h at 20°C. De-staining was performed overnight with a washing buffer (containing 10% ethanol, 7.5% acetic acid, and Milli Q water) until the background colour of the gel was removed.

4.2.6 Determination of Maillard reaction markers by LC-MS/MS

The levels of furosine, Nε-(carboxymethyl)-$_L$-lysine (CML), and Nε-(carboxyethyl)-$_L$-lysine (CEL) were determined using a high-pressure liquid chromatograph (HPLC) (Ultimate 3000, Thermo Scientific, USA). The HPLC was coupled with a TSQ Quantum triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, CA, USA). Prior to sample loading, 200 µL of 5 mg/mL of LP, LP+Fr0, LP+Fr1, and LP+Fr2 were hydrolysed under a fume hood in a capped heating tube containing 4 mL 6 M HCl [14]. The headspace of the tubes was saturated with nitrogen to limit oxidation. The tubes were incubated at 110°C for 22 h. After incubation, the sample was filtered with 0.2 µm PTFE filters, and 500 µL of the filtrate was transferred to an Eppendorf tube and dried completely with nitrogen gas. The dried samples were reconstituted in 490 µL of Milli Q water and mixed with 10 µL of internal standard mix to reach 25 ppm of d_4-Fur, d_4-CML and d_4-CEL [15]. Solid-phase extraction was performed, as described. [16]. The elutes were evaporated at 35°C and reconstituted in 200 µL 50% acetonitrile. The solution was centrifuged at 800 g and 20°C for 1 min. The supernatant was transferred into a vial, and 10 µL was loaded into the LCMS/MS system, as previously reported [14]. A reversed-phase core shell HPLC column was used for separation. The levels of furosine, CML, and CEL were determined using a calibration curve of known concentrations (0.025–10 ppm) standard against the corrected area (analyte peak area/internal standard peak area) [14,15].

4.2.7 Simulated *in vitro* gastrointestinal digestion

LP and glycated LP samples were subjected to the COST INFOGEST method of simulated *in vitro* gastrointestinal digestion [17]. In the oral phase, each 5 g of LP and glycated LP samples was mixed with 3.5 mL of simulated salivary fluid, 25 µL of 0.3 M $CaCl_2$, and 1.475 mL of MilliQ water. The mixture was incubated at 37°C for 2 min. Thereafter, the bolus (10 mL) was mixed with 7.5 mL simulated gastric fluid, and pH was adjusted to 3 using 1M HCl. A 1.6 mL aliquot of pepsin stock solution (25000 U/mL) was added to reach a concentration of 2000 U/mL. Additionally, 5 µL of 0.3 M CaCl2 was added. The final volume of the bolus was adjusted to 20 mL using MilliQ water and incubated at 37°C for 2 h with constant

shaking. After the gastric phase, the pH was adjusted to 7 using 1 M NaOH. For the intestinal phase, 11 mL of simulated intestinal fluid and 40 µL of 0.3 M $CaCl_2$ were mixed with the gastric chyme. Additionally, 2.5 mL of fresh bile stock solution and 5 mL of pancreatin stock solution were added to the chyme. The final concentrations of bile and pancreatin in the chyme were 10 mM and 100 U/mL (based on trypsin activity), respectively. The volume of the intestinal chyme was adjusted to 40 mL using MilliQ water and incubated for 2 h at 37°C with constant shaking. At the end of the intestinal phase, the digesta were immediately stored in a –20°C freezer to halt enzymatic activities.

4.2.8 Determination of degree of hydrolysis (DH)

The degree of hydrolysis after the intestinal phase of *in vitro* digestion was determined using the *o*-phthaldialdehyde (OPA) method [18]. First, the OPA reagent was prepared by adding 3.81 g sodium tetraborate to a flask containing 80 mL Milli Q water. Subsequently, 0.088 g dithiothreitol, 0.1 g sodium dodecyl sulphate, and 0.080 g OPA dissolved in 2 mL ethanol were added to the solution. Milli Q water was further added to the flask, resulting in a volume of 100 mL. L-serine (0.15–0.0125 mg/mL) was prepared and used for a standard curve. A 400-µL aliquot of the standard, blank (Milli Q water), or digested samples were added to 3 mL of the OPA reagent, mixed for 5 s, and incubated at room temperature for 2 min. The absorbance of the resulting solution was measured at 340 nm using a spectrophotometer (Varian Cary® 50 UV-Vis). DH was then determined as free amino groups from digestion/free amino groups from acid hydrolysis and expressed as a percentage. Acid hydrolysis was performed by mixing 5 g of samples (LP, LP+Fr0, LP+Fr1, and LP+Fr2) with 40 mL of 6 M HCl and incubating at 100°C for 24 h.

4.2.9 Acid precipitation of digesta

The pH of LP+Fr0, LP+Fr1, and LP+Fr2 digesta were adjusted to 4 under constant stirring for 30 min. The suspension was then centrifuged for 30 min at 7000 g and 20°C. The sediment (recovered as insoluble and high molecular weight peptides) was freeze-dried. The recovered insoluble high molecular weight hydrolysates of LP+Fr0, LP+Fr1, and LP+Fr2 were hereafter referred to as LP+Fr0-H, LP+Fr1-H, and LP+Fr2-H, respectively.

4.2.10 Batch fermentation

The high molecular weight hydrolysates from the glycated LPs (LP+Fr0-H, LP+Fr1-H, and LP+Fr2-H) were used in a batch fermentation set-up to evaluate their effect on the metabolism of gut microbiota. Buffered colon medium was prepared with 5.22 g K_2HPO_4, 16.32 g KH_2PO_4, 2 g NaHCO3, 2 g yeast extract, 2

g peptone, 1 g mucin, 0.5g L-cysteine HCl, and 2 mL Tween-80 in 1 L deionized water. A phosphate buffer was prepared with 8.8 g K_2HPO_4, 6.8 g, 0.1 g KH_2PO_4, and 0.1 g sodium thioglycolate in 1 L of deionized water. The medium, phosphate buffer, and penicillin bottles and their caps were sterilized before use.

Preparation of faecal inoculum:
Faecal samples were donated by three non-smoking healthy donors with normal body mass indices and no recent histories of antibiotic use. The faecal inoculum was prepared by mixing fresh faecal samples with phosphate buffer at 200 mg/mL in a stomacher bag. The mixture was homogenized with a mixer for 10 min at 300 rpm. The suspension was centrifuged at 500 g for 2 min at 20°C. The supernatant was recovered as the faecal inoculum. The faecal inoculum was prepared in a biosafety cabinet.

Thereafter, 21.5 mL medium and 10 mL sterile deionized water were added to the penicillin bottles and tightly closed with rubber caps and aluminium [19]. LP+Fr0-H, LP+Fr1-H, and LP+Fr2-H were added to reach a concentration of 1% w/v. Phosphate buffer was used as control. The bottles were flushed with nitrogen gas for 30 min to initiate anaerobic conditions. The faecal inoculum (3.5 mL) was added to the penicillin bottles. The bottles were incubated at 37°C under mild rotation for 48 h. The samples were obtained at 0, 6, 24, and 48 h for further analysis of microbial metabolites.

4.2.11 Simulator of the human intestinal microbial ecosystem (SHIME®)

From the levels of metabolites obtained from the batch fermentation, LP+Fr0-H and LP+Fr2-H were selected to evaluate the effect of partially digested glycated LPs in the proximal and distal colon. The SHIME® system (PRODIGEST, Belgium) was used to simulate the colon, as previously described [20]. Briefly, the triple SHIME® comprising two connected double jacketed vessels was used to simulate the proximal and distal colon. Three sets of PC and DC vessels were used for the basal medium (as control), LP+Fr0-H, and LP+Fr2-H.

Pre-stabilized faecal inocula from two non-smoking adult donors were used. Both donors had no history of probiotic or antibiotic use over the previous 6 months and no history of irritable bowel syndrome. The faecal inoculum was adapted to the SHIME® basal medium for 3 days before treatments were added. The basal medium comprised 1.2 g/L arabinogalactan, 2.0 g/L pectin, 0.5 g/L xylan, 0.4 g/L glucose, 3.0 g/L yeast extract, 1.0 g/L peptone, 3.0 g/L mucin, 0.5 g/L L-cysteine-HCl, and 4.0 g/L starch. PC vessels contained 500 mL at pH 5.6–5.9, and DC vessels contained 800 mL at pH 6.6–6.9. During the adaptation period, 140

mL of basal medium was added to the vessels every 8 h. After the adaptation, the treatment period of 10 days was initiated [21]. Growth media for the treatments were prepared by replacing 2.5 g/L starch in the basal medium with 2.5 g/L of either LP+Fr0-H or LP+Fr2-H. Every 8 h, 140 mL of growth medium was added to the system. Samples were taken on day 0, 1, 2, 3, 4, 7, 8, 9, and 10 at same time of the day. The aliquots were centrifuged at 9000 g, 4°C for 5 min. Supernatants and pellets were stored separately at –20°C until further analysis.

4.2.12 Determination of levels of short- and branched-chain fatty acids produced

Supernatants obtained from fermentation were filtered using 15 mm of 0.2 µm RC filters. Calibration standard solutions (10–750 ppm) of acetic acid, propionic acid, butyric acid, valeric acid, isobutyric acid, and isovaleric acid were prepared. The internal standard containing 0.45 mg/mL of 2-ethylbutyric acid (in 0.3 M HCl and 0.9 M oxalic acid) was used. The filtered samples (100 µL) were mixed with 50 µL of internal standard in vials. The levels of short chain fatty acids in the samples were measured using the Shimadzu GC2014 gas chromatograph (Kyoto, Japan) coupled with a flame-ionization gas detector (FID). Additionally, 1 µL of the mixture was injected into a capillary fatty acid-free Stabil wax-DA column (1 µm × 0.32 mm × 30 m) (Restek, Bellefonte, PA, USA).

4.2.13 Determination of levels of ammonia produced

Levels of ammonia produced were measured according to the microplate assay procedure provided by the kit manufacturer. Briefly, 210 µL of distilled water, 30 µL of assay buffer, and 20 µL of NADPH solution were added to blank wells. To the sample and standard wells, 10 µL of sample (supernatants obtained from fermentation) or standard, 200 µL of distilled water, 30 µL of assay buffer, and 20 µL of NADPH were added. The plates were mildly shaken to mix the solutions in the wells and incubated for 2 min. The absorbance at 340 nm was read after the initial incubation. Glutamate dehydrogenase solution (2 µL) was added to each well and incubated again for 5 min. The final absorbance was read at 340 nm. Ammonia levels present were calculated as indicated in the manufacturer's manual.

4.2.14 Determination of population profile

DNA was isolated from pellets obtained from the SHIME® samples using QIAamp PowerFecal Pro DNA Kit (Qiagen). The yield was quantified using Qubit HS Fluorescence (Invitrogen). Afterwards, 2.5 µL of the DNA material was used to amplify bacterial 16S rRNA gene variable V3 and V4 regions by polymerase chain reaction. The primers, Pro341F CCTACGGGNBGCASCAG and Pro805R GACTACNVGGGTATCTAATCC, were used with universal extension, as recommended by

the 16S Metagenomic Sequencing Library Preparation guidelines from Illumina, USA. After initial amplification of the 16S target region, amplicons were purified and analysed on a Bioanalyzer with DNA1000 chips. Indexing PCR was performed using Nextera UD index adapters from Illumina, USA. Barcoded amplicons were quantified using Qubit HS Fluorescence and the equimolar pooled. Sequencing was performed on the Illumina MiSeq instrument using v2 flow cell and chemistry with 4 pM library loading concentration. Paired end reads sequencing was obtained by 2*251 cycles. Base calling and subsequent data demultiplexing were performed using bcl2fastq v2.20.0.422. The Qiime2 platform (https://qiime2.org) was used to identify and classify taxonomic units. Diversity analyses were performed using the qiime2 diversity tool.

4.2.15 Statistical Analysis

Mean ± standard deviation of triplicate experiments was reported. Repeated measure analysis of variance (two-way) with post hoc Tukey test was used to determine significant differences between mean values at $P < 0.05$.

3. Results and Discussion

4.3.1 Particle size of glycated and non-glycated proteins

The average particle sizes of the native protein and glycated samples were measured to determine possible modification of their particle characteristics. Compared to the particle size of LPs, the particle size of the glycated samples increased as incubation time increased (Figure 4.1A). The highest increase occurred in LP+Fr2, where the average particle size was about twice that of LP particles. LP+Fr0 particles were comparable to those of LP. Considering the similarity in particle size of LP and LP+Fr0, the presence of fructose residues did not significantly influence the size of the proteins as much as the incubation temperature. The observed increase in LP+Fr1 and LP+Fr2 were likely due to aggregation from the incubation temperature of 60°C. This was further confirmed by the increase in average particle size as incubation time increased.

Feng *et al.* reported that due to aggregation, soy protein-glucose conjugates had larger particles than the unheated soy protein-glucose conjugates [14]. In another study, Yang *et al.* measured absorbance as an indicator of protein aggregation, and observed that absorbance of ovalbumin-glucose conjugates decreased as degree of glycation increased [11]. Wang *et al.* also observed that the particle size of egg white-isomaltooligosaccharide conjugates were larger than that of

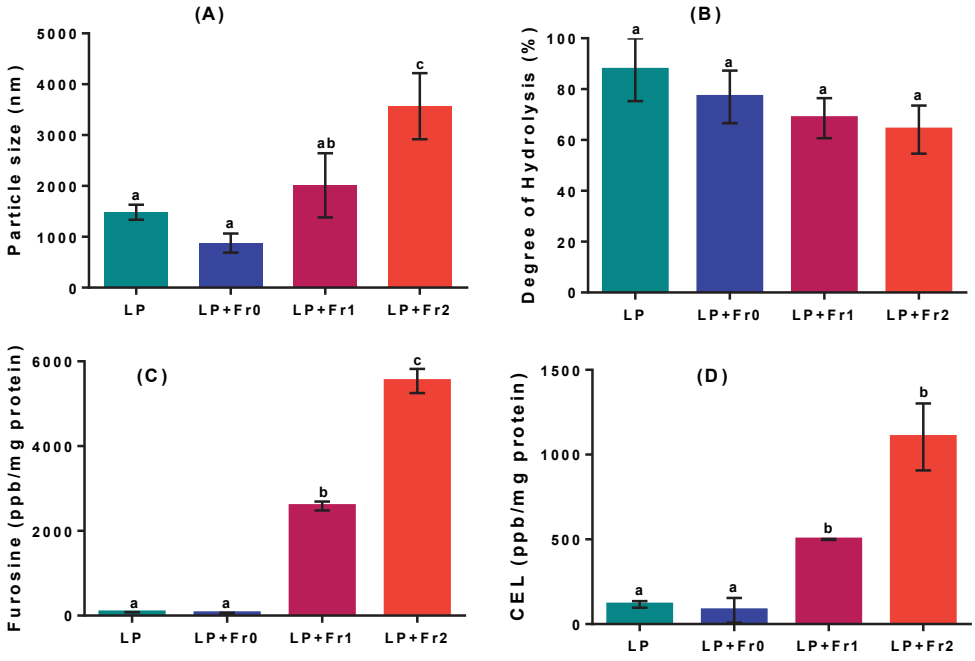

Figure 4.1. (A): Particle size of lentil protein and lentil protein-fructose conjugates **(B):** Degree of hydrolysis (%) of lentil protein-fructose conjugates after simulated ***in vitro*** gastrointestinal digestion **(C):** Levels of furosine in lentil protein-fructose conjugates **(D):** Levels of CEL in lentil protein-fructose conjugates.

Conjugates were prepared with dry lentil protein+fructose (1:1) mixture incubated at 60°C for 0, 24, or 48 h (referred to as LP+Fr0, LP+Fr1, and LP+Fr2). Different letters represent statistically significant differences ($P < 0.05$).

the native egg protein due to aggregation. However, the hydrodynamic diameter of conjugates was smaller than that of the native protein due to high electrostatic repulsion within the egg native protein [22]. The molecular weight range, hydrophilicity, and solubility of the proteins tend to favour the trend observed by Wang *et al.*

4.3.2 Maillard reaction markers

Condensation between the amino side chain of proteins and carbonyl group of reducing sugars forms Amadori products at the initial stage of the Maillard reaction. Acid hydrolysis of these products produces furosine which is then used as an indicator of the early stage of the Maillard reaction. CML and CEL are formed at the advanced stages of the reaction [23]. Therefore furosine, CML, and CEL were measured as indicators of the extent of Maillard reaction in the glycated LP samples.

The levels of furosine increased as incubation time increased, such that levels recorded in LP+Fr1 and LP+Fr2 were 30 times and 70 times higher than that of LP, respectively (Figure 4.1C). About a two-fold increase was observed from 24 h incubation to 48 h incubation, confirming that glycation of proteins is dependent on time and temperature of incubation. The levels of CEL in LP+Fr1 and LP+Fr2 were about 5 times and 10 times that of LPI, respectively (Figure 4.1D). Therefore, doubling the incubation time produced a two-fold increase in CEL levels, as observed in LP+Fr1 and LP+Fr2. CML levels were below detectable limits.

During the early stage of the Maillard reaction, the electrophilic carbonyl carbon of a reducing sugar binds to a free non-protonated amino group, such as ε-amino group of lysine, to form an imine. The imine, also known as Schiff's base, rearranges into more stable products known as Amadori or Heyns products, if the carbonyl group is from an aldose or ketose, respectively [24]. Furosine [ε-N-(furoylmethyl)-l-lysine] is an example of these stable products formed. After protein glycation in the early stage of the Maillard reaction, several reactions, including degradation, dehydration, and fission reactions, transform the stable Amadori/Heyns products into reactive compounds that further degrade, cyclise, enolise, oxidise, or rearrange to produce advanced glycation products [23]. CEL is from methylglyoxal and lysine and can be used as an indicator of the advanced stage of the Maillard reaction [3].

The extent of the Maillard reaction depends on several factors, including water activity, molecular weight, pH, temperature, and incubation time [14,25]. Our results show that the levels of Maillard reaction products can correspondingly increase with incubation time. Increasing the heating time allows for more interaction between the reducing sugar and free amino group. Moreover, considering the globular structure of the predominant proteins in lentils, heating can unfold the proteins, expose the reactive amino groups, and speed up conjugation. The onset temperature of thermal denaturation of lentil proteins have been reported to occur around 70 °C and peak around 80 °C [26]. The reactive form of sugars, which is the open chain form, also increases at high temperatures. The unfolding effect of heat is confirmed by the comparable levels of furosine and CEL in LP and LP+Fr0. These samples were not exposed to heat as the other samples. Additionally, the pH of all the samples was neutral (pH~7); hence, both the carbonyl groups and free amino groups existed in their reactive forms. The neutral pH eliminates the possible marked contribution from different pH conditions in samples. Feng *et al.* also observed a substantial increase in furosine and CEL levels in a soy protein-glucose system produced under similar conditions as the present study [14].

4.3.3 Degree of protein hydrolysis after simulated *in vitro* gastrointestinal digestion

The extent of hydrolysis after digestion was measured by determining the fraction of amino groups that has been cleaved from the protein backbone. The DH of LP was the highest (87.62% ± 12.33), followed by LP+Fr0. The lowest was observed in LP+Fr2, with DH of 64.10% ± 9.44. DH decreased as the incubation period increased, although without statistical significance. The increase in particle size of LP+Fr1 and LP+Fr2 and the corresponding decrease in their DH suggest that aggregation contributed to the decrease in digestibility.

From our results, the presence of fructose slightly reduced the DH after *in vitro* digestion. Condensation reactions that occur during glycation, even at the initial stage of the Maillard reactions, involve covalent interactions and cross-linkages that yield products that are less susceptible to proteases [27,28]. The structural change around the reacting amino group likely reduces the proteins' susceptibility to proteases in the pancreatin used in the intestinal phase. For instance, trypsin is known to cleave the carboxyl end of lysine and arginine. However, the ε-amino groups of lysine are known as the main amino groups involved in the Maillard reaction amongst others, such as the guanidine group in arginine, imidazole in histidine, and indole from tryptophan [25]. Additionally, leguminous proteins have high levels of arginine and lysine. Hence, glycation at the lysine residues can decrease the digestibility of the glycated samples. Although the possible mechanism by which digestibility was reduced in our study was not evaluated, it is likely that access of proteases to peptide bonds was reduced by modification of cleavage sites involving carboxyl groups of lysine and arginine, and steric hindrance caused by the condensation and crosslinking reactions. Glycation has been reported to reduce digestibility of casein and ovalbumin such that ovalbumin's digestibility decreased proportionally to the extent of glycation [11,29].

DH in the heat-incubated samples (LP+Fr1 and LP+Fr2) were slightly lower than the levels in the unheated samples (LP and LP+Fr0). High temperatures have been reported to enhance the rate of the Maillard reaction to produce more digestion-resistant end products. The reactive open form of sugars commonly occurs at high temperatures, and globular proteins also unfold at elevated temperatures. The incubation temperature (60 °C) used in our study is slightly below the onset temperature of thermal denaturation reported in literature (~70 °C) [26], however it is likely that unfolding of the globular proteins was initiated during the 24-h and 48-h incubation period, and thus proteins were slightly unfolded. Therefore, the decreased DH in the heated samples could be due to the

presence of more Maillard reaction products and possible aggregation at the incubation temperature.

3.4 Molecular weight profile of glycated and non-glycated proteins by SDS-PAGE

The molecular weight profile of LP shows bands at 17–100 kDa (Figure 4.2A). Bands observed around 25 kDa and 48 kDa correspond to basic and acidic subunits of legumin, respectively. The band that is slightly above 63 kDa corresponds to vicilin trimers held by hydrophobic interactions. Convicilin was observed around 75–100 kDa [30]. The profile of LP+Fr0 was comparable to that of LP. LP+Fr0 was mixed with fructose but was not heated. However, after incubating at 60 °C for 24 h and 48 h, predominant bands corresponding to 63–75 kDa were faint in LP+Fr1 and even fainter in LP+Fr2. The incubation of these large proteins likely caused the globular structure to unfold and expose the reactive sites for glycation. The glycation likely progressed to the advanced stages to produce large MRPs that do not bind to SDS nor migrate through the gel, hence the reduced intensity of the bands. Comparably, disappearance of bands in a pea protein-gum arabic complex has been reported [7]. Bands for the low molecular weight proteins (17–25 kDa) in LP+Fr1 and LP+Fr2 almost disappeared. The decrease of the 17–25 kDa bands suggests that these low molecular weight proteins were likely involved in the condensation reaction between their free amino groups and the carbonyl groups of fructose.

Condensation between the proteins and fructose and the possible interprotein cross-linkages are expected to cause an increase in the molecular weight of the proteins. However, the advanced stage of the Maillard reaction involves complex reactions that can yield either low or high molecular weight products. The observed brown colour of the glycated samples indicates the presence of melanoproteins which are formed by cross-linkage between proteins and MRPs [31,32]. Melanoidins can possess high molecular weights and low solubility; hence, it is likely that they did not migrate along the gel [7]. Overall, these results show that glycation modified the molecular weight profile of the proteins.

Digested LP (LP-dig) showed bands around 11 kDa, and these bands were not present in the undigested sample. Additionally, prominent bands were observed around 25 kDa and 63 kDa. The prominent bands at 63–75 kDa in the undigested sample disappeared after digestion. However, the only prominent band in the digested glycated samples (LP+Fr1 and LP+Fr2) was around 25 kDa and became fainter as incubation time increased.

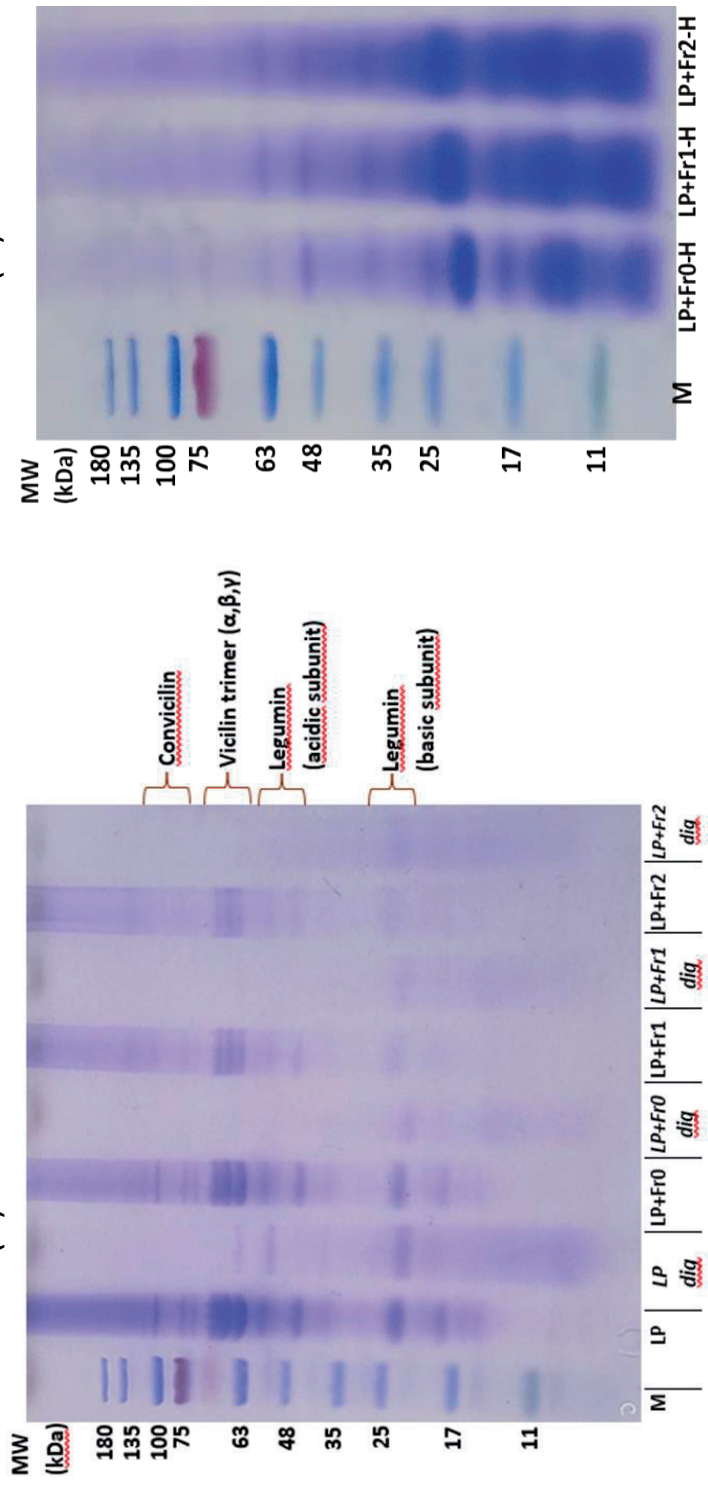

Figure 4.2. Molecular weight (MW) profile of **(A):** non glycated lentil protein (LP), glycated lentil proteins (LP+Fr0, LP+Fr1, and LP+Fr2), and their digests (LP *dig*, LP+Fr0 *dig*, LP+Fr1 *dig*, and LP+Fr2 *dig*). **(B):** Insoluble hydrolysates obtained from acid precipitation of digested LP+Fr0, LP+Fr1, and LP+Fr2 labelled as LP+Fr0-H, LP+Fr1-H, and LP+Fr2-H.

MW was evaluated with SDS-PAGE under non-reducing conditions.

4.3.5 Metabolites produced after batch fermentation of glycated and non-glycated lentil proteins

Insoluble high molecular weight hydrolysates from *in vitro* digestion of the glycated LPs (LP+Fr0, LP+Fr1, and LP+Fr2) were subjected to batch fermentation to evaluate how the extent of glycation affects metabolites produced by the gut microbiota. The insoluble hydrolysates were used as a representation of the fraction reaching the colon. Same amount of hydrolysates (1% w/v) was added to the fermentation vessels irrespective of the extent of the glycation and degree of hydrolysis.

The levels of SCFAs and BCFAs produced from the glycated proteins over the 48-h fermentation period were comparable, except for the slight differences observed in propionic acid and butyric acid at 48 h (Table 4.1**)**. Production of BCFAs evidently began after 6 h. BCFA production indicates that proteins are the only available carbon source in the substrate [33,34]. Carbohydrates are the primary and preferred substrate for the gut microbiota; therefore, when carbohydrates are available, protein fermentation is restricted. This confirms the relative delay in production of BCFAs until the carbohydrates in the medium were likely exhausted [35].

The levels of ammonia increased significantly at 6 h and 24 h, after which levels remained fairly constant. The increase observed at 24 h was higher in LP+Fr0-H than in the other hydrolysates. Overall, the effect of time was statistically significant (P <0.0001), whereas the effect of treatment was not (P = 0.71).

Dominika *et al.* observed a higher production of acetic acid after *in vitro* fermentation of non-glycated pea proteins than glycated pea proteins, although the difference was not statistically significant [12]. Additionally, Yang *et al.* recorded a higher production of acetate and propionate after *in vitro* fermentation of 24-h glycated fish protein hydrolysates [36]. However, our results do not show any remarkable differences between the glycated and non-glycated proteins. This difference could be because we used the insoluble fraction after digestion, whereas these studies used the whole digests. The insoluble fractions were obtained as pellets from acid precipitation of the digested samples, mainly consisting of high molecular weight polypeptides. Large peptides are initially hydrolysed by proteolytic bacteria to produce free amino acids before subsequent catabolism into bacterial metabolites, unlike in the case of amino acids in the whole digests used in the other studies, [37].

Table 4.1: Increase in short- and branched-chain fatty acids recorded after supplementing growth medium with LP+Fr0-H, LP+Fr1-H, and LP+Fr2-H in a 48 hour-batch fermentation.

Concentration (mM)	Time (h)	Control (Basal medium)	LP+Fr0-H	LP+Fr1-H	LP+Fr2-H
Short-chain fatty acids					
Acetic acid	6	2.41 ± 0.51^{a}	6.09 ± 1.93^{a}	5.56 ± 1.38^{a}	5.62 ± 1.44^{a}
	24	0.76 ± 0.74^{a}	7.31 ± 2.47^{ab}	6.87 ± 1.29^{ab}	6.89 ± 1.25^{ab}
	48	1.69 ± 0.60^{a}	10.14 ± 3.00^{b}	10.53 ± 2.36^{b}	10.93 ± 1.95^{c}
Propionic acid	6	0.34 ± 0.15^{a}	0.13 ± 0.08^{a}	0.16 ± 0.05^{a}	0.18 ± 0.11^{a}
	24	0.68 ± 0.19^{a}	3.53 ± 0.83^{b}	3.22 ± 0.39^{b}	2.98 ± 0.35^{b}
	48	0.46 ± 0.65^{a}	5.68 ± 1.68^{b}	5.33 ± 1.58^{b}	5.35 ± 1.01^{b}
Butyric acid	6	<0.01	<0.01	0.01 ± 0.01	0.01 ± 0.01^{a}
	24	0.01 ± 0.01^{a}	0.07 ± 0.11^{a}	0.05 ± 0.08^{a}	0.05 ± 0.08^{a}
	48	nd	0.50 ± 0.42^{a}	0.38 ± 0.63^{a}	0.59 ± 0.53^{b}
Valeric acid	6	nd	nd	nd	nd
	24	nd	0.16 ± 0.28^{a}	0.12 ± 0.21^{a}	0.11 ± 0.20^{a}
	48	nd	0.70 ± 1.20^{a}	0.82 ± 1.42^{a}	0.81 ± 1.40^{a}
Total SCFA	6	2.75 ± 0.62^{a}	6.23 ± 2.01^{a}	5.72 ± 1.44^{a}	5.80 ± 1.56^{a}
	24	1.45 ± 0.92^{a}	11.07 ± 3.40^{b}	10.26 ± 1.70^{b}	10.03 ± 1.55^{b}
	48	2.00 ± 1.12^{a}	17.01 ± 3.23^{c}	15.29 ± 3.11^{c}	17.68 ± 1.08^{c}
Branched-chain fatty acid					
Isobutyric acid	6	nd	nd	nd	nd
	24	nd	nd	0.12 ± 0.20^{a}	0.10 ± 0.18^{a}
	48	nd	nd	0.46 ± 0.80^{a}	0.46 ± 0.80^{a}
Isovaleric acid	6	0.04 ± 0.07^{a}	nd	0.16 ± 0.28^{a}	nd
	24	0.07 ± 0.12^{a}	0.57 ± 0.90^{a}	0.62 ± 0.52^{a}	0.47 ± 0.55^{a}
	48	0.38 ± 0.67^{a}	1.48 ± 1.79^{a}	1.65 ± 2.23^{a}	1.73 ± 2.10^{a}
Total BCFA	6	0.04 ± 0.07^{a}	nd	0.16 ± 0.28^{a}	nd
	24	0.07 ± 0.12^{a}	0.57 ±0.90^{a}	0.73 ± 0.72^{a}	0.57 ± 0.73^{a}
	48	0.38 ± 0.67^{a}	1.48 ± 1.78^{a}	2.12 ± 3.03^{a}	2.19 ± 2.89^{a}

LP+Fr0-H, LP+Fr1-H, and LP+Fr2-H are the insoluble hydrolysates obtained from acid precipitation of digested LP+Fr0, LP+Fr1, and LP+Fr2, respectively. Basal growth medium was used as control. Different superscripts indicate statistical difference ($P < 0.05$) between hydrolysates at different timepoints. nd: not detected

Table 4.2: Increase in levels of ammonia produced after supplementing growth medium with LP+Fr0-H, LP+Fr1-H, and LP+Fr2-H in a 48 hour-batch fermentation.

Time (h)	Concentration (mg/mL)			
	Control (basal medium)	LP+Fr0-H	LP+Fr1-H	LP+Fr2-H
6	15.28 ± 10.04[a]	24.00 ± 12.58[a]	20.56 ± 10.41[a]	18.51 ± 9.91[a]
24	28.68 ± 12.35[b]	44.41 ± 11.94[b]	31.44 ± 13.77[b]	33.24 ± 4.98[b]
48	30.72 ± 13.49[b]	37.57 ± 13.77[b]	36.40 ± 11.99[b]	35.23 ± 15.28[b]

LP+Fr0-H, LP+Fr1-H, and LP+Fr2-H are the insoluble hydrolysates obtained from acid precipitation of digested LP+Fr0, LP+Fr1, and LP+Fr2, respectively. Basal growth medium was used as control. Different superscripts indicate statistical difference ($P < 0.05$) between hydrolysates at different timepoints.

Proteins can escape complete hydrolysis due to reduced access of the proteases. These undigested or partially digested peptides move to the large intestines where the microbiota can further metabolize them. Conversion of peptides to microbial metabolites involves microbial peptidase activity, deamination, and decarboxylation [38]. The predominant pathway involves deamination of the amino acids to produce SCFAs and ammonia. BCFAs are formed from deamination of branched-chain amino acids, such as leucine, isoleucine, and valine. The microbiota can also decarboxylate amino acids to produce amines and carbon dioxide [39,40]. Acetate can be produced from glutamate, glycine, proline, histidine, cysteine, and lysine. Propionate is mainly produced from aspartate and threonine, whereas butyrate can be formed from glutamate, alanine, methionine, and lysine. Additionally, butyrate can be produced from glutamate, alanine, lysine, serine, and methionine (Fan et al., 2015). Considering that lysine and arginine can serve as substrates for SCFAs, condensation between these amino acid residues and reducing sugars can reduce levels of SCFAs produced from fermentation. However, our results do not confirm the hypothesis that LP+Fr2 should have low SCFA due to the extent of glycation. This finding is in line with recent studies showing that Maillard reaction products resist digestion, can be transported to the colon [10], and are degraded within 24 h of fermentation [41]. Additionally, Bui *et al.* reported that an isolated pure culture produced butyrate from N-ε-fructoselysine [42]. This suggests that the Maillard reaction products in LP+Fr2 were fermented by the gut microbiota.

4.3.6 Population profile and diversity of the gut microbiota using the SHIME®

The effect of glycation of LPs on the population profile of the gut microbiota in the proximal and distal colon selections were evaluated by fermenting LP+Fr0-H and LP+Fr2-H for 10 days in the SHIME®. LP+Fr0-H and LP+Fr2-H were selected

to evaluate the effect of extent of glycation, since no sizeable differences in metabolites were observed among LP+Fr0-H, LP+Fr1-H, and LP+Fr2-H. The richness and abundance of the population were evaluated by Alpha diversity based on phylogenetic diversity (Figure 4.3A) and Beta diversity based on Bray-Curtis distance (Figure 4.3B). No significant differences were observed between the diversity in the population from fermentation of LP+Fr0-H and LP+Fr2-H. However, notable differences were observed between the colon sections, such that the alpha diversity index of the proximal colon was lower than the median in both treatments and timepoints. Bray-Curtis distances also clustered based on the colon section (Figure 4.3B). Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria were the predominant phyla observed in all samples (Figure 4.4A).

The relative abundance of Firmicutes reduced in both proximal and distal colon sections in Donor 1. The decrease, however, was higher in the proximal section of LP+Fr2-H, with about 20% relative decrease compared to about 3.7% decrease observed in LP+Fr0-H. In the distal colon of Donor 2, slight increases were observed. LP+Fr0-H and LP+Fr2-H caused about 26% and 42% increase, respectively, in Bacteroidetes in the proximal colon of Donor 1. Firmicutes are known to preferably ferment oligosaccharides rather than peptides, hence the decrease observed [43]. Although starch might be the primary and preferred substrate, peptides provide fermentable carbon substrates in the distal colon. The distal colon has low carbohydrate levels due to their depletion in the proximal colon [35,44], hence the low abundance of Firmicutes. Consequently, the distal gut is colonized by amino acid fermenting groups, such as *Bacteroides*, *Clostridium*, *Fusobacterium*, *Streptococcus*, *Lactobacillus*, and *Peptococcus sp.* that hydrolyze peptides that reach the large intestine [35].

At the family level, Bacteroidaceae increased by about 25% and 40% in LP+Fr0-H and LP+Fr2-H, respectively (Figure 4.4B). LP+Fr2-H seems to favour the growth of Bacteroidetes. The increase conforms to findings from Aguirre *et al.,* where Bacteroidetes increased significantly during fermentation of a high-protein diet [45] . Our results show that LP+Fr0-H and LP+Fr2-H differentially modulated the bacteria population profile in both colon sections depending on the donor. The differences observed after fermentation of LP+Fr0-H and LP+Fr2-H were mainly in the relative abundance of Firmicutes, Bacteroidetes, and Verrucomicrobia. Additionally, Yang *et al.* observed a modulatory effect of glycated fish protein where relative abundance of Firmicutes and Bacteroidetes reduced compared to the non-glycated fish protein [46]. This effect was attributed to higher levels of sugar substrates in the glycated proteins than in the native fish protein. Notably, Yang *et al.* fermented the whole digesta, whereas the insoluble undigested

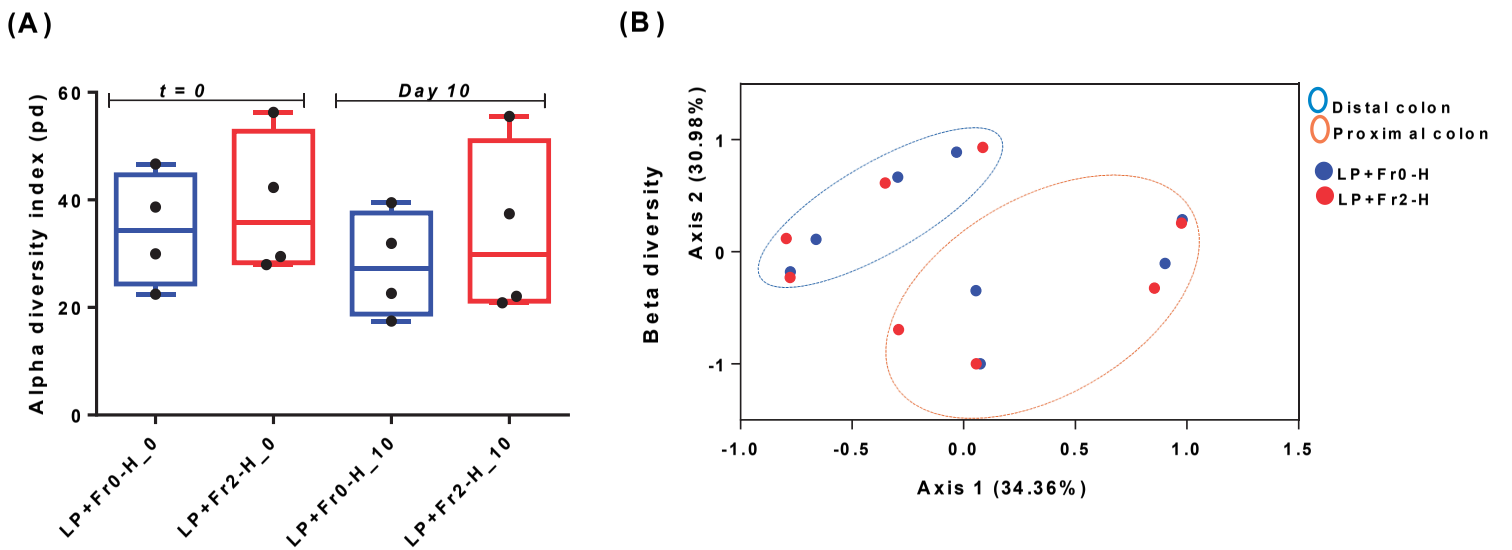

Figure 4.3. Diversity measures from 16S rRNA gene sequencing results after supplementing growth medium with insoluble hydrolysates (LP+Fr0-H and LP+Fr2-H) in a 10 day-fermentation with SHIME®. **(A):** Alpha diversity based on phylogenetic diversity of microbial population **(B):** Beta diversity based on Bray-Curtis distance between treatments.

LP+Fr0-H and LP+Fr2-H are the insoluble hydrolysates obtained from acid precipitation of digested LP+Fr0 and LP+Fr2, respectively. *LP+Fr0-H_0* are results from initial sampling (t=0) whereas *LP+Fr0-H_10* are from final sampling (day10).

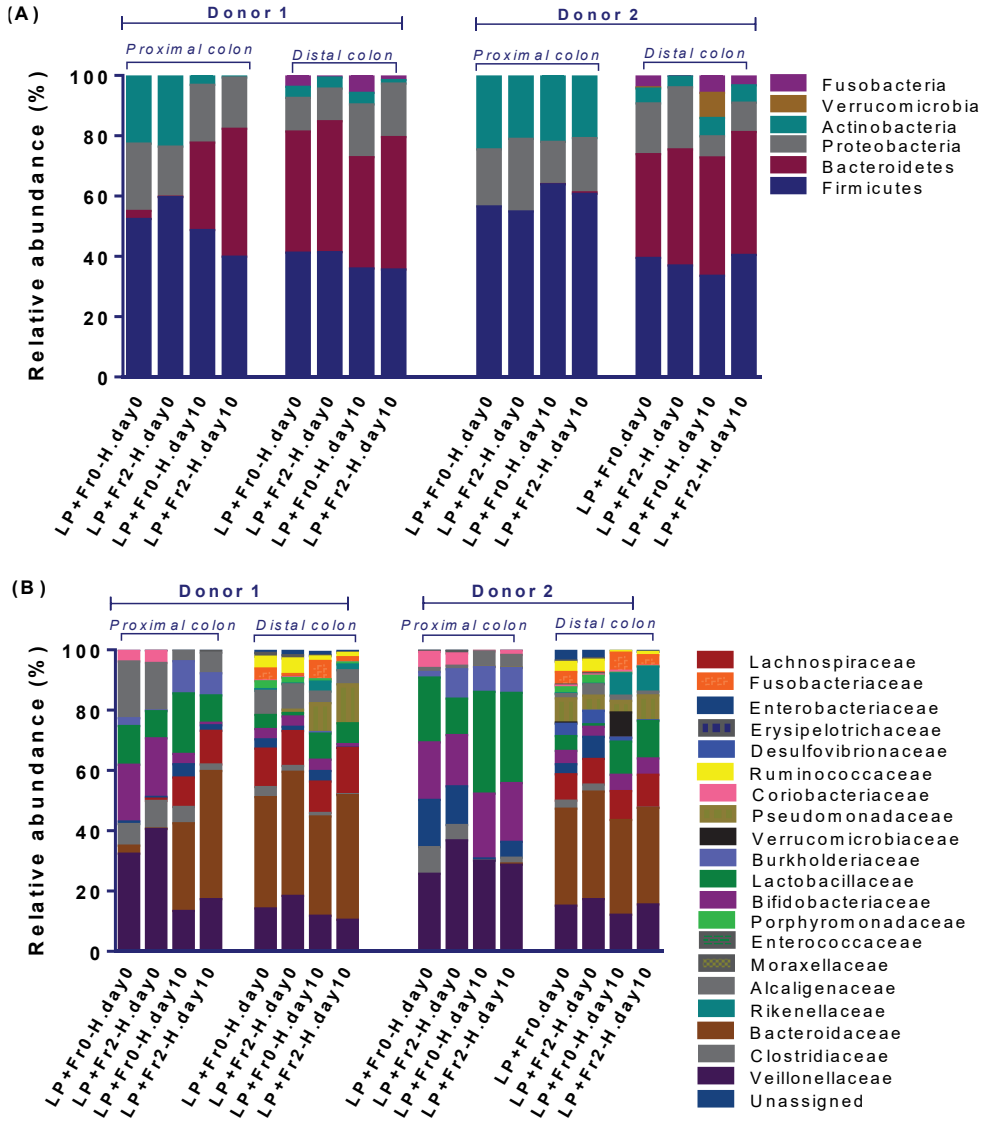

Figure 4.4. Population profile from 16S rRNA gene sequenced after supplementing growth medium with insoluble hydrolysates (LP+Fr0-H and LP+Fr2-H) in a 10 day-fermentation with SHIME®. **(A):** Relative abundance at phylum level **(B):** Relative abundance at family level.

LP+Fr0-H and LP+Fr2-H are the insoluble hydrolysates obtained from acid precipitation of digested LP+Fr0 and LP+Fr2, respectively.

fraction (from acid precipitation) of the digesta was used in our study to mimic the fraction that reaches the colon. The main difference between LP+Fr0-H and LP+Fr2-H could be the type of peptides produced post-digestion due to the extent of glycation and the levels of Maillard reaction products. The SDS-PAGE

showed that bands around 25 kDa were more prominent and slightly higher for LP+Fr2-H than the same bands in LP+Fr0-H (Figure 4.2B).

Similar to the batch fermentation, no significant differences in the levels of SCFA, BCFA, and ammonia were observed between LP+Fr0-H and LP+Fr2-H (Figures S1, S2, and S3 in Supplementary material). This suggests that either the substrates from these hydrolysates were similar and were catabolized similarly or the Maillard reaction products in LP+Fr2 were catabolized to produce SCFA, BCFA, and ammonia. The sugar residues were likely cleaved prior to bacterial proteolytic activity [47]. Bui *et al.* reported that isolated Intestinimonas strain AF211 is able to convert fructoselysine into butyrate [42]. In another study by Borelli and Fogliano (2005), Bifidobacteria recorded substantial proliferation compared to Clostridia, Streptococci, and Enterobacteriacea when exposed to melanoidins. Although inconclusive, the authors suggest that Bifidobacteria use these melanoidins as source of carbon and nitrogen for their metabolism, hence the growth recorded [48].

Considering the conditions used in the SHIME® fermentation set-up in this study, a growth medium that contains only glycated protein hydrolysates and no other source of nitrogen, such as peptone would have given further insights into the effect of glycation on gut microbiota metabolism and the population profile. This study supplemented the SHIME® growth media with glycated protein hydrolysates at a standardized concentration. The difference between *in vitro* protein digestibility of the LP+Fr0 and LP+Fr2 did not substantially influence the amount of insoluble pellets produced after digestion, thus, we mainly focused on how extent of protein glycation affected gut microbiota population profile and metabolites.

4.4 Conclusion

In this study, we evaluated the effect of glycation on plant protein digestibility. We further studied the effect of glycated plant protein hydrolysates that might reach the colon on gut microbiota metabolites and population using batch fermentation and SHIME®. Lentil protein was selected as a model plant protein. As the incubation period of glycation increased, the digestibility decreased, though no statistical significance was recorded. A decrease in digestibility is mainly attributed to the aggregation of the proteins. The molecular weight profile was modified by glycation of the proteins. However, this modification in the protein profile did not cause a significant difference in the levels of metabolites

produced during in vitro colonic fermentation. Additionally, no significant differences were observed in the diversity of the microbial population after a 10-day fermentation of the insoluble high molecular weight hydrolysates. The 16S rRNA gene sequencing showed that the hydrolysates differentially modulated donors' microbiota profile in the proximal and distal colon sections.

The main difference among the hydrolysates is the type of peptides produced post-digestion due to the extent of glycation and the amount of Maillard reaction products. Therefore, we postulate that the substrates from these hydrolysates are similar and catabolized similarly or the Maillard reaction products from the highly glycated proteins were also catabolized to produce SCFA, BCFA, and ammonia. Our findings show that the extent of glycation does not affect the metabolites produced from their fermentation in the colon, yet the effect on the microbiota population is dependent on the host and colon section. Higher temperature and longer incubation time might produce more extensively cross-linked glycated proteins and Maillard reaction products that might be utilized differently by the gut microbiota. Evaluation of the effect of harsher glycation conditions on the gut microbiota is needed. Additionally, further studies of how these metabolites affect the host's metabolism are warranted.

Ethics statement

Authors declare that no human or animal subjects were included in this study.

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Declaration of Competing Interest

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

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Supplementary material:

Glycated lentil protein hydrolysates modulate the gut microbiota and not their metabolites *in vitro*

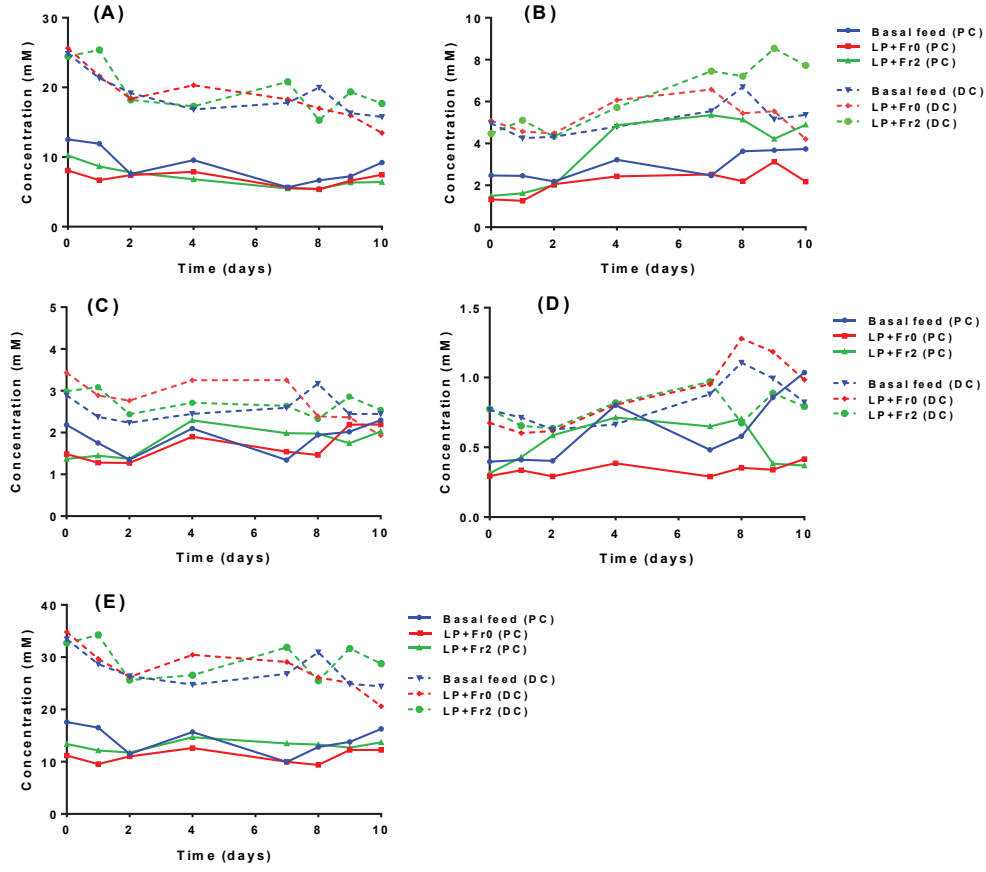

Figure 4.S1: Short-chain fatty acids produced after supplementing growth medium with insoluble hydrolysates (LP+Fr0-H and LP+Fr2-H) in a 10-day fermentation with SHIME. **(A):** Acetic acid **(B):** Propionic acid **(C):** Butyric acid **(D):** Valeric acid **(E):** Total Short chain fatty acids.

LP+Fr0-H and LP+Fr2-H are the insoluble hydrolysates obtained from acid precipitation of digested LP+Fr0 and LP+Fr2, respectively. Basal growth medium was used as control. PC: Proximal colon DC: Distal colon. For all short-chain fatty acids measured, no significant difference ($P > 0.05$) was observed between hydrolysates at different timepoints.

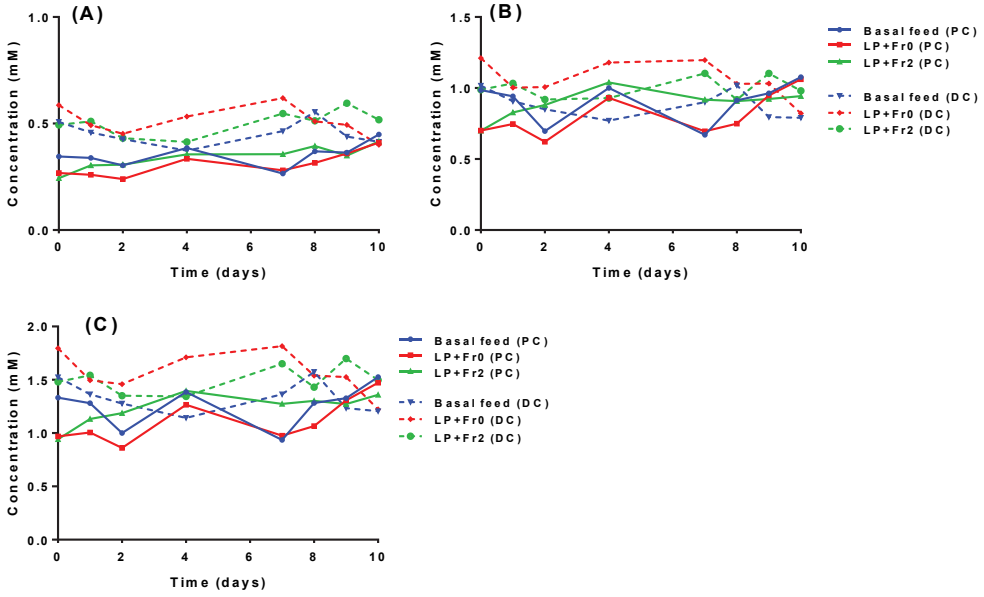

Figure 4.S2: Branched-chain fatty acids produced after supplementing growth medium with insoluble hydrolysates (LP+Fr0-H and LP+Fr2-H) in a 10-day fermentation with SHIME. **(A):** Isobutyric acid **(B):** Isovaleric acid **(C):** Total branched chain fatty acids.

LP+Fr0-H and LP+Fr2-H are the insoluble hydrolysates obtained from acid precipitation of digested LP+Fr0 and LP+Fr2, respectively. Basal growth medium was used as control. **PC:** Proximal colon **DC:** Distal colon. For both branched-chain fatty acids measured, no significant difference ($P > 0.05$) was observed between hydrolysates at different timepoints.

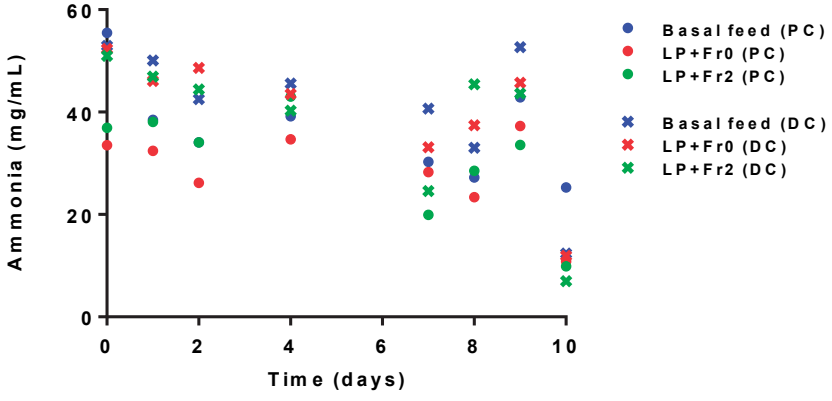

Figure 4.S3: Levels of ammonia produced after supplementing growth medium with insoluble hydrolysates (LP+Fr0-H and LP+Fr2-H) in a 10-day fermentation with SHIME.

LP+Fr0-H and LP+Fr2-H are the insoluble hydrolysates obtained from acid precipitation of digested LP+Fr0 and LP+Fr2, respectively. Basal growth medium was used as control. **PC:** Proximal colon **DC:** Distal colon. No significant difference ($P > 0.05$) was observed between hydrolysates at different timepoints.

5

Chapter 5

Partially digested proteins from yellow peas and lentils stimulate CCK and GLP-1 secretion in STC-1 intestinal cells

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Abstract

Pulse proteins can stimulate production of satiety hormones in the small intestine. However, a significant fraction of plant proteins remains undigested and move to the colon. In this study, we investigated the effect of undigested high molecular weight (HMW) peptides (>3 kDa) from pea and lentil proteins on cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) secretion in an enteroendocrine cell model, the intestinal secretin tumour cell line (STC-1). STC-1 cells were exposed to 0.05, 0.1, and 0.5 mg/mL of HMW peptides obtained from *in vitro* digestion of lentil and pea proteins for 1 h or 2 h. The HMW fraction from both pea and lentil proteins dose-dependently stimulated the release of CCK in the STC-1 cells. Compared to the HEPES blank, no significant levels of CCK and GLP-1 were secreted at 0.05 mg/mL of HMW peptides from both pea and lentil. However, about a five-fold relative increase in GLP-1 secretion was observed at 0.1 mg/mL and 0.5 mg/mL. The difference between 1 and 2-h incubation was not significant for both CCK and GLP-1. Our results show the ability of these HMW peptides to induce CCK and GLP-1 secretion, and thus, their potential to produce a similar response in enteroendocrine cells *in vivo*. These findings unveil the physiological relevance of undigested protein fraction and suggest that a high protein diet might have the potential to influence glucose metabolism and enhance satiety.

Keywords: Pulse proteins, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), STC-1, undigested proteins, food proteins

5.1 Introduction

Consumption of plant proteins is increasing substantially because they are more sustainable than animal proteins. Plant proteins, particularly pulse proteins, are a major protein source globally, because pulses are accessible and affordable (1). Food proteins have been extensively investigated for their nutritional properties and the production of bioactive peptides after gastrointestinal digestion (2). Several studies show that animal proteins can stimulate secretion of cholecystokinin (CCK) and glucagon-like peptide-1 (GLP-1) and inhibit dipeptidyl peptidase-IV (DPP-IV) activity ((3–5). DPP-IV inhibition prevents the cleavage and inactivation of GLP-1, which reduces the risk of hyperglycemia and consequently Type 2 Diabetes (6–8). GLP-1 affects glucose metabolism by enhancing insulin secretion and consequently reducing postprandial blood glucose levels (9,10). GLP-1 secretion also enhances satiety and consequently reduces food intake, whereas CCK secretion reduces gastric emptying to halt further food intake (11).

Considering the contribution of plant proteins to global protein demand, it is imperative to explore their effects on health-related patterns modulated by the bioactive peptides produced in the intestinal lumen. Recent reports show that pulse proteins have several health benefits, including antihypertensive, antioxidant, antimicrobial, anti-inflammatory, and antidiabetic activities (12–16). Pulse proteins have also been reported to reduce risk of obesity and Type 2 Diabetes by stimulating production of gut hormones, such as CCK and GLP-1, in the small intestine (17). CCK is secreted by enteroendocrine I cells in the duodenum, whereas GLP-1 is secreted by L-cells in the jejunum, ileum, and colon (18–20).

Secretion of gut hormones is dependent on the rate of exposure to nutrients produced from digestion and the location of secretory cells (21). CCK and GLP-1 secretion is mainly stimulated by amino acids and oligopeptides through amino acid and peptide transporters in the apical membrane of enteroendocrine cells. The apical membrane of enteroendocrine cells detects luminal oligopeptides and amino acids through different sensory pathways involving G protein-coupled receptors (GPCRs) or transporters, such as the proton-coupled peptide transporters and Na^+-coupled amino acid transporters (11). CCK can also be secreted *in vivo* by negative feedback from pancreatic proteases. The CCK releasing factor remains active to mediate CCK secretion when the proteases rather bind or hydrolyze the luminal proteins and polypeptides (22,23).

Studies that have reported the ability of peptides/protein hydrolysates to stimulate secretion of CCK and GLP-1 have mostly focused on the whole digest or low

molecular weight fraction of protein digestion end-products (24–26). However, about 12–18 g of partially digested dietary proteins and endogenous proteins are unabsorbed and transported to the colon daily (27). These polypeptides end up in the colon because of their low susceptibility to digestive enzymes, which can be due to different reasons. Dietary proteins can undergo structural changes during processing, or they can be modified from their interaction with other dietary components, and thus become less susceptible to digestive enzymes (28).

It is unclear if this digestion-resistant fraction can directly stimulate production of CCK and GLP-1 in enteroendocrine cells in the distal gut. Previous studies have shown that gut hormone secretions in the distal gut is stimulated by short-chain fatty acids produced by gut microbiota (29–31). Therefore, the aim of this study was to investigate the direct effect of high molecular weight peptides (>3 kDa) obtained from simulated *in vitro* digestion of pea and lentil proteins on *in vitro* secretion of CCK and GLP-1 in enteroendocrine cells, using the intestinal secretin tumour cell line (STC-1). These high molecular weight peptides were used to represent the digestion-resistant fraction.

5.2 Materials and Methods

5.2.1 Materials

Yellow pea (*Pisum sativum*) and lentil (*Lens culinaris*) protein concentrates were obtained from Roquette, France and Ingredion Germany GmbH, respectively. Pepsin (from porcine gastric mucosa, ≥250 units/mg solid) and pancreatin (from porcine pancreas, 8× USP specification) were purchased from Merck Life Science NV, The Netherlands. NuPAGE® LDS Sample buffer (4X), SDS Running Buffer (20X), and 12% Bis-Tris Gel (1.0 mm, 10 Well) were purchased from ThermoFisher Scientific, The Netherlands. BlueRay Prestained Protein Marker 10-180 kDa was purchased from Jena Bioscience GmbH, Germany, and Coomassie Brilliant Blue R-250 Staining Solution was purchased from Merck Life Science NV, The Netherlands. Centrifugal filter units were obtained from Merck Life Science NV, The Netherlands, and VWR International B.V., The Netherlands. Corning culture flasks (75 cm^2) and Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L of glucose, L-glutamine, and 25 mM HEPES were purchased from Sigma Aldrich, The Netherlands. Phosphate-buffered saline (without $CaCl_2$ and $MgCl_2$) and Trypsin–EDTA (0.25%) were purchased from Sigma Aldrich, The Netherlands. Hyclone foetal bovine serum was purchased from Fisher Scientific, The Netherlands. STC-1 cells (passage 34) were obtained from American Type Culture Collection (ATCC) through LGC Standards GmbH, Germany. CCK (26–33) non-sulfated-EIA

kit was purchased from Phoenix Pharmaceuticals, and GLP-1 (Active) ELISA kit (EGLP-35K) was purchased from Sigma Aldrich, The Netherlands.

5.2.2 Methods

2.2.1 Preparation of pea and lentil proteins:

The commercial protein concentrate powders were subjected to alkaline extraction and isoelectric precipitation to increase the protein concentration. Protein content of the commercial pea and lentil protein concentrates were 71.01 ± 0.25% and 48.55 ± 0.06%, respectively, as determined by the Dumas method. The concentrate powders were mixed with de-ionized water (10%, w/v) and stirred constantly at 20°C for 4 h to solubilize proteins while maintaining pH at 10 with 3 M NaOH. After 4 h, the suspension was centrifuged at 7000 g for 30 min at 20°C. The supernatant was recovered and constantly stirred at a pH of 4 for 2 h to precipitate the proteins. pH was adjusted with 3 M HCl. After 2 h, the suspension was centrifuged at 7000 g for 30 min at 20°C, and the proteins were recovered as the pellet. The pellet's pH was changed to 7 with 3 M NaOH and lyophilized. The protein content of the resulting pea protein concentrate was 68.93 ± 0.34%, and that of lentil protein was 70.08 ± 0.01%, as determined by the Dumas method.

5.2.2 Simulated in vitro gastrointestinal protein digestion:

Pea and lentil proteins were digested using the COST INFOGEST method of static simulated *in vitro* gastrointestinal digestion (32) with slight modifications, as reported by Ariëns et al. (33). The proteins (5 g) or 5 mL MilliQ water (as digestion control) were mixed with 3.5 mL of simulated salivary fluid, 25 µL of 0.3 M $CaCl_2$, and 1.475 mL MilliQ water. The mixture was incubated at 37°C for 2 min. Simulated gastric fluid (7.5 mL) and 0.3 M $CaCl_2$ (5 µL) were added. Aliquots of 1 M HCl were gradually added to the mixture to adjust the pH to 3. Pepsin stock solution (25000 U/mL) was prepared, and a 1.6 mL aliquot was added to the mixture (the final concentration of pepsin was 2000 U/mL). MilliQ water was added to make a final volume of 20 mL, and the gastric phase was incubated at 37°C for 2 h with constant mild shaking on a rotating device. The gastric phase was stopped by changing the pH to 7, using 1 M NaOH. Simulated intestinal fluid (11 mL) and 40 µL of 0.3 M $CaCl_2$ were added to the gastric chyme. A 5 mL aliquot of pancreatin stock solution with 800 U/mL trypsin activity was added to the chyme, and MilliQ water was added to reach a final volume of 40 mL. The final concentration of pancreatin was 10 U/mL, based on trypsin activity, as reported by Ariëns et al. (33). The chyme was incubated at 37°C for 2 h with constant mild shaking on a rotating device. After the intestinal phase, the digesta were vortexed and immediately stored at –20°C to inhibit further pancreatin activity. Samples were

collected at the end of the gastric phase and the 1 and 2 h time points of the intestinal phase.

5.2.3 Fractionation of digesta:

Samples of the digesta obtained from the simulated *in vitro* gastrointestinal digestion of pea and lentil proteins and digestion blank were fractionated with centrifugal filter units. First, the samples were centrifuged at 4000 g and 20°C for 10 min. The resulting supernatant was subjected to stepwise filter-centrifugation with molecular weight cutoffs of 30, 10, and 3 kDa at 4000 g. Although the fraction of interest was >3 kDa, stepwise filter-centrifugation was performed to prevent large peptides from concentrating and blocking the 3-kDa filter membrane. The >3-kDa fraction, which includes the pellets from the initial centrifugation and retentates from the filter-centrifugation, were mixed, lyophilized, and stored at –20°C until further use. The >3-kDa fractions obtained from the gastric phase of digestion of pea protein, lentil protein, and digestion blank are hereafter referred to as PP-gas, LP-gas, and BP-gas, respectively. Fractions from 1 h intestinal digestion of pea protein, lentil protein, and digestion blank are hereafter referred to as PP-Int 1h, LP-Int 1h, and BP-Int 1h, respectively. Fractions from 2-h intestinal digestion of pea protein, lentil protein, and digestion blank are hereafter referred to as PP-Int 2h, LP-Int 2h, and BP-Int 2h, respectively.

5.2.4 Determination of the molecular weight profiles of glycated proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE):

Molecular weight profiles of the >3-kDa fraction obtained from the digested samples were assessed by SDS-PAGE under denaturing non-reducing conditions, following the NuPAGE® electrophoresis system manufacturer's instruction. Briefly, the dried HMW (>3 kDa) peptide powders were mixed with MilliQ water to reach a protein concentration of 0.5–1 mg/mL. The protein content of the HMW peptides was determined using the Lowry method. HMW peptide solutions (2 µL) were mixed with 15 µL MilliQ water and 5 µL of LDS sample buffer (4X). The resulting solution was centrifuged for 1 min, 2000 rpm at 20°C. The solutions were heated at 70°C for 10 min and centrifuged again for 1 min, 2000 rpm at 20°C. The supernatants were recovered, and 10 µL aliquots were loaded on 12% Bis-Tris Gel wells pre-mounted in the electrophoresis system. A pre-stained protein marker (11–180 kDa) was also loaded in another well. The loaded gel was run for 45 min at 200 V, gently rinsed twice with water, and stained with Coomassie Brilliant Blue R-250 Staining Solution for 1 h at 20°C. After staining, the background color of the gel was sufficiently removed with a washing buffer

overnight. The washing buffer contained 10% ethanol and 7.5% acetic acid in MilliQ water.

5.2.5 Cell culture conditions:

STC-1 cells were cultured in Dulbecco's modified Eagle's medium (containing 4.5 g/L of glucose, L-glutamine, and 25 mM HEPES) supplemented with 10% heat-inactivated fetal bovine serum. The cells were maintained in 75 cm^2 culture flasks of up to 70–80% confluence in a 5% CO_2 atmosphere at 37°C and 90% humidity.

5.2.6 Cell viability assay:

Cells cultured under the abovementioned conditions were used for the cell viability assay, as reported by Lei et al. (34). A 500 µL aliquot of STC-1 cell solution (3×10^5 cells/mL) was seeded in 48-well plates and incubated for 48 h at 37°C, 5% CO_2, and 90% humidified atmosphere. After the 48-h incubation period, the medium was removed, and the cells were washed twice with 500 µL HEPES buffer. The HEPES buffer contained 20 mM HEPES, 1.2 mM $CaCl_2$, 10 mM glucose, 4.5 mM KCl, 1.2 mM $MgCl_2$, and 140 mM NaCl at a pH of 7.4 (35). The cells were pre-incubated in 500 µL HEPES buffer for 1 h to induce starvation, after which the solution was removed and the cells were incubated with 500 µL HMW peptides for 2 h at test concentrations of 0.5, 0.1, and 0.05 mg/mL. Peptides were pre-mixed in HEPES buffer. HEPES buffer was used as control. After the incubation, the peptides were removed, and 125 µL of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) solution (0.5 mg/mL) was added to each well and incubated for 2 h at 37°C and 5% CO_2 humidified atmosphere. The MTT solution was then removed, and 125 µL of dimethyl sulfoxide-ethanol (1:1) solution was added to each well. The plate was mildly shaken for 15 min, and the absorbance of the resulting solution was read at 570 nm. Viability of cells were expressed as a percentage of the control (HEPES buffer). The MTT assay measures the ability of cells to convert MTT, which is a tetrazolium salt, to formazan. The mitochondrial dehydrogenases of a cell that is metabolically active reduces the tetrazolium salt to formazan (36,37). This conversion translates to a colour change from yellow to purple.

5.2.7 CCK and GLP-1 secretion studies:

Cells cultured in DMEM at 37°C and 5% CO_2 humidified atmosphere, as mentioned above, were used for the hormone secretion studies. The cells were seeded in 48-well plates with 500 µL of a 3×10^5 cells/mL cell stock solution. The plates were incubated for 48 h at 37°C and 5% CO_2 humidified atmosphere. At the end of the 48-h incubation period, the medium was removed, and the wells were washed twice with 500 µL HEPES buffer. The cells were then pre-incubated with 500 µL

HEPES buffer for 1 h at 37°C and 5% CO_2. HMW peptides (>3 kDa) were mixed at 0.5 mg/mL, 0.1 mg/mL, and 0.05 mg/mL in HEPES buffer. A 500 µL aliquot of the peptide solution was added to each well. The HEPES buffer was used as control. Cells were incubated with HMW peptides and HEPES control for either 1 h or 2 h. At the end of the incubation, the supernatants were transferred into tubes pre-filled with a protease inhibitor, phenylmethylsulfonyl fluoride (PMSF). The final concentration of the PMSF was 0.6 mM. Levels of CCK and GLP-1 were measured according to the instructions provided in the commercial ELISA kits. Measurements of each biological duplicate were performed in technical duplicates.

5.2.8 Statistical analysis:

Levels of CCK and GLP-1 were reported as mean ± standard deviation of biological duplicate measurements. Two-way ANOVA (treatment x concentration) and Tukey post hoc test were used to determine significant difference ($P < 0.05$).

5.3 Results and Discussion

5.3.1 Molecular weight profile of HMW peptides from *in vitro* digestion of pea and lentil proteins

After simulated *in vitro* digestion of pea and lentil proteins, the digested samples were fractionated to obtain >3-kDa peptides as a representation of the undigested proteins. The molecular weight profile of peptides is shown in Figure 5.1. The HMW fractions obtained from the gastric phase of pea and lentil protein digestion (PP-gas, LP-gas) comprised large peptides with sizes up to about 100 kDa. Fractions obtained at 1 h and 2 h timepoints during intestinal digestion demonstrated that the large peptides in the gastric phase were extensively hydrolysed, such that prominent bands were only observed around 11 kDa in PP-Int 1h and PP-Int 2h. Similarly for lentil protein, although faint bands were observed at 17–63 kDa, most prominent bands in LP-Int 1h and LP-Int 2h were observed around 11 kDa.

5.3.2 Cell viability in >3 kDa peptides:

In this study, we assessed the ability of >3-kDa peptides from gastrointestinal digestion of pea and lentil proteins to stimulate secretion of CCK and GLP-1 by exposing STC-1 cells to these peptides. The cells were incubated in the peptides for either 1 or 2 h. Before incubation of the cells, their mitochondrial activity in the test concentrations was evaluated using the MTT assay. Preliminary tests showed that exposing cells to HMW peptides at concentrations of 5 and

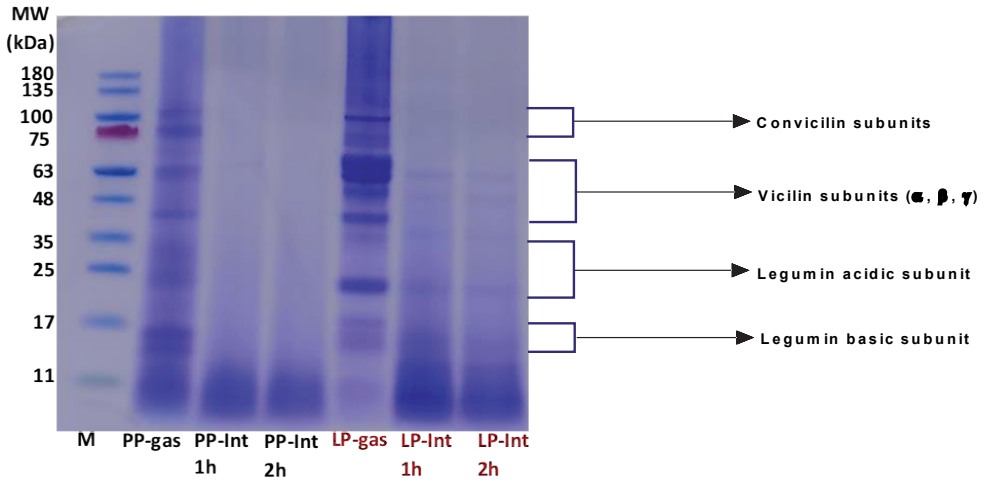

Figure 5.1: SDS-PAGE molecular weight profile of >3 kDa peptides produced from *in vitro* digestion of pea and lentil proteins.

PP-gas: pea protein gastric phase, LP-gas: lentil protein gastric phase, PP-Int 1h: pea protein 1 h intestinal digestion, LP-Int 1h: lentil protein 1 h intestinal digestion, PP-Int 2h: pea protein 2 h intestinal digestion, LP-Int 2h: lentil protein 2 h intestinal digestion

10 mg/mL resulted in low cell mitochondrial activity (<50%). Additionally, cells detached from the bottom of the 48-well plates due to residual trypsin activity in the digested samples. Therefore, samples were heated at 100 °C for 10 min to denature trypsin. The HMW peptide samples were further diluted to 0.05, 0.1, and 0.5 mg/mL. Percent mitochondrial activity of the resulting samples was over 90%, indicating that the test samples did not substantially alter mitochondrial dehydrogenase activity of the cells, compared to the blank, as shown in Figure 5.2.

5.3.3 CCK secreted by STC-1 cells after incubation in >3-kDa peptides:

The aim of this study was to evaluate the stimulatory effect of the undigested fraction produced after *in vitro* gastrointestinal digestion. Hence, cells were exposed to >3-kDa peptides produced from 2-h intestinal digestion. The cells were incubated in 0.05, 0.1, and 0.5 mg/mL of the HMW peptides (>3 kDa) for 1 or 2 h. Levels of CCK released after exposing the STC-1 cells to these peptides were measured using competitive enzyme immunoassay (Figure 5.3). The digestion blank sample (BP-Int 2h) in our study, which contained pepsin and pancreatin, also stimulated CCK release at all tested concentrations, although at lower release levels. Considering that this digestion blank had no dietary protein but only the digestive enzymes, our finding shows that digestive enzymes, such as proteases, can also exert a satiating effect, although minimal.

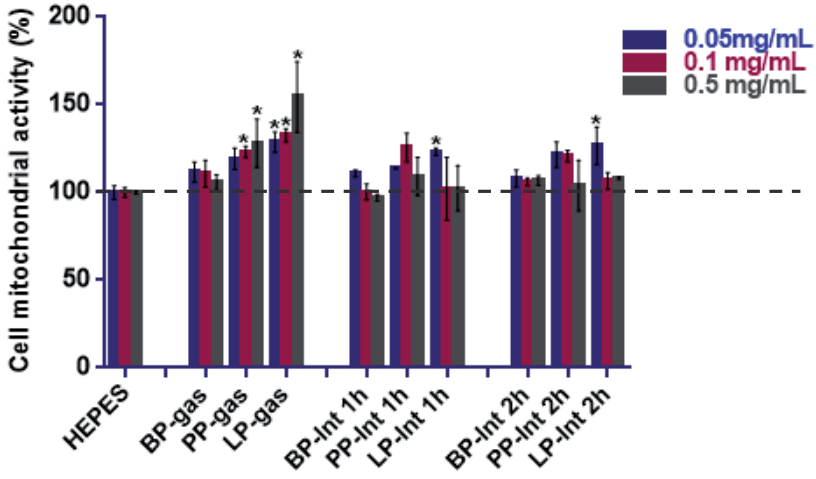

Figure 5.2: Percent mitochondrial activity of STC-1 cells in >3-kDa peptides at tested concentrations measured with the MTT assay.

HEPES buffer was used as control. BP-gas: digestion blank gastric phase, PP-gas: pea protein gastric phase, LP-gas: lentil protein gastric phase, BP-Int 1h: digestion blank 1 h intestinal digestion, PP-Int 1h: pea protein 1 h intestinal digestion, LP-Int 1h: lentil protein 1 h intestinal digestion, BP-Int 2h: digestion blank 2 h intestinal digestion, PP-Int 2h: pea protein 2 h intestinal digestion, LP-Int 2h: lentil protein 2 h intestinal digestion. Samples were incubated in 0.05, 0.1, and 0.5 mg/mL of the >3 kDa peptides. *Significantly different from HEPES.

The difference in CCK level released after 1-h and 2-h incubation was not statistically significant. Both pea and lentil peptides dose-dependently stimulated the release of CCK (Figure 5.3). No significantly different secretion levels were stimulated by the least tested concentration (0.05 mg/mL) of the digestion blank, pea and lentil protein, compared with the experimental blank (HEPES). However, a significant difference was observed among the peptides from the digestion blank, pea protein, and lentil protein at the highest concentration (0.5 mg/mL). Our results are consistent with findings from several studies in which intestinal digests induced CCK release (3,35,38,39).

Santos-Hernández et al. demonstrated that casein and whey peptides from the intestinal phase dose-dependently induced CCK release at a ten-fold increase, compared to the control (HEPES buffer) (39). Considering that peptides from the intestinal phase contain oligopeptides and free amino acids, the main inducers were unclear. However, when the cells were incubated in a mixture of free amino acids, no substantial difference was observed in comparison with the control, indicating that the observed stimulatory effect was attributable to the oligopeptides rather than free amino acids. Another study reported that CCK secretion

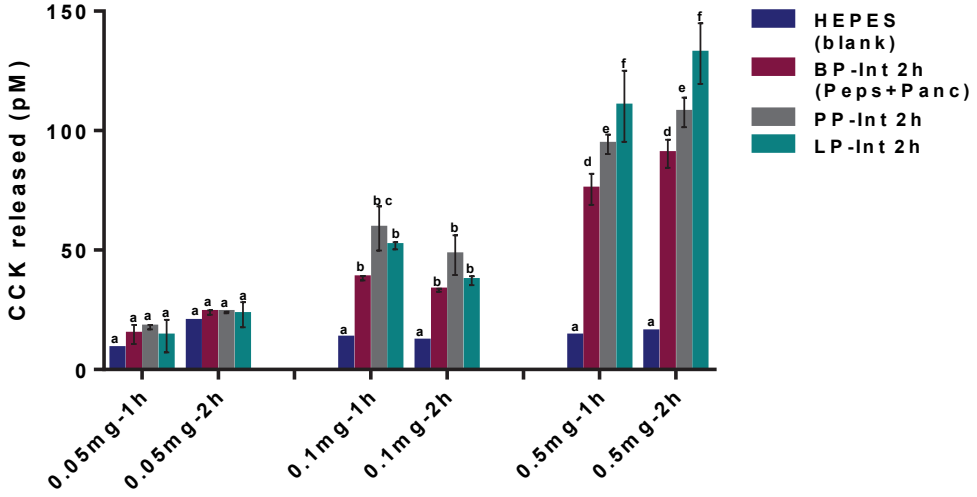

Figure 5.3: Levels of CCK released after incubating STC-1 cells in >3-kDa peptides produced from gastrointestinal *in vitro* digestion of pea and lentil proteins.

BP-Int 2h: digestion blank 2 h intestinal digestion, PP-Int 2h: pea protein 2 h intestinal digestion, LP-Int 2h: lentil protein 2 h intestinal digestion. HEPES buffer was used as experimental blank. Samples were incubated in 0.05, 0.1, and 0.5 mg/mL of the >3-kDa peptides. Different letters indicate statistical difference ($P < 0.05$).

was induced by peptides with 4–11 amino acid residues (40,41). In our study, the STC-1 cells were incubated in larger peptides (>3 kDa), suggesting that these large peptides can also act as inducers of CCK secretion. In a study by Geraedts et al., pea and egg hydrolysates produced comparable levels of CCK (38). Contrastingly, other reports show that pea and whey hydrolysates could not activate CCK receptor-expressing cells, although both hydrolysates stimulated release of CCK (41). The hydrolysates used in these studies were from commercial producers, and authors did not provide the hydrolysis conditions under which these hydrolysates were obtained. However, the differences observed could be due to hydrolysis conditions used, extent of hydrolysis, absence of specific amino acid residues, and sequence of the peptide chain.

In our study, incubation of the STC-1 cells with the >3 kDa peptides simulated the luminal exposure of enteroendocrine cells to undigested peptides. CCK secreting cells, I-cells, are predominantly located in the duodenum, yet mural colonic enteroendocrine cells can produce and store active forms of CCK (42). Nakajima et al. demonstrated that peptides >500 Da can use the calcium sensing receptor (CaSR) to induce CCK secretion (43). Other reports show that peptide-induced stimulation of CCK release is mediated by CaSR and G protein-coupled receptor-93 (GPR93) mediate of CCK (40,44) and can be expressed in the caecum and

proximal colon (45). Therefore, the ability of these HMW peptides to induce CCK secretion suggests their potential to produce a similar response in the distal gut *in vivo*.

5.3.4 GLP-1 levels released by STC-1 cells after incubation in >3-kDa peptides:

Levels of GLP-1 released after incubating STC-1 cells in >3-kDa peptides from pea and lentil protein digestion were measured by immunologic quantification of biologically active forms of GLP-1. Results are shown in Figure 5.4. Relative to the lowest concentration used (0.05 mg/mL), we observed a five-fold increase in secretion levels after incubation in 0.1 mg/mL of pea and lentil peptides. However, no substantial increases were observed between 0.1 mg/mL and 0.5 mg/mL (Figure 5.4). The digestion blank (containing digestive enzymes) recorded the lowest release levels without any concentration effects. Longer exposure periods did not yield an increase in level of GLP-1 released.

A similar stimulatory effect of large (>3 kDa) and medium (3–0.5 kDa) peptides produced after *in vitro* gastrointestinal digestion of egg white was reported in 0.0075–0.5 mg/mL test concentrations (35). Additionally, Cordier-Bussat et al.

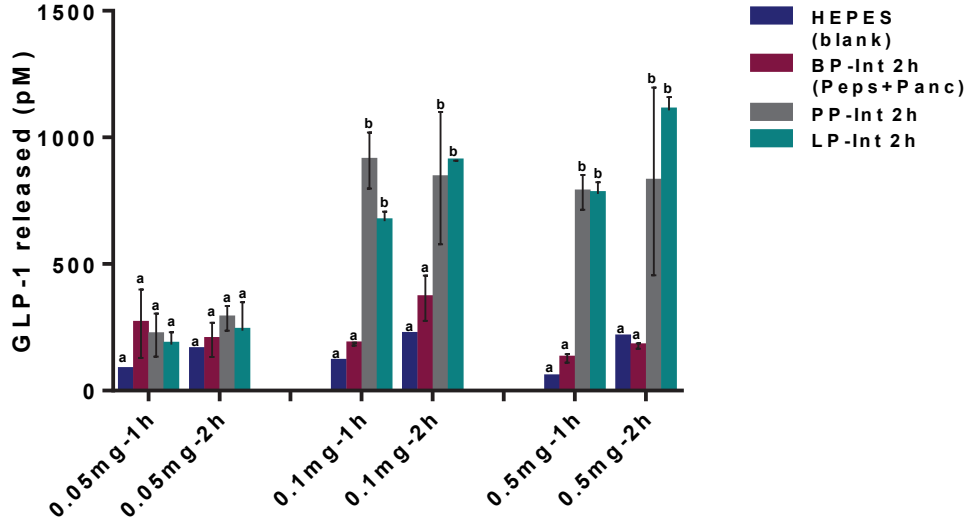

Figure 5.4: Levels of GLP-1 released after incubating STC-1 cells in >3-kDa peptides produced from gastrointestinal *in vitro* digestion of pea and lentil proteins.

BP-Int 2h: digestion blank 2 h intestinal digestion, PP-Int 2h: pea protein 2 h intestinal digestion, LP-Int 2h: lentil protein 2 h intestinal digestion. HEPES buffer was used as the experimental blank. Samples were incubated in 0.05, 0.1, and 0.5 mg/mL of the >3-kDa peptides. Different letters indicate statistical significance ($P < 0.05$).

reported that peptones dose-dependently induced GLP-1 release, whereas a mixture of amino acids or an intact protein (bovine serum albumin) had no effect (46). Contrastingly, Geraedts et al. demonstrated that intact casein, egg, pea proteins, and wheat proteins induced GLP-1 release. However, not all their respective hydrolysates induced GLP-1 release. In that study, different commercial pea protein powders were used. All the intact pea proteins were active in stimulating GLP-1 release, whereas only one of three pea hydrolysates induced GLP-1 release (38). In our study, the contribution of amino acids to GLP-1 secretion was minimal, because we used the >3-kDa fraction.

Several studies suggest that intact proteins, peptides, and amino acids can stimulate production of GLP-1 through different signalling pathways (47–49). Miguens-Gomez et al. postulate that the signalling pathway specifically depends on the amino acids present, amino acid sequence, and peptide chain length (50). Intact proteins, amino acids, and peptides with either HMW or low MW can reach luminal chemo-sensors and activate GLP-1 secretion pathways (50). Therefore, the key factor is the presence of a motif that acts as the inducing site in the stimulant. In a recent study, a specific amino acid sequence was indicated to be essential in the release of GLP-1, such that modification of the N-terminus of peptides caused lower secretion levels (40).

Until now, the clearest mechanism by which protein digestion products stimulate GLP-1 is demonstrated to occur either after their uptake into cells as free amino acids through sodium-coupled transport or after uptake as peptides by peptide transporter 1 (PepT1) (51–53). Although data on the mechanism of large peptides is lacking in literature, protein hydrolysates have been reported to increase intracellular calcium ion level, which then induces the secretion of GLP-1 (54). Therefore, the significantly higher GLP-1 release from pea and lentil peptides than from the blank points to the ability of these peptides to activate the intracellular calcium ion-mediated GLP-1 release.

5.4 Conclusion

The stimulatory effect of HMW peptides (>3 kDa) from gastrointestinal digestion of pea and lentil proteins on CCK and GLP-1 secretion in enteroendocrine cells was evaluated in STC-1 cells. We observed that the HMW fraction from both pea and lentil proteins stimulated release of CCK and GLP-1 in the STC-1 cells. A dose-dependent effect was observed in the levels of CCK and GLP-1 produced. Prolonging cell exposure time from 1 h to 2 h did not significantly increase secre-

tion levels. Our results show that the unabsorbed and undigested large peptides that are produced after gastrointestinal digestion have the potential to influence satiety regulation. To the best of our knowledge, this study is the first to investigate the role of HMW peptides or undigested pulse proteins in satiety regulation. Additionally, the >3-kDa fraction of digestive enzymes (pepsin and pancreatin) can stimulate release of CCK, suggesting the stimulatory effect of endogenous proteins. These findings need to be further investigated *in vivo* to confirm the satietogenic effect of these peptides. However, our findings unveil the physiological relevance of undigested protein fraction and suggest that a diet with significant amount of low digestible proteins might have the potential to enhance satiety due to the long-term action of the undigested peptides reaching the colon.

Ethics statement

Authors declare that no human or animal subjects were included in this study.

Declaration of Competing Interest

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

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Chapter 6

General Discussion

6.1 Summary of findings

The global population is rising rapidly at a rate of 0.9% annually, which represents about 80 million people every year [1]. This poses a challenge in meeting the food security needs of everyone including their protein demands. Hence, food researchers are exploring more sources of food proteins beyond the traditional animal sources. This is particularly necessary because the energy requirements of animal production systems have detrimental effects on the environment and must reduce [2]. Current alternative sources of food proteins include algae, insects, legumes and pulses, cereals and pseudo-cereals, oilseeds, and tubers. Figure 6.1 shows emerging alternatives and their common food applications.

Plant proteins are more suitable because they are more sustainable, have health benefits, and are already consumed in areas with low food security. However, the structural difference between animal and plant proteins poses a challenge to their digestibility when used in food applications [3]. Moreover, processing and interaction with other dietary components can affect plant protein digestibility. In Chapter 2 and 3, we investigated the effect of protein-β-glucan and

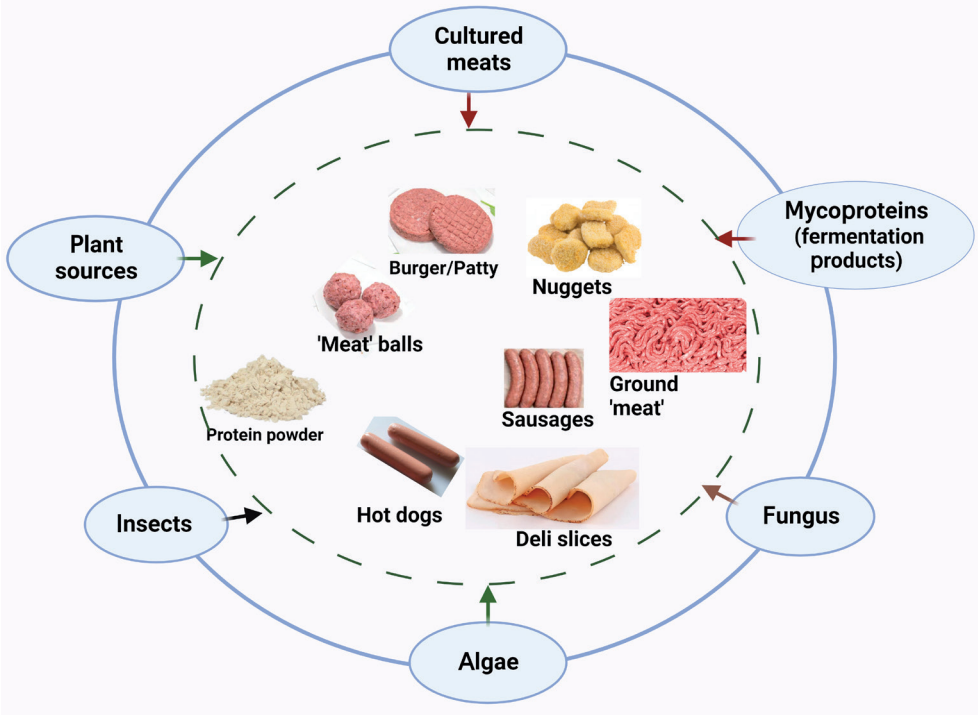

Figure 6.1: Current commercial food products from sustainable protein alternatives

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protein-tannic acid interaction on particle characteristics and digestibility of plant proteins. β-glucan and tannic acid were selected because they are commonly occurring in plant-based foods and added to functional food formulations for their health benefits. Chapter 2 focused on proteins isolated from lentil and yellow pea seeds whereas Chapter 3 and Chapter 4 focused on lentil proteins as model plant proteins. In Chapter 4, we evaluated how plant protein glycation during Maillard reaction also affect protein digestibility. Maillard reaction occurs frequently during processing of high protein foods and has been suggested to enhance techno-functionality of plant proteins [4]. An intense discussion is ongoing among researchers on the effect of dietary advanced glycation end-products (dAGEs). A review of the available literature indicates that dAGEs have potential detrimental effects on health, however findings from clinical studies are inconclusive [5].

Considering that glycation can alter protein digestibility, the equilibrium between the peptides that are absorbed in the small intestine epithelium and those reaching the colon can also be affected and consequently determine the type of peptides transported to the colon. After protein digestion in the small intestine, partially digested fractions transported to the colon can be sensed by enteroendocrinal cells and possibly induce gut hormone secretions [6]. These partially digested residues can also influence the gut microbiota. Therefore, in Chapter 4, we further assessed how undigested glycated lentil protein residues that potentially reach the colon affect the gut microbiota. We used batch fermentation and the Simulator of Human Intestinal Microbiome Ecosystem (SHIME®). Chapter 5 investigated the effect of high molecular weight pea and lentil protein hydrolysates on Cholecystokinin (CCK) and Glucagon Like Peptide 1 (GLP-1) secretion in intestinal endocrine cells, using Secretin Tumour Cell line (STC-1) cell model.

The results from Chapter 2 – 5 are summarized in Figure 6.2. Results from Chapter 2 and 3 show that interaction between pulse proteins and food components such as dietary fibre and phenolic compounds can produce complexes that alter the particle characteristics and structure of the proteins. Chapter 4 also demonstrates that glycation of these proteins modifies the particle characteristics. Consequently, the structural modification reduces digestibility of the proteins. From Chapter 4, the extent of protein glycation during processing does not affect the short chain fatty acids (SCFAs), Branched Chain Fatty Acids (BCFAs), and ammonia produced in the colon, while the effect on the microbiota population is dependent on the host and colon section. Chapter 5 demonstrates that undigested pea and lentil protein residues that reach the colon can stimulate production of satiety hormones CCK and GLP-1.

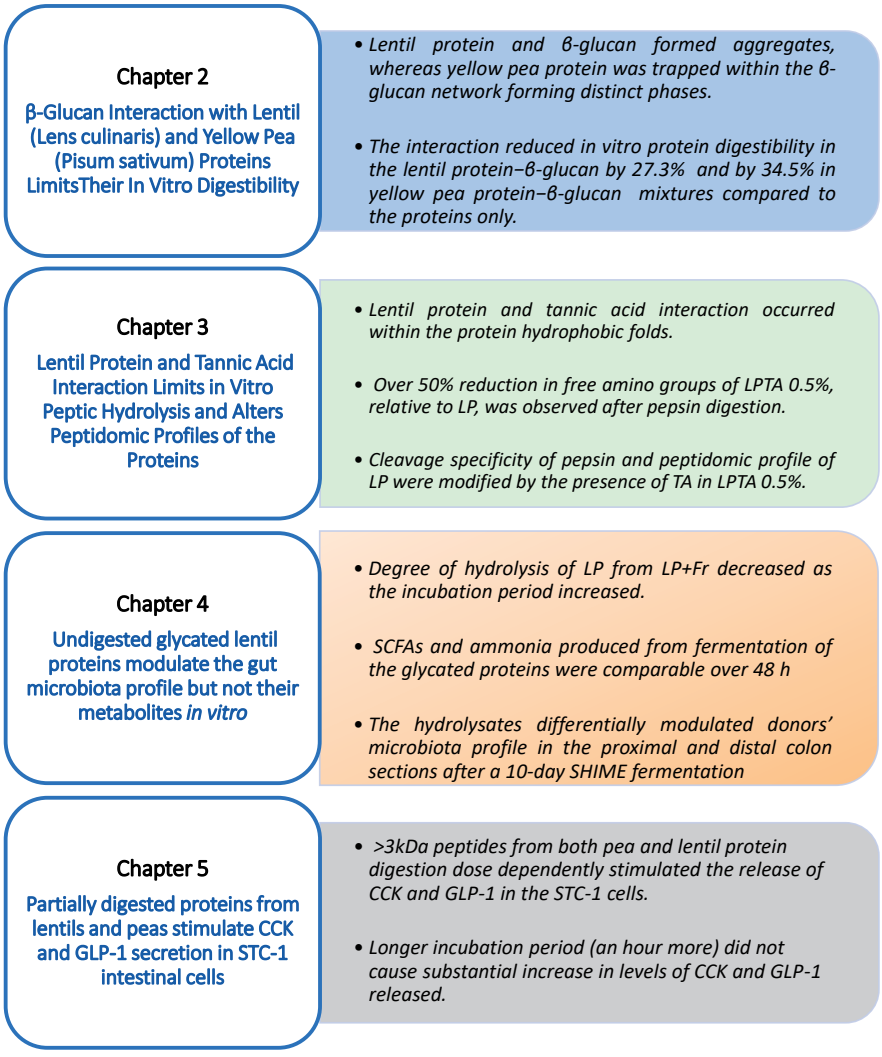

Figure 6.2: Summary of findings from research chapters.

LP: Lentil protein, LPTA 0.5%: Lentil protein-Tannic acid mixture containing 1% w/v LP and 0.5% TA. LP+Fr: Lentil protein-fructose mixtures. SCFAs: Short-chain fatty acids

6.2 Novelty of this thesis

In this thesis, we evaluated how processing and the interaction between pulse proteins and other dietary components affect protein digestibility. As food scientists explore sustainable protein alternatives, it is important that we explore relevant factors such as their fate along the digestive tract. Figure 6.1 shows examples of food application of protein alternative sources and in-depth under-

standing of fate of the proteins along the digestive tract is necessary because this determines the effect on the consumer's health. This thesis shows different mechanisms that influence protein digestibility of pulse proteins. Chapter 2 shows that when dietary fiber is present in a food product along with pulse proteins, electrostatic interactions can occur between them to form complexes, or the dietary fiber can physically hinder the protein and then reduce protein digestibility. This finding provides an additional mechanism by which dietary fibre can reduce access of the digestive enzymes to proteins, beyond the effect of their viscosity, which is the mechanism most often mentioned in the scientific literature. Chapter 3 shows that phenolic compounds can form both soluble and insoluble complexes with proteins that limit pulse protein digestibility. Also, Chapter 3 shows that these complexes can modify the cleavage specificity of pepsin and alter the peptides released. This is the first study to characterize the peptides released after pepsin hydrolysis of lentil proteins.

This thesis proceeded further to study how the undigested protein fraction that potentially reaches the colon affects the gut microbiota and the intestinal cells. We recovered the large peptides from the digested samples as a representative sample of the fraction that potentially reaches the colon. To the best of our knowledge, this study is the first to use the large peptide fractions from plant proteins (as a representative sample of the fraction that reaches the colon) to evaluate the potential of stimulating satiety hormones in the colon. Most studies use native proteins or whole digests which limit their physiological relevance. The >3-kDa fraction of digestive enzymes (pepsin and pancreatin) used in Chapter 5 also stimulated release of CCK, suggesting the stimulatory effect of endogenous proteins. This provides more insights into the role of endogenous proteins in host satiety.

Additionally, Chapter 4 shows that structural modification caused by Maillard reaction reduces digestibility of proteins and potentially limit bioavailability of essential amino acids such as lysine. Chapter 4 provides more insight into how protein structural changes from processing influences the fate of proteins along the digestive system. The study enhances our knowledge on how the gut microbiota ferments glycated proteins in the colon. Consideration of the fate of pulse proteins in the colon and their contribution as a sustainable protein alternative is novel and has not been extensively studied in the past. Although STC-1 cells are entero-endocrinal cells and not native to the colon, their ability to produce CCK and GLP-1 when exposed to undigested proteins suggest the potential of these undigested proteins to stimulate similar responses in colon entero-endocrinal

cells. Findings in Chapter 5 advance the knowledge on satiety enhancing effect of food proteins along the digestive tract.

6.3 Pulse protein digestibility: effect of protein interactions and processing

6.3.1 Effect of protein interactions on protein digestibility

The major molecular forces in protein interactions are hydrogen bonds, electrostatic interactions, hydrophobic forces, and covalent disulfide bonds which involve the protein's reactive side chains and functional groups [7]. Proteins interact with other proteins through aggregation or even phase separation, depending on the net forces of the proteins involved [7,8]. For instance, electrostatic attraction among native proteins due to the opposite surface charge can result in aggregation of these proteins. When the concentration of the more reactive protein is low, phase separation can occur. pH is key in these interactions; therefore, at the isoelectric point of the proteins, they aggregate. However, at other pH, the side chains form both intramolecular and intermolecular complexes [9]. The nature of protein interactions is determined by molecular size, pH, hydrophobicity, amino acid residues, and spatial orientation. Other relevant factors include the physical state of the matrix and the matrix components.

The reactivity of the side chains of the protein's amino acid residues determines their interaction with other dietary components and as such the presence of other reactive food components can induce formation of protein complexes. This interaction can in turn alter protein secondary structure and consequently modify their digestibility. In Chapter 2, the decrease in percent transmittance of the protein–β-glucan complexes suggested the formation of insoluble complexes. The pH of the protein-β-glucan mixtures was around neutral which means that the net charge of the proteins was negative, favouring electrostatic repulsion between the proteins and β-glucan. At low concentrations, the phosphate group of β-glucan is considered to impart a negative charge. The lower (less negative) ζ-potential recorded in the protein-β-glucan mixtures compared to the protein controls (with no β-glucan), indicates that β-glucan covers the protein surface and the charged residues. Hence, the complexes display a lower electrostatic repulsion and can easily aggregate. Consequently, compared to the protein controls, the protein-β-glucan complexes recorded lower degree of protein hydrolysis. This confirms previous reports that insoluble complexes and their aggregates can hinder sufficient access of pepsin and pancreatin to the proteins [10,11].

Based on fluorescence imaging of the aqueous mixtures, lentil protein and β-glucan formed aggregates, whereas yellow pea protein was trapped within the β-glucan network forming distinct phases. This caused a more extensive decrease in the degree of hydrolysis in the pea protein- β-glucan complexes. Although lentil and yellow pea have similar protein types, the relative amounts of the fraction with different molecular features are different. Lentil proteins are more glycosylated which make them more soluble and prone to hydrophilic interactions.

In Chapter 3, fluorescence quenching of the protein in the presence of tannic acid (TA) indicates binding of tryptophan residues or binding of other residues that alter the conformation of the protein and expose the tryptophan residues. Tryptophan residues are usually located in the hydrophobic core of the protein, thus, decrease in the tryptophan fluorescence intensity suggests an interaction within the hydrophobic core. This possibly occurred through hydrogen bonding and strong hydrophobic interactions. The bimolecular quenching rate constant also indicates about 1:1 lentil protein-tannic acid molecules in the complexes formed. As a result, the secondary structure was modified. Increasing TA content caused an overall increase in the regular and distorted α-helix fractions whereas the β-structures remained fairly constant. Few tannic acid concentrations (0-0.005% w/v) were selected for the secondary structure determination based on the fluorescence quenching ability. Therefore, it is unclear if the highest TA concentration (0.5%) used in this study could have altered the proportion of β-structures. However, the β-structures were more abundant than the α-helical structures, hence, the low digestibility of the proteins. B-structures reduce access of proteases to the proteins and consequently reduce their digestibility [12,13]. The *in vitro* protein digestibility by pepsin, as measured by the level of free amino groups in the digesta shows a substantial decrease (56.8%) in the LPTA 0.5% mixture compared to the lentil protein control (without TA). LPTA 0.5% is the Lentil protein-Tannic acid mixture containing 1% w/v LP and 0.5% TA

Findings from Chapter 2 and 3 show that pulse proteins can interact with other food biomolecules through several mechanisms; electrostatic interactions, hydrogen bonding, and hydrophobic interaction depending on the food biomolecules. The type of interaction also determines how protein digestibility will be affected. For instance, in protein-β-glucan interaction, the negative surface charge of the proteins supports electrostatic repulsion within the complexes. However, bulk hindrance from β-glucan likely contributed to reducing access of pepsin and pancreatin enzymes. Other reports also suggest that dietary fibres can increase the viscosity of the mixtures and reduce sufficient mixing of the

enzyme [14]. Results from Chapter 2 confirm that complexes formed from the electrostatic interaction between proteins and dietary fibre is an additional mechanism by which access of the digestive enzymes can be reduced. In the case of protein-tannic acid, both soluble and insoluble complexes were formed depending on the levels of TA. Protein-TA interaction begins with hydrogen bonding to produce soluble complexes and proceeds further to hydrophobic interactions to produce aggregates depending on the levels of TA present. Results from Chapter 3 show that soluble complexes were formed at 0.1% TA whereas insoluble complexes were formed at 0.5% TA. These complexes reduced peptic hydrolysis of the proteins. Two possible mechanisms for this outcome could be that: (1) structural changes that occurred from the interaction reduced the protein's susceptibility to pepsin hydrolysis. (2) TA could have bound to the pepsin and reduced its hydrolytic effect. However, previous studies show that reduction in digestibility of proteins in the presence of TA is predominantly due to obstruction of the protein surface [15].

6.3.2 Effect of processing on protein digestibility

Protein structure is a core determinant of protein digestibility. Thus, factors that affect the protein structure consequently alters the digestibility. Digestibility of food proteins are determined by intrinsic and extrinsic factors. The intrinsic factors comprise the protein structure such as disulphide bonds, cross-linkages, β-sheets, and in plants sources, anti-nutritional factors that might be present. The extrinsic factors include the other biopolymers present in the food or the formulation, pH, temperature [16,17]. During food processing, both intrinsic and extrinsic factors can be altered to enhance or decrease protein digestibility.

Protein structural changes caused by food processing can also influence protein digestibility and the type of peptides released. Food processing methods are used to enhance edibility and improve the functionality of the proteins, yet their structures can be modified during processing [17]. The native structure of proteins can be altered upon heat treatment, change in pH and ionic strength. Heating, change in pH and ionic strength can disrupt secondary structure and cause the proteins to unfold. After unfolding, proteins can easily aggregate because they can reform inter- and intra- molecular bonds such as disulphide bonds and cross-linkages [18]. Possible structural changes and modifications that occur after native proteins unfold has been presented in Figure 6.3. Structural changes such as increase in β-structures improve thermal stability of the proteins, yet this change can reduce their digestibility.

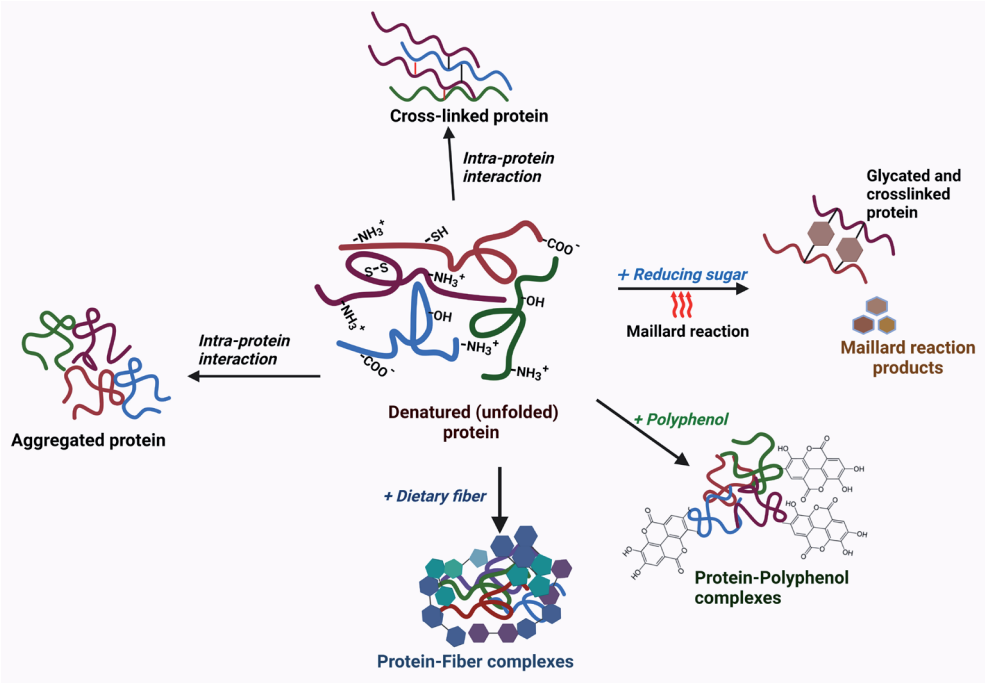

Figure 6.3: Possible structural changes and modifications that occur after native proteins unfold.

Figure was created in BioRender.com

In Chapter 4, protein digestibility of thermally treated samples was slightly lower than that of unheated samples. Digestibility of lentil proteins reduced by 20% after incubation at 60°C for 48 hours. Although the difference was not statistically significant, the decrease confirms the possibility of thermal processing to alter digestibility of proteins. Thermal treatment can also inactivate trypsin inhibitors and reduce their inhibitory effect on the digestibility of plant proteins. However, pulse proteins used in this thesis were extracted by alkaline extraction and isoelectric precipitation which reduces levels of trypsin inhibitor by about 70% [19]. This means that effect of trypsin inhibitors on protein digestibility is significantly reduced, and rather pointing to the structural changes of the proteins.

Globular proteins unfold at high temperatures, such as 60 °C (24) and become more reactive and accessible to digestive enzymes. As a result, digestibility of the denatured (unfolded) proteins increases. However, after protein unfolding, they can also form interprotein cross-linkages or undergo Maillard reaction to form digestion-resistant end products as shown in Figure 6.3. Condensation reactions that occur during glycation, even at the initial stage of the Maillard

reactions, involve covalent interactions and cross-linkages that yield products that are less susceptible to proteases (25,26). The structural change around the reacting amino group likely reduces the proteins' susceptibility to digestive enzymes. Therefore, the overall effect of thermal treatment can increase or decrease the protein digestibility depending on the chemical composition of the various foods.

Thermal processing is a common processing method used in making pulse proteins edible [20,21]. For instance, during thermal processing, the proteins can be denatured such that the globular structure can unfold and make the proteins more accessible to the digestive enzymes and increase digestibility. On the other hand, unfolded proteins can aggregate and reduce accessibility of digestive enzymes [22]. Also, in the presence of other reactive food molecules such as sugars, Maillard reactions can occur. These insights are relevant in the application of pulse proteins as functional ingredients or for their health benefits. When added to product formulations for their health benefits, profound attention should be given to the processing method used and other dietary components in the formulation in a way that protein digestibility is either improved or unaffected. Their digestibility is significant in determining if they are a sustainable source of food proteins. It is important that the benefits of their food applications outweigh their environmental impact. Additionally, given the impact of processing on protein digestibility, food product design can be used to target the digestibility of proteins based on protein requirements of different groups in the population. This is feasible because the processing conditions determine the digestibility of proteins and consequently, the amount of free amino acids absorbed and the type of peptides that move to the large intestine. Thus, protein digestion, absorption, and colonic fermentation can be modulated with the appropriate product formulation.

6.4 Contribution of undigested pulse protein residues in the colon

In assessing the health benefits of dietary proteins consumed, the primary interest of researchers was initially focused on the effect realized after their absorption and the outcome after uptake into the systemic circulation. More recently, the fate of the undigested fraction that moves to the large intestine is receiving research attention, especially considering the long residual time. The colon cells and gut microbiota are the key determinants of the contribution of peptides that reach the colon as shown in Figure 6.4. The figure shows the fate of pulse proteins

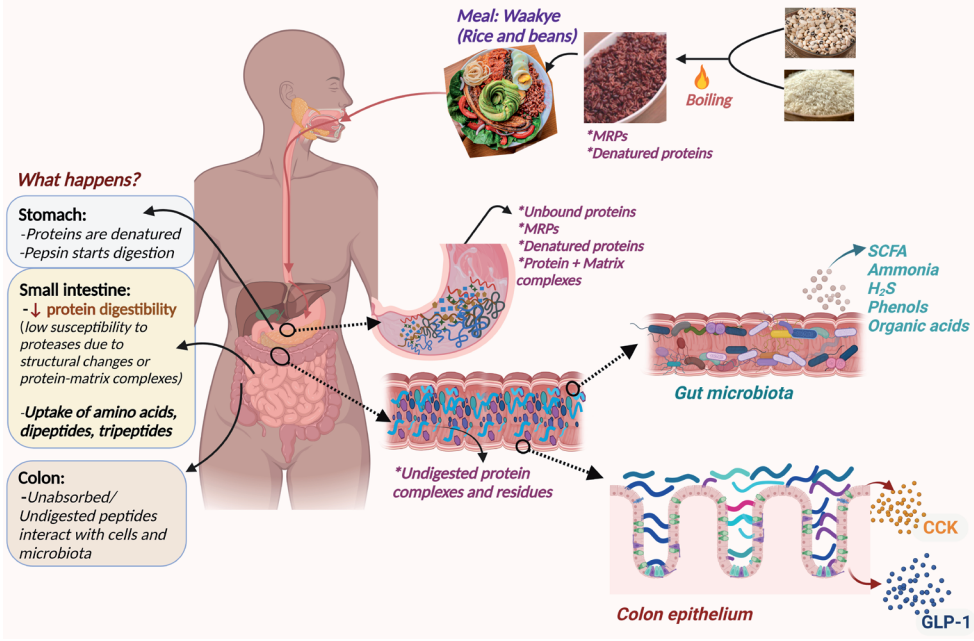

Figure 6.4: Fate of pulse proteins from legume-based meals along the gastrointestinal tract.
Figure was created in BioRender.com

along the intestinal tract after consumption of a thermally treated pulse-based meal such as Waakye (rice and beans). The proteins are likely denatured from boiling of the beans. In the presence of other dietary components, these denatured (unfolded) proteins can also interact with these components to produce complexes with low susceptibility to the digestive enzymes. Hence, the digestibility of the proteins decreases, and the undigested proteins are transported to the colon where they can interact with the gut microbiota and colon cells. The following sub-sections explain the possible effect of the undigested proteins on the gut microbiota and the entero-endocrinal cells in the colon.

6.4.1 Gut microbiota

Proteolytic fermentation by the gut microbiota produces short-chain fatty acids, branched chain fatty acids, ammonia, amines, indoles, phenols, and hydrogen sulphide [23,24]. These metabolites can be absorbed and enter the systemic circulation [25]. Short fatty acids and branched fatty acids are reported to play crucial role in maintaining amino acid balance and host metabolism and signalling [26]. Additionally, short-chain fatty acids produced by the gut microbiota has been reported to induce GLP-1 secretion and this has been confirmed by the presence of SCFA receptor G-protein-coupled receptor 43 (GPR43) in L-cells [27,28]. On the other hand, other metabolites such as ammonia and hydrogen

sulphide can limit oxygen uptake in the mitochondria of the colon cells, depending on the levels present in the colon [29]. The gut microbiota can influence overall health outcome of host through the interplay between the metabolites and the gut-brain axis [30].

The type of metabolites produced by the gut microbiota is dependent on the type of substrates it is exposed to. Processing methods enhance functionality of proteins, yet during processing, the native structure of proteins can be altered upon heat treatment, change in pH, and ionic strength. This means that it can alter susceptibility of the proteins to proteases, thereby determining amount of free amino acids released and absorbed in the small intestine epithelium, and the amount and nature of peptides transported to the colon. Processing can also change the susceptibility of the peptides to bacterial proteases and possibly make them less fermentable substrate for the gut microbiota. Thus, we investigated how glycation of proteins during processing affects the gut microbiota in the colon. The levels of short- and branched-chain fatty acids produced upon 48 hours fermentation were the same for samples with different levels of glycation (incubated with fructose at 60°C for 0 and 48 hours). Our findings show that the extent of glycation does not affect the metabolites produced in the colon. However, the microbiota population was modulated differently based on the host and colon section. It was expected that fermentation of highly glycated peptides will yield less SCFA and BCFA because steric hindrance and possible structural changes will limit microbial protease action, as reported [31,32]. Xu et al reported slight reduction (about 10%) in total SCFA [31] and Dominika et al reported no statistical difference [32].

Previous studies have indicated that MRPs can either have detrimental or beneficial effect on gut microbiota and host metabolism [33,34]. However, from Chapter 4, the levels of SCFAs, BCFAs, and ammonia produced from highly glycated proteins were similar to those produced from less glycated proteins. Our findings, along with Xu et al and Dominika et al show no increase in the concentration of potentially harmful ammonia after fermentation of glycated proteins compared to the unglycated proteins. This shows that the gut microbiota is able to breakdown the MRPs at levels comparable to the unglycated proteins. Different metabolic pathways might be used, but similar levels of metabolites are produced. For instance, certain bacterial strains can remove glucose residues from glycated proteins. *Aspergillus* sp. and *Corynebacterium* sp. have fructosyl amine oxidase which can 'deglycate' fructosyl-amino acids [35,36]. However, we did not observe a higher SCFA concentration (end products of carbohydrate utilization by bacteria) after fermentation of the glycated proteins. Amadori products can

be converted to butyric acid by *Intestinimonas* AF211 and its related gut bacteria [37]. Bifidobacteria has also been reported to use melanoidins as source of carbon and nitrogen for their metabolism [38].

Fermentation of digestion resistant MRPs can also produce bioactive metabolites with antioxidant capacity [39]. Proteins and MRPs can also enhance the microbial population profile diversity [40] and protect host against inflammation of the epithelial lining of the colon [41–43]. The effect of MRPs on gut microbiota is determined by the stage of Maillard reaction [33]. Our findings show that Maillard reaction does not alter end-products of protein fermentation by the gut microbiota.

It is clear that the *in vitro* models used in Chapter 4 is not able to fully mimic the physiology and anatomy of the human digestive tract. However, this provides insights into possible outcomes *in vivo*. It is also possible that higher temperature and longer incubation time might produce more extensively crosslinked glycated proteins and Maillard reaction products that might be utilized differently by the gut microbiota. Yet, Chapter 4 provides a clear understanding that processing affects protein digestibility and consequently influence the fate of proteins along the digestive system. More interestingly, the study provides additional knowledge on how the gut microbiota ferments glycated proteins in the colon which has not been extensively studied in the past.

6.4.2 Gut hormones: CCK and GLP-1

As discussed earlier, food processing can cause structural changes to the protein and facilitate interactions with other proteins or other components in the food matrix. This structural change and interaction can reduce the digestive enzyme's access to the proteins, thus, reduce protein digestibility. When the undigested fraction reaches the colon, they can interact with enter-endocrinal cells as shown in Figure 6.4.

The colon epithelium is densely populated with intestinal cells that differentiate into entero-endocrinal, goblet, paneth, tuft, and absorptive cells [44,45]. The entero-endocrinal cells have subtypes that are located amidst other intestinal cell types such as the absorptive cells. These subtypes include K, I, and L cells which secrete Glucose dependent Insulinotropic Polypeptide (GIP), CCK, and GLP-1, respectively. The I-cells are predominantly located in the duodenum and jejunum; however, I-cells are also located in the colon. CCK was initially known for controlling gall bladder contraction but has recently been found to modulate gastric emptying and reduce further food intake [46]. L-cells are found along the

entire gastrointestinal tract with elevated levels in the distal ileum and colon [44,47–49]. Entero-endocrinal cells are open cells with their microvilli reaching the lumen and thus, can detect and interact with the content of the lumen. The entero-endocrine cells sense luminal peptides through calcium sensing receptors and initiate a calcium dependent stimulation of the secretion of the gut hormones [50,51].

In Chapter 5, we incubated entero-endocrinal cells in >3kDa peptide fraction from digestion of pea and lentil protein. The >3kDa peptide fraction was used as a representation of partially digested proteins. The digestion blank (which contained pepsin and pancreatin) stimulated release of CCK although lower than levels recorded for the pea and lentil proteins. These results suggest that endogenous proteins can influence satiety in the host. The ability of the large peptides used in Chapter 5 to also stimulate secretion of CCK and GLP-1 in STC-1 cells within an hour demonstrate their potential to contribute to satiety. More interestingly, these findings suggest that partially digested proteins that are not absorbed into systemic circulation can still provide beneficial effects for the host. These benefits include regulation of host energy metabolism through the ability of these gut hormones to stimulate secretion of insulin, promote β-cell proliferation, control glucagon production, and regulate appetite and food intake [52,53].

6.5 Methodologies used: their suitability and limitations

6.5.1 Model food systems

Model food systems can be used to study possible interaction between co-existing food components, the effect of their pH, temperature, concentration, and physical state on this interaction [54,55]. These systems are easy to formulate to study multiple interactions with no need for specialized equipment [56]. However, model systems show the interaction occurring, and not necessarily the kinetic and thermodynamic aspect of the interaction [54]. An additional benefit of model systems is the ease of isolating an effect by modifying the conditions used. The model system formulated in Chapter 2 and 3 showed the type of interaction occurring between the proteins and β-glucan and tannic acid.

6.5.2 Simulated *in vitro* digestion model

In understanding the effect of food matrix interaction and processing on the digestibility of pulse proteins, it is essential to evaluate their fate along the digestive tract. The entire digestion process from the mouth, stomach, small intestine, and large intestine can be studied *in vitro* or *in vivo*. *In vivo* methods might be

more physiologically relevant; however, their use is limited by inter-individual differences, cost, and ethical considerations. Animal studies also have many limitations and there is a strong societal pressure to minimize the use of animal experiments. *In vitro* methods are used due to the low cost involved, simplicity, ease of mechanistic study of separate sections [57]. In the last few years, several complex *in vitro* systems have been developed to mimic the human physiological conditions especially *in vitro* intestine systems.

The *in vitro* methods can either be static, semi-dynamic, or dynamic and the suitability of the method depends on the research question. The aim of this thesis was to evaluate the effect of other food components and processing on the digestibility of pulse proteins and so the COST INFOGEST static *in vitro* method was used due to the availability of a standardized method with harmonized digestion conditions. The COST INFOGEST static *in vitro* digestion method provides a physiologically relevant protocol with uniform conditions such as pH, temperature, and enzyme activity [57–59]. However, a major challenge with this method is that it does not consider the body-food feedback system that happens when food interacts with various parts of the digestive system [58]. On the other hand, a major advantage of *in vitro* models of digestion is that the effect of inter-individual variation is removed.

The COST INFOGEST method involves a 2-min oral phase, 2-hour gastric phase, and 2-hour intestinal phase. The pH suggestion for the gastric phase is 3 whereas that of the intestinal phase is 7 [58]. Unlike in most *in vitro* methods, digestion *in vivo* is dynamic such that the gastric phase starts with an initial mixing of the food with hydrochloric acid and gradually adjust pH to 2, after suppressing the buffering effect of the food. The enzymes are also released periodically to reach an enzyme-substrate ratio halfway through the gastric phase. All these conditions are determined *in vivo* by the feedback system depending on the individual, dietary pattern, the consistency of the meal, and nutrient density of the food consumed. Therefore, the main difference between the static models and the dynamic models occurs in the gastric phase [60].

Considering this difference in relation to the *in vitro* method used in our study, the gastric transit time of protein complexes would be different from the protein controls. For instance, it is likely that in the *in vivo* system, the protein controls would exit the stomach faster than the protein- β-glucan complexes due to the viscosifying effect of β-glucan. However, the amount of β-glucan used in this study and the simulated salivary and gastric fluids added during the *in vitro* digestion produced a chyme with low viscosity. Moreover, the contribution of

peptic hydrolysis to overall protein digestibility is reported to be minimum and so the difference in gastric transit time would not have caused remarkable difference in protein digestibility trends observed in our study. Pepsin activity however determines the polypeptides transported to the small intestine for trypsin digestion [61]. Thus, although the trend would not differ significantly, it cannot be excluded that different levels of protein digestibility could be observed *in vivo*.

Additionally, the correlation between the *in vitro* and *in vivo* conditions used remains debatable [59]. Previous reports indicate that dynamic *in vitro* methods can better mimic physiological conditions [62,63]. In vitro models such as the The Dynamic Gastric Model (DGM) [64], human gastric simulator [65], and TNO gastro-intestinal model [66] address this challenge. The TNO gastro-intestinal model comprises separate compartments to mimic separate sections of the digestive tract, simulate peristaltic movements, control transit time, control flow rates, and collect digested materials and metabolites. Thus, comparable to *in vivo* conditions.

6.5.3 Estimation of protein digestibility

Amino acids, the end-product of dietary proteins, are involved in the energy production cycle and are essential components in protein synthesis in the body. Thus, the quality of a protein is determined by its amino acid composition and its digestibility along the digestive tract. Food and Agriculture Organization (FAO) recommends the digestible indispensable amino acid score (DIAAS) as a better representation of protein quality than the protein digestibility corrected amino acid score (PDCAAS). DIAAS focuses on ileal digestibility of the essential amino acids instead of fecal digestibility of the protein.

Protein digestibility measures the release of free amino groups upon hydrolysis of the native proteins [67] and so digestibility is influenced by structure of the protein and how easy the peptide bonds can be cleaved [68]. From this, various *in vitro* methods are used to estimate the digestibility of dietary proteins. The focus of these *in vitro* methods is to determine the proportion of amino acids and peptides produced after the enzyme's hydrolytic activity on the protein. It can also be considered as the fraction of peptide bonds cleaved after hydrolysis.

Free amino groups can be measured as an estimate of the bonds that have been cleaved with the TNBS (trinitrobenzensulfonic acid) and OPA (o-phthaldialdehyde) method or as the level of soluble nitrogen in aqueous trichloroacetic acid [69]. Gel electrophoresis can also show the change in molecular weight of

peptides in the digesta as a qualitative measure of digestibility. The released peptides can also be characterized to determine digestibility. Other researchers also measure the decrease in pH from cleavage of peptide bonds [70] or use the osmometer to detect the change in the freezing point depression during the hydrolysis [69]. These *in vitro* methods are useful when multiple samples must be evaluated. However, their comparability to the actual conditions *in vivo* has not been confirmed. These *in vitro* methods can underestimate the actual digestibility since oligopeptide fractions might not be accounted for in the determination. Under *in vivo* conditions, oligopeptides (tripeptides and dipeptides) are further hydrolyzed by the mucosal and brush border membrane peptidases which is not included in *in vitro* methods [71].

In Chapter 2 and 3, we measured digestibility using the OPA method as suggested by Nielsen (2001) [69]. In this method, dithiothreitol and primary amino groups react with OPA to produce an isoindole derivative [69]. This derivative is colored and can be measured with a spectrophotometer at 340 nm. This method is specific for primary amino groups. Thus, OPA gives a good estimate of cleaved bonds. Compared to other *in vitro* methods, the OPA method is more reliable based on the low variability in degree of hydrolysis obtained [69]. As expected in colorimetric methods, background color of samples might interfere with absorbance readings which is challenging when comparing samples with different colors. Levels of tannic acid (0.001-0.5% w/v) used in lentil protein-tannic acid complexes were considerably low to cause a drastic change in color of entire digesta. Additionally, preliminary tests proved that at 340 nm, tannic acid does not interfere with absorbance readings.

Mendes et al reported a strong correlation between *in vitro* and *in vivo* methods, however the pH drop method was used and thus not widely applicable [72]. Therefore, correlation between *in vitro* methods and *in vivo* measures such as protein digestibility corrected amino acid score (PDCAAS), digestible indispensable amino acid score (DIAAS) remains unclear [73]. Additionally, protein digestibility obtained from *in vitro* measurements does not entirely indicate the proportion that will be absorbed for metabolic use by the body. For instance, the OPA measures the fraction of cleaved bonds. A large polypeptide can have a cleaved bond but will not be absorbed since only tripeptides, dipeptides, and amino acids are usually absorbed by the enterocytes [68]. However, these *in vitro* methods are appropriate for kinetic studies, comparing protein digestibility among samples, and evaluating the role of protein structure and reactivity during digestion. Thus, the OPA method was selected for this thesis.

6.5.4 *In vitro* colonic fermentation models

The contribution of the gut microbiota and its metabolites gives a full picture of the health benefits of foods consumed. The colon is highly colonized by gut bacteria that can further break down undigested or partially digested food components that are transported there [74]. As the undigested fraction passes through the caecum and the ascending colon, they are mixed and further transported to the distal parts of the colon. Various parts of the colon have distinct pH, substrate availability, bacterial population, and density [75]. Therefore, colonic fermentation can be compared to a multistage continuous culture system [76].

In mimicking colonic fermentation, the feed or substrate fractions used must represent digestion residues in the ileal effluents that are transported to the colon *in vivo*. In chapter 4, the whole digesta produced from *in vitro* digestion was subjected to acid precipitation at pH 4 to recover insoluble high molecular weight fractions to represent the unabsorbable, undigested, or partially digested glycated lentil proteins. HCl was used in pH adjustment during acid precipitation, instead of trichloroacetic acid to reduce possible exposure to traces in the gut microbiota culture. Whole digesta can also be dialyzed to obtain the pellet [77] but simple precipitation and centrifugation was used because it is less time consuming and laborious.

Several *in vitro* set-ups have been used to mimic the colon. It is necessary that the set-up closely simulates physiological conditions such as pH conditions, anerobic conditions, and complex gut microbiota. A physiologically relevant model should also consider fluid uptake, dissolved oxygen levels, a mucus layer, and epithelial cells [78]. The *in vitro* colonic fermentation methods available have varying levels of these factors involved. Current methods span from simple batch fermentation models to dynamic models. Dynamic models include the Reading model, Simulator gastro-intestinal model (SIMGI), Polyfermentor Intestinal model (PolyFermS) [79] and small-scale models such as MiniBio model, Copenhagen MiniGut [80], smallest intestine *in vitro* model (TSI) [81,82].

In Chapter 4, we used batch fermentation to assess the metabolites (SCFA, BCFA, and NH_3) produced from glycated lentil proteins. In batch fermentation, single vessels are inoculated with pure or mixed cultures grown under anaerobic conditions and fed with the substrate of interest. They are exposed to the substrate for periods of up to 72 hours. This method is simple and can be used in evaluating several substrates [82]. The fermentation vessels are closed and sampled at desired timepoints. This means that possible exhaustion of substrate makes it difficult to do long incubation times. Also, there is no means to remove the

metabolites produced. Hence, the metabolites can build up and possibly affect further microbial activity. Thus, it is only suitable for short fermentation periods to assess the microbial metabolites from substrates or compare fermentation end products of different dietary components.

On the other hand, dynamic methods comprise multiple vessels to represent different segments of the gut and are incubated at predetermined pH [83]. Current dynamic methods include the TNO In Vitro Model of the Colon (TIM-2) and the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). Each of these has its advantages and limitations. For instance, the TIM-2 like TIM-1 have peristaltic simulations and a dialysis system [84]. SHIME includes gastric and intestinal simulations and can further be adapted to focus on one colon segment [85]. SHIME is very flexible and so can be customized to a parallel set up or even involve mucosal and luminal microbiome in the M-SHIME setup. The proximal and distal colon sections were simulated, and three vessels were assigned to each section, allowing treatments to run concurrently. These vessels were assigned to the basal feed (as control) and the two protein treatments (hydrolysates from lentil proteins incubated for 0 and 48 h) used in Chapter 4.

SHIME fermentation can be conducted over longer periods to monitor possible modulation in the gut microbiota. Thus, the SHIME was used to evaluate how glycated lentil proteins influence the gut microbiota population profile. A clear limitation of SHIME is the lack of peristaltic simulations and dialysis systems. However, the controlled periodic waste removal from the last vessel avoids accumulation of metabolites over the long incubation period. Another limitation of both SHIME and TIM-2 is lack of contribution from epithelial cells. However, these models can be connected to a host-gut microbiota interaction model. Additionally, although challenging, metabolites produced from these *in vitro* methods can be exposed to intestinal cell models. The inoculation method used is also a crucial factor in the reproducibility [74]. To reduce variability in inoculum, pooling in fecal samples has been suggested. However, the pooled samples result in a new 'microbiota' [86].

The population profile and changes can be assessed with many meta-omics approaches depending on the target or desired information. Next-generation sequencing techniques such as shotgun metagenomics and the 16s rRNA gene amplicon sequencing can be conducted to identify the composition. The aim of Chapter 4 was to evaluate possible changes in population profile and so 16s rRNA gene amplicon sequencing was selected to provide a qualitative measure of how the microbial population was affected by the glycated proteins. Beyond the

composition, DNA metagenomics and meta-transcriptomics can be done to delve into the transcription and translation mechanism of the bacteria. Metabolomics can also provide insights into the metabolism of bacteria [87]. However, 16s rRNA gene amplicon sequencing was adequate in showing changes in population profile and thus, was selected.

6.5.5 *In vitro* cell models

In an attempt to mimic the *in vivo* interactions of the intestinal epithelium, several methods have been developed. Currently, organoids, primary intestinal monolayers, organoid derived monolayers, isolated perfused intestine, Ussing Chambers, and isolated entero-endocrinal cells are used. Relevant factors that must be considered in the selection of appropriate models include the throughput, cell-cell contact and interaction, ability to secrete hormones of interest, and experiment time window [88]. Although models such as the isolated intestinal perfusion possess better similitude to the intestinal epithelium, their low throughput, time consideration, and need for specialized techniques limit the ease of use. In vitro cell models like STC-1 cells are simplified and have limited physiological relevance because they are used as monolayers without the total influence of the intestinal cell conditions [89]. They originate from tumors, and this might present deviations in their polarity, morphology, and gene expressions. However, STC-1 cells have demonstrated significant similarities with *in vivo* cells at the transcriptomic level [88]. Their high throughput and ease of use also facilitate their use in understanding intestinal responses to multiple stimuli.

Our primary interest in Chapter 5 of this thesis was to study the ability of peptides to directly stimulate entero-endocrinal cells to release CCK and GLP-1. Secretion of these hormones *in vivo* are mediated by cytoplasmic cAMP, intracellular calcium, G-protein coupled receptors, and PepT1 [45]. Previous studies have demonstrated the ability of STC-1 to express these receptors [90,91], thus STC-1 cell model was appropriately selected. A further step to understand the pathway of stimulation would have been challenged by the lack of cell-cell interaction. Also, interexperimental and interlaboratory differences have been reported and attributed to heterogeneity of the STC-1 cells. However, we addressed this challenge by using cells that have been passaged over ten times as suggested by McCarthy et al [89].

6.6 Implication and Societal relevance of findings

Findings from this study demonstrate that pulse proteins can interact with other dietary components and these interactions can limit protein digestibility. Additionally, large peptides produced from partial digestion of the pulse proteins and their complexes can play significant roles in the colon when transported there. The large peptides can modulate the gut microbiota and their metabolites. The entero-endocrinal cells also secrete satiety hormones in response to these large peptides. The satiety hormones CCK and GLP-1 studied in this thesis play significant roles in energy metabolism and homeostasis. CCK regulate digestion by delaying gastric emptying to halt further food intake. GLP-1 stimulates insulin release from β-cells of the pancreas when blood glucose levels increase. GLP-1 can also enhance satiety, and thus reduce food intake. This means that in addition to their nutritional function, pulse proteins can also prolong satiety when the undigested fractions reach the colon. Initially, the satiety enhancing effect of pulses was mainly associated with dietary fibre [92,93] because of its bulking capacity which reduces food intake and elongates gastric transit time. Recent studies demonstrate that pulse proteins can also enhance satiety [94,95]. This thesis demonstrates that pulse proteins can stimulate production of satiety promoting hormones *in vitro* which can reduce further caloric intake. Consequently, this can reduce the risk of developing obesity, Type 2 diabetes, and related metabolic disorders. Our findings provide insights into plant protein utilization in food applications and food product designs and will also inform development of functional food formulations.

Pulse proteins can be used in food formulations for their satiety promoting roles in the small intestine and the colon. However, from this thesis, it can be deduced that digestibility of the proteins can be impacted by processing and interaction between proteins and other dietary components. Therefore, these factors should be considered in the product design or improvement process because they can influence the type of peptides and amino acids released. Essential amino acids might not be bio-accessible due to the structural modifications caused by processing. In this thesis, we observed that glycation did not alter production of short-chain fatty acids and ammonia when the glycated pulse proteins were subjected to *in vitro* fermentation. However, clear differences were observed in population diversity in the colon sections of the different donors.

From this thesis, it is shown that in the case of reduced protein digestibility, they can still provide satiety-related benefits in the colon. Their effect on satiety is however beneficial or detrimental depending on the physiological conditions

like age and health condition. For instance, in the case of the elderly, prolonged satiety from these peptides might be detrimental. However, in adults, prolonged satiety is beneficial in the maintenance of a healthy body weight.

Focusing on sustainability, these findings support the use of plant proteins as a sustainable alternative. Although protein quality has typically been based on its bioavailability, our study shows that the contribution in the colon should be factored in the sustainability equation. Due to the low digestibility of pulse proteins when compared to traditional animal proteins, it is expected that large quantities of pulse proteins will not be digested or absorbed, but rather move to the colon. This thesis shows that pulse proteins have beneficial roles beyond those that occur after intestinal absorption because the undigested pulse proteins have the potential to prolong satiety through their ability to stimulate satiety hormones in entero-endocrinal cells. This benefit might outweigh the relatively low protein yield and digestibility. Therefore, cultivation of underutilized pulses such as lentils can be increased to make healthy foods more accessible to people of all socio-economic groups. This, in turn, will improve global food and nutrition security and contribute to the United Nation's Sustainable development goals, especially Goal 2 which aims to end hunger, achieve food security, improve nutrition, and promote sustainable agriculture [96].

6.7 Future directions

The contribution of pulse proteins towards meeting the increased protein demands of the growing global population warrants the need to explore sustainable protein alternatives with added health benefits beyond their nutritional role in the body. Our findings in Chapter 2 of this thesis points to the ability to modulate protein digestibility by changing the physical characteristics of the food matrix. Additionally, Chapter 3 demonstrates that levels of other dietary components in the food matrix determines the nature of interaction and consequently, the effect on digestibility. Therefore, plant protein interactions with other food biomolecules should be further explored considering the significant role of matrix interactions on protein quality. Furthermore, the proposed evaluations should be conducted under frequently used domestic and industrial processing methods, and under emerging novel processing methods. This will provide a clearer relationship between food matrix, the processing methods, and protein quality.

The optimum conditions and the necessary modifications needed for desired health benefits should be explored in different age groups and health states. For

instance, the satietogenic effect produced in the colon as observed in Chapter 4 might be beneficial in a healthy adult but not in the elderly nor infants. Additionally, the physiological relevance of CCK and GLP-1 levels released in response to the test peptides used in this thesis should be investigated *in vivo* with more physiologically relevant evaluation methods to confirm the findings of these studies *in vivo*.

In Chapter 4 of this thesis, we found that structural modification of the proteins does not alter the metabolites (short-chain fatty acids and ammonia) produced in the gut microbiota. However, it is unclear if the same is true for other protein fermentation products like hydrogen sulphide, carbon dioxide, phenols, and organic acids. Therefore, effect of these metabolites on host energy metabolism should be explored to ascertain whether these peptides impart beneficial or detrimental attributes in the colon. It is also important to further identify protein substrate levels that provide metabolites with beneficial roles rather than produce detrimental effects. The effect of harsher glycation conditions that are used in thermally processed foods should also be evaluated since the extent of protein structural modification might differ from what was observed in the model system used in this thesis. The effect of dAGEs produced in actual food products on the gut microbiota might differ from the effect in model systems.

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Summary

The global population is rising rapidly with about 80 million people being added every year. This poses a challenge to global food security and food scientists are currently exploring means of addressing this challenge. Particularly, meeting the protein demands of the global population requires that beyond the traditional animal sources, we explore more sources of food proteins which have less detrimental effects on the environment. Current sustainable protein alternatives include legumes and pulses, cereals and pseudo-cereals, oilseeds, tubers, algae, and insects. Of these protein alternatives, plant proteins such as pulses contribute significantly to global protein demand because they are more sustainable, have health benefits, and are already consumed in areas with low food security. However, the structural difference between animal and plant proteins poses a challenge to their digestibility when used in food applications. Processing and interaction with other dietary components can modify the native plant protein structure and affect digestibility of the proteins. This thesis evaluated how processing conditions such as thermal treatment and interaction with other dietary components influence plant protein digestibility. Lentil and yellow pea proteins were selected as model plant proteins because these pulses are commonly consumed, and their proteins are gaining attention for use in food applications.

In **Chapter 2**, we investigated the influence of β-glucan interaction on the particle characteristics and *in vitro* digestibility of proteins isolated from lentil and yellow pea seeds. In **Chapter 3**, we evaluated the effect of tannic acid interaction on particle characteristics, structure, thermal stability, peptide profile, and in vitro peptic digestibility of lentil proteins. Results from Chapter 2 and 3 show that interaction between pulse proteins and food components such as dietary fibre and phenolic compounds can produce complexes that alter the particle characteristics and structure of the proteins. These structural changes consequently limit digestibility of the proteins. In **Chapter 4**, we evaluated how plant protein glycation during Maillard reaction also affects protein digestibility. Maillard reaction occurs frequently during processing of high protein foods and has been suggested to enhance techno-functionality of plant proteins. Chapter 4 demonstrated that glycation of these proteins modifies the particle characteristics and consequently reduce digestibility of the proteins.

After digestion, residual proteins which are partially hydrolyzed polypeptides potentially move to the colon and can interact with the intestinal entero-endocrine cells and the gut microbiota. To investigate this, in **Chapter 4** we assessed how glycated lentil protein hydrolysates that reach the colon affect the metabolites

and population of the gut microbiota using batch fermentation and the Simulator of Human Intestinal Microbiome Ecosystem (SHIME®). We observed that the extent of protein glycation during processing does not affect the short-chain fatty acids (SCFAs), branched-chain fatty acids (BCFAs), and ammonia produced in the colon, while the effect on the microbiota population is dependent on the host and colon section.

Several studies have reported that food proteins and peptides can stimulate secretion of satiety hormones such as CCK and GLP-1. However, it is unclear if digestion-resistant fractions also lead to a similar outcome. Hence, **Chapter 5** explored the effect lentil protein hydrolysates have on CCK and GLP-1 secretion in intestinal endocrine cells, using Secretin Tumor Cell line (STC-1). We used high molecular weight peptides (>3 kDa) to represent the digestion-resistant fraction. The peptides from both pea and lentil stimulated production of satiety hormones CCK and GLP-1.

A general discussion of the summary of the findings of this thesis and their relevance, limitations, and future directions is reported in **Chapter 6.** This thesis shows that digestibility of pulse proteins can be limited by processing and interaction with other dietary components, yet the undigested fraction proteins have the potential to modulate the gut microbiota and prolong satiety through their ability to stimulate satiety hormones in entero-endocrine cells. This benefit might outweigh the relatively low protein yield and digestibility. Therefore, cultivation of underutilized pulses such as lentils can be increased to make healthy foods more accessible to people of all socio-economic groups. Although the *in vitro* methods used in this study limit their physiological relevance, our findings provide insights into plant protein utilization in food applications and guide food product designs in the functional food industry.

Acknowledgement

My PhD journey has been a memorable one because it was filled with moments of joy, laughter, and love, but also challenging moments. Altogether, they have inspired and motivated me to reach out for more. It has been an amazing time of personal growth. Finishing this PhD further reminded me that every detail of my life is continually woven together for good. I am forever grateful to God for His love and for being my source of hope and strength through the entire PhD journey.

Firstly, I would like to thank my supervisors, Prof Vincenzo Fogliano, Dr Chibuike Udenigwe, Dr Edoardo Capuano, and Dr Teresa Oliviero, for their dynamic supervision and seamless teamwork. I am glad I got the opportunity to work with each of you because beyond your in-depth research expertise, you all had peculiar attributes that made our work easier. Vincenzo, your practical questions about feasibility of timelines and relevance of research methods helped me focus on important aspects of my work. Thank you for your objectivity and mentorship. Dr Udenigwe, thank you for recommending the collaboration with the Food Quality and Design Group in WUR. I also appreciate the support your family gave me when I moved to Ottawa. You were my primary supervisor during the first half of the project, and you provided great guidance, which was useful for the entire program.

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I would like to thank all my co-authors, Mieke, Ogadimma, Nico, Shannan, Jurriaan, Monic, and Raliat, for their contribution in producing such significant research papers we can share with the world. I would like to thank all my students, Mieke, Hristiyanna, Nienke, and Kirsten, for their contributions to this thesis. I learnt a lot from you all because you came in ready to learn and gave off your

best. Mieke, I would like to specially thank you for ensuring that I settled in well in Wageningen and for the many trips you organized to ensure that I saw much of the Netherlands as possible. To my assessment committee members, Prof. Vincken, Dr de Vries, Dr Camps, and Dr Deglaire, I appreciate your timely evaluation of my thesis.

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I moved to Wageningen amid the COVID-19 pandemic, which meant that I did not meet a lot of people from the onset. However, I got all the support I needed from the Food Quality and Design group, WUR. Thank you Corine, Lisa, and Ciska for being welcoming and resourceful. I would like to thank Elisa again for connecting me with Marianna who provided helpful responses to my many questions during my early days in Axis. As time went on, I got to meet the FQD technicians who have been readily available to provide training and guidance whenever needed. Erik, not only did you help with my SHIME experiments, but you were also available for our insightful conversations about my experimental plans. Yajing, I did not know we will become friends later, but you were a great PhD buddy. Our PhD trip to Spain brought us even closer, and we even recruited Feilong. Momo, thank you for your regular check-ins and coffee time and the amazing food we enjoyed together. Fiametta, you always had a nice and inviting smile. You regularly asked about the progress of my work, and I saw how genuine your interest was.

I am grateful to all FQDers for the diverse contributions they made to the success of my time in FQD. We had fun potlucks, FQD days, lab and office conversations, coffee, and many more. Thank you Diana, Cristina, Nayara, Melania, Vincenzo C, Ranti, Sara P, Swantje, Carole, Mostafa, Ruth N, Niels, Luc, Lise, Fleur, Lucia, Lotte, Lijiao, Pieter, Lintianxiang, Yao, Araceli, Julie, Zongyao, Tomer, Jiaying, Qing, Annelies, Zhijun, Rutger, Peiheng, Qing, Sofia, Marialena, Hongwei, Yongkai, David,

Laura, Renske, Seren, David & Fabio, Martina, Arnau, Folake, Siwei, Xiangnan, Keqing, Kangni, Rutger, Vincenzo D, Luigi, Zekun, Qing, Zhao, Peiheng, Fabiola, Pengfei, Bei, Obaid, Zhan, Yifan, Michelle, Juliana, Andrea, Jolien, Marielle, Abbey, and Maryia. Again, thank you to FQD staff: Anita, Catriona, Jenneke, Matthijs, Pieternel, Bea, Kasper, Ruud, Charlotte, Thom, Saskia, Markus Nicoletta, Josep, Christos, Pien, Sjoera, Trang Anh, Geert, Xandra, Jelle, Nienke, Frans, Erik, Corine, Lotte, Dieuwerke, Etske, Pieter, Sara E, Tamara, Blerina, Ciska, Deniz, Lisa, Anastasia, and Ebru. To the VLAG office, thank you for your regular emails to check my progress.

Thank you Shiksha and Mohammad for accepting to be my paranymphs. Shiksha, our friendship started with few words here and there in Axis until our long conversations in the office, which also progressed to our overfilling dinners at home. You are warm-hearted, and I am glad we got to be friends in Axis and at home. I look forward to being friends beyond Wageningen. Thank you Thisun for our long lunches and conversations in Axis. I see the effort you put into making everyone around you feel belonged. You took time to organize many trips; although I could join a few, you invited me anyways. I enjoyed our King's Day trip and May 5 hangout. I appreciate your help with designing the cover of my thesis.

I am also thankful for ANFGC Halifax and ANFGC Ottawa. I made valuable connections, and I knew that I could count on you for support. In Ottawa, I would like to specifically thank Dr Veronica Adu-Bobie for the warm reception and support you gave me when I was in Ottawa. It was amazing how I always left your office happier and more motivated. I would like to also thank Naomie and Deisy for making our home in Kanata comfortable and peaceful; the environment you helped create was conducive for resting and rejuvenation after the long lab hours. I would like to thank AGP Wageningen for the warmth, smiles, hugs, and the place of growth you provided. Pastors Farai and Busi, your willingness to help is admirable. I am glad I joined AGP. I would like to thank the entire Sikaman community, especially the patrons and leadership. I enjoyed our hangout events where we had good Ghanaian dishes and reconnected with our local languages and music. I also made great friends from Sikaman, and I am glad I met you all.

During this PhD, I enjoyed the affectionate care from many friends. I appreciate your contribution to my life, and I hope that we will be connected for the long haul, shaping each other to be better! If things turn out differently, remember that we were there for each other when needed and when we could. Thank you to the Owusu, Mpi, and Kasaji families. Thank you Emmanuel, Augustina, Jacob,

Halimat, and the many more whose names do not appear here. I appreciate you all.

Finally, I would like to thank my family for their unwavering support. Particularly, I would like to thank Dada, Mama, Johnmark, Patience, and Pauline. Dada, thank you for your encouraging words especially towards the end of the PhD work. It was reassuring to know that you could relate to my ordeal and you were readily available to talk. Mama, Nyame nhyira wo sɛ wokɔ so a bɔ mpaeɛ na wosan hyɛ menkuran. Mmerɛ biara a mɛfrɛ a mewerɛ aho anaa sɛ nnoɔma nkɔ sɛ nea mepɛ no, wode Nyame asɛm hyɛ me nkuran. Wohwɛ sɛ wone me bɛkasa ntɛmtɛm so. Medaase paa. Mani sɔ nea woyɛ nyinaa. Johnmark, you started Med school in the same 2019 and I know how busy the program is but every time I needed you to proofread my papers, you made time to do it tactfully and thoroughly. You also challenged me with questions about the next steps and exploring all my options. I am grateful. Patience, thank you for always checking in with me to make sure I am doing well. I know how much you care about me and all your well wishes. Pauline, thank you for the long and periodic check-in calls and messages. You all believed in me and were very understanding and empathetic. I love you all dearly.

About the author

Ruth Takyiwaah Boachie was born on May 6, 1991, to John Kwabena Boachie and Comfort Sarfo. She has three siblings: Johnmark, Patience, and Pauline. Ruth grew up in Kumasi, Ghana, where she also had her basic and high school education. She obtained a BSc Nutrition and Food Science degree in 2013 from the University of Ghana, Accra, Ghana. Thereafter, Ruth worked as a Research Assistant for 2 years in the Food Process Engineering Department of the same University. During her undergraduate program, she developed an interest in further understanding how food affects our body and overall health, and so she pursued a MSc Applied Human Nutrition at Mount Saint Vincent University, Halifax, Canada in 2017. Her MSc thesis focused on the effect of hydrothermodynamic (HTD)-processed blueberries on postprandial blood glucose control and antioxidant status in human adults.

Right after her Masters, Ruth explored the application of research in industry, since she believes that research findings can effectively improve the quality of life when researchers, policy makers, and industry work together. Hence, she worked as a Research Associate in the Applied Research Department of Nova Scotia Community College from 2016 to 2017. She also worked as an R&D and Quality Coordinator in the nutraceuticals industry, specifically with Nova Scotia Organics from 2017 to 2018.

While working, Ruth always had an interest in conducting advanced research in nutrition and food science. Therefore, in February 2019, she began her PhD study with the Food Quality and Design group in Wageningen University and Research. Her PhD project was a collaboration between the Food Quality and Design group and School of Nutrition Sciences in University of Ottawa, Canada. Hence, she began her research with the Udenigwe Lab in University of Ottawa from February 2019 and moved to Wageningen in January 2021. In the same year, she was awarded a Postgraduate Scholarships-Doctoral (PGS-D) by the Natural Science and Engineering Research Council, Canada. She was a member of the 2021-2022 VLAG PhD council in Wageningen University and Research.
Ruth likes to have good meals and conversations with her family and friends. She also enjoys watching movies and volunteering at her church.

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List of Publications

List of Publications from this thesis

1. **Boachie RT**, Commandeur MM, Abioye RO, Capuano E, Oliviero T, Fogliano V, Udenigwe CC. β-Glucan Interaction with Lentil (Lens culinaris) and Yellow Pea (Pisum sativum) Proteins Suppresses Their In Vitro Digestibility. Journal of Agricultural and Food Chemistry. 2021 Sep 2.
2. **Boachie RT**, Okagu OD, Abioye R, Hu̇ttmann N, Oliviero T, Capuano E, Fogliano V, Udenigwe CC. Lentil Protein and Tannic Acid Interaction Limits in Vitro Peptic Hydrolysis and Alters Peptidomic Profiles of the Proteins. Journal of Agricultural and Food Chemistry. 2022 May 20.
3. **Boachie RT**, Capuano E, Oliviero T, Udenigwe CC, Fogliano V. Undigested glycated lentil proteins modulate the gut microbiota profile but not the metabolites in vitro. Journal of Functional Foods. 2023 Aug 1;107:105667.
4. **Boachie RT**, Capuano E, Bastiaan-Net S, Tomassen M, Jurriaan M, Oliviero T, Udenigwe CC, Fogliano V. Undigested proteins from lentils and peas stimulate CCK and GLP-1 secretion in STC-1 intestinal cells. 2023 (*Manuscript in preparation*)

Conference papers from this thesis

1. **Boachie RT**, Commandeur MMB, Abioye R, Capuano E, Oliviero T, Fogliano V, Udenigwe CC. Inhibitory Effect of β-Glucan Interaction on In vitro Digestibility of Proteins Isolated from Lentil (Lens culinaris) and Yellow Pea (Pisum sativum) Seeds. Virtual 2021 AOCS Annual Meeting & Expo. DOI: 10.21748/am21.170
2. **Boachie RT**, Okagu OD, Abioye RO, Huttmann N, Oliviero T, Capuano E, Fogliano V, Udenigwe C. Formation of lentil protein-tannic acid complexes limits in vitro peptic hydrolysis and alters peptidomic profiles of the protein. In Journal of The American Oil Chemists Society 2022 Oct 1 (Vol. 99, pp. 143-144). 111 River St, Hoboken 07030-5774, Nj Usa: Wiley.
3. **Boachie RT**, Okagu OD, Abioye R, Hu̇ttmann N, Oliviero T, Capuano E, Fogliano V, Udenigwe CC. Lentil Protein and Tannic Acid Interaction Limits in Vitro Peptic Hydrolysis and Alters Peptidomic Profiles of the Proteins. Presented virtually at: NIZO Plant Protein Functionality Conference 2022; 2022 October 11-12.
4. **Boachie RT**, Okagu OD, Abioye RO, Huttmann N, Oliviero T, Capuano E, Fogliano V, Udenigwe C. Lentil protein and tannic acid interaction limits *in vitro* peptic hydrolysis and alters peptidomic profiles of the proteins. Presented at: Polyphenols Applications 2022, Valencia, Spain: 2022 September 28-30

Overview of completed training activities

Name of the course/meeting	Organizing institute (s)	Location	Year
Category A: Discipline specific activities			
AOCS Annual Meeting & Expo[b]	American Oil Chemists' Society	Virtual	2020
AOCS Annual Meeting & Expo[a]	American Oil Chemists' Society	Virtual	2021
Bridge2Food Research Conference Plant-Based Foods & Proteins Europe	Bridge2Food	Virtual	2021
Chemometrics	VLAG	Wageningen, Netherlands	2021
Polyphenols Applications Congress[a]	Polyphenols Applications	Valencia, Spain	2022
2nd NIZO Plant Protein Functionality Conference[b]	NIZO	Virtual	2022
Intestinal Microbiome of Humans and Animals	VLAG	Wageningen, Netherlands	2023
Healthy Food Design	VLAG	Wageningen, Netherlands	2023
Healthy and Sustainable Diets	VLAG	Wageningen, Netherlands	2023
Category B: General courses			
Presenting with impact	Wageningen Graduate School	Wageningen, Netherlands	2021
Writing grant proposals	Wageningen Graduate School	Wageningen, Netherlands	2021
Introduction to R	VLAG	Virtual	2021
Applied Statistics	VLAG	Virtual	2021
Reviewing a scientific manuscript	Wageningen Graduate School	Wageningen, Netherlands	2021
Critical thinking and argumentation	Wageningen Graduate School	Wageningen, Netherlands	2021
Project & Time management	Wageningen Graduate School	Wageningen, Netherlands	2021
Scientific writing	Wageningen Graduate School	Wageningen, Netherlands	2022
Philosophy and Ethics of Food science and Technology	VLAG	Wageningen, Netherlands	2022

Category D: Other activities			
Writing Research Proposal	Food Quality and Design	Ottawa, Canada	2019
Annual FQD PhD Tour	Food Quality and Design	Spain	2022
VLAG PhD Council	VLAG PhD Council	Wageningen, Netherlands	2021-2022
Supervision of MSc and BSc students	Food Quality and Design	Ottawa, Canada & Wageningen, Netherlands	2019-2022
Food Quality and Design Weekly Colloquia	Food Quality and Design	Wageningen, Netherlands	2021-2023

[a]Oral presentation
[b]Poster presentation
VLAG: Graduate School for Nutrition, Food Technology, Agrobiotechnology and Health Sciences
FQD: Food Quality and Design

Colophon

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