Preparation, characterisation, and application of plant protein-dairy protein hybrid nano assemblies

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Massey University, Auckland, New Zealand

Imaan Thind

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Abstract

For centuries, pulses have played a significant role in human nutrition due to their high protein, dietary fibre, vitamins and minerals content. Faba bean is a good source of protein with a well-balanced amino acid profile and offers environmental advantages such as nitrogen fixation and adaptability to colder climates.

Despite being rich in nutrients, faba bean proteins exhibit poor functionality, including low solubility, a high tendency to form aggregates, and the presence of antinutritional factors. These challenges limit the commercial use of faba bean proteins in food systems that require strong emulsification and gelation. Combining faba bean proteins with the dairy proteins is a promising approach to improve the functionality of faba bean protein isolate (FPI) while maintaining sustainability.

This research is based on this background, which investigates how faba bean protein isolate (FPI) and whey protein isolate (WPI) can be combined to create a functional hybrid which addresses the growing sustainable needs. WPI is well known for its excellent functional properties, such as solubility, interfacial behaviour, and gelation, but it is not particularly sustainable. Combining FPI and WPI is a promising approach, but understanding their different behaviours and the best way to incorporate them is essential for practical use in food systems.

In this study, FPI, WPI, and their blends were processed using heat treatment, cold sonication, and thermosonication to investigate the effects of these treatments on particle size, structural organisation, zeta potential, emulsification, and gelation properties. The untreated sample, especially those with high FPI content such as WPI: FPI (2:8 and 0:10), showed larger particle sizes due to stronger hydrophobic interactions. Cold sonication disrupted intermolecular bonds, resulting in a smaller particle size. In contrast, heat treatment was most effective on higher WPI-ratio mixtures (WPI: FPI (10:0 and 8:2)) rather than higher FPI-ratio mixtures (WPI: FPI (2:8 and 0:10)), resulting in higher turbidity and aggregation. Thermosonication was effective in producing smaller, more uniform particles across all samples.

Zeta potential results showed that WPI-rich solutions (WPI: FPI (10:0 and 8:2)) exhibited higher negative charge and greater stability. SDS-PAGE confirmed structural changes in mixtures of different WPI: FPI ratios. The results showed that samples with higher FPI (WPI: FPI (2:8 and 0:10)) exhibited greater protein aggregation, particularly under non-reducing

conditions. Among all the treatments, thermosonication showed the most precise and most organised band patterns, thus justifying the fact that it induces the reorganisation and unfolding of proteins more than any other treatment.

Thermosonication significantly improved ($P<0.05$) emulsifying properties, especially of the higher FPI-ratio blends (WPI: FPI (2:8 and 0:10)). Under oscillatory rheology, these emulsions showed smaller oil droplet sizes and a stronger gel-like structure. Cold-sonication showed less improvement in emulsion stability and gel-like behaviour than thermosonication, and heat treatment was more effective on the blends with the higher WPI ratio, especially WPI: FPI (10:0). Overall, thermosonication enhanced interfacial behaviour, improved adsorption, and led to the formation of a stronger interfacial film.

Acid-induced gels were formed using glucono-δ-lactone to study the gelation properties of the proteins. Untreated and cold-sonicated gels were weaker, while heat-treated gels were stronger, especially in higher WPI-ratio systems, particularly WPI: FPI (10:0). Among the treated gel samples across the WPI: FPI ratios studied, thermosonicated gels were the strongest as confirmed by confocal microscopy, which revealed uniform, dense structures. Water-holding capacity results further confirmed that the thermosonicated gels retained water better, even in higher FPI-ratio samples (WPI: FPI (5:5 and 0:10)). This can be explained by the combined effect of thermosonication's mechanical disruption and protein unfolding.

These findings demonstrate that thermosonication, as a processing method, can help overcome the limitations of the FPI by forming a hybrid system with the WPI. The study shows that hybrids of these two proteins behave differently from the individual proteins, and this behaviour can be altered by using the proper processing method. Thermosonication produced the best and most consistent overall results by reducing particle size, improving emulsifying and gelation behaviour, and increasing water-holding capacity. This study highlights the potential of combining WPI and FPI to form hybrid systems that yield high-performing food formulations and support sustainability. This research provides the pathway to develop new protein formulations for the future of sustainable food production.

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1. Introduction

1.1. Overview of the research

Animal proteins have been popular because they are essential for human growth. They are also known for their superior quality to plant proteins because of their high amino acid score, better digestibility and solubility (Balandrán-Quintana et al., 2019). Proteins are widely used in the food industry as functional ingredients, serving as gelling agents, emulsifiers, and foaming agents. Milk proteins are renowned for their exceptional functional properties, which justify their widespread use in the industry. With an increasing population, the demand for protein has also risen; however, meeting this demand has environmental consequences. In today's world, plant proteins are increasingly recognised as alternatives to meat and dairy proteins. They serve as functional agents or meat substitutes, offering a range of benefits. This shift is primarily driven by a growing awareness of the need for a more sustainable world.

Plant proteins offer physiological benefits to humans, such as improved bone density, an increase in leg power and an increase in lean body mass (Hertzler et al., 2020). Plant proteins have also shown results in weight reduction, improved metabolic features and reduced pro-inflammatory status in the obese individuals (Jain & Goomer, 2020).

Pulses are the primary source of plant proteins, which have been providing essential nutrition to people worldwide for centuries. They are high in protein, dietary fibre, minerals, and vitamins, low in fat, and a good source of energy. They are high in lysine, which gives them an advantage over cereals. Pulses comprise dry non-oil seed legume crops, including lentils, chickpeas, beans, and dried beans (Tyler et al., 2017).

Due to this growing awareness and shift towards plant proteins from animal proteins, many researchers have worked on plant proteins, such as soy protein (Gallagher et al., 2004; Hou et al., 2015), pea protein (X. Du et al., 2023; Krentz et al., 2022), quinoa protein (Luo, 2022; Patole, 2022; Yang et al., 2022) and faba bean (Boukid & Castellari, 2022; Hu, Cheng, Gilbert, Lee, et al., 2024; Johansson et al., 2022; Neofytos et al., 2025). Soybeans have been one of the most dominant plant proteins in the food industry. However, other plant proteins

have been widely researched due to the problems associated with growing soybeans worldwide. Faba beans are widely studied as a source of plant protein due to their high protein content. Faba beans are easier to grow than soybeans. Faba beans also perform better environmentally because they can fix nitrogen symbiotically, reducing the need for fertilisers (Köpke & Nemecek, 2010). Faba beans, botanically known as *Vicia faba*, are an annual legume. This legume prefers cool, moist conditions for growth, but it tolerates frost. Faba beans are partially allogamous, and bees play an essential role in cross-pollination. Faba beans are an excellent source of protein, carbohydrates, vitamins, and minerals, with protein content ranging from 20% to 41% across varieties and sources (Yang et al., 2018).

However, plant proteins generally have poorer physicochemical properties than milk proteins, which limits their use in food systems (Rashwan et al., 2025; R. Yu et al., 2024). Plant-based proteins also usually lack some essential amino acids; therefore, partially replacing plant protein with animal protein can be a practical option. A plant-dairy protein hybrid offers an innovative solution that enables the use of plant proteins as a functional ingredient while reducing the reliance on milk protein, with sustainability in mind. However, there is a limited understanding of the interactions between plant and dairy proteins and how their interactions will influence the functionality of their hybrid systems (Neofytos et al., 2025).

In this study, the focus has been on faba bean protein and whey protein, exploring their emulsifying and gelation properties and how these properties can be utilised to create an innovative product. This research design begins with a detailed examination of faba bean protein isolate (FPI) and whey protein isolate (WPI), which is necessary to understand before combining these two proteins to assess their functional and sensory properties. This understanding is crucial before developing the hybrid systems using these proteins. This could help build a foundation for a hybrid system utilising these two proteins, with a focus on sustainability and functionality. This research planned to employ processing techniques such as heat treatment, cold sonication, and primarily thermosonication to understand how these processing techniques influence the properties and functionality of proteins. Little research has been conducted on FPI- and WPI-based hybrids that can be utilised commercially. This gap highlighted the significance of the study to investigate the properties of these blended proteins and their commercial use in food systems to address the problems the world is currently facing.

Both WPI and FPI are made of different protein fractions which might behave differently during processing and formation of the hybrids. WPI mainly consists of β-lactoglobulin and α-lactalbumin which are known for their magnificent solubility, interfacial behaviour and gelation properties. On the other hand, FPI mainly contains globulin proteins such as vicilin and legumin contributing to its nutritional quality but may also result in greater tendency to form aggregates hence decreasing solubility. The interactions between these proteins can result in structural organisation, therefore, influencing the emulsification and gelation properties (Hu, Cheng, Gilbert, Lee, et al., 2024; Neofytos et al., 2025). Understanding the interactions between these individual protein fractions is crucial in developing a stable and functional plant-dairy hybrid protein.

The hypothesis of this study is that combining WPI and FPI by using different processing methods can alter protein-protein interactions leading to improved functional properties of the hybrid systems as compared to the untreated proteins. It is expected that thermosonication will lead to greater unfolding and rearrangement due to its combined mechanical and thermal effects compared to the cold sonication and heat treatment. These changes in the structure are expected to improve interfacial behaviour, particle size distribution, emulsifying properties and gelation characteristics.

1.2. Thesis Objective:

The objective of this research is to:

1. To develop a whey protein isolate (WPI) and faba bean protein isolate (FPI) hybrid using thermosonication and compare it with cold sonication and heat treatment.

2. To compare the physicochemical properties of the WPI, FPI and their hybrids made using different treatments.

3. To investigate and compare the properties of the emulsions of WPI, FPI and their hybrids.

4. To investigate and compare the properties of the gels of WPI, FPI and their hybrids.

5. To investigate how different protein fractions of WPI and FPI influence the formation and functionality of the hybrid systems under different processing techniques.

6. To determine whether thermosonication is the best method to improve the structural and functional properties of the WPI-FPI hybrids as compared to the cold sonication and heat treatment.

2. Literature Review

2.1. Introduction of faba bean:

Pulses have been an essential component of human nutrition worldwide. They provide a valuable source of protein, dietary fibre, minerals, and vitamins, and are low in fat and high in energy. Pulses are comprised of dry and non-oil seed legume crops, including lentils, chickpeas, beans, and dried beans (Tyler et al., 2017). Compared to cereals, they are relatively high in lysine. This improves the overall amino acid balance of the diet, providing pulses with a nutritional advantage (Khvostenko et al., 2024; Martineau-Côté et al., 2022). Legume crops are among the high-protein foods recognised for their ecological and economic benefits.

The faba bean (*Vicia faba L.)* is a protein-rich legume belonging to the Fabaceae Family. It is also known as broad bean, fava bean and horse bean and is also one of the oldest cultivated crops (Mínguez & Rubiales, 2021). Most of the faba beans production occurs in Asia, Africa and Europe. It is used as a human food source in Asia/Africa, and also as animal feed in some regions of Europe (Badjona et al., 2024; Dhull et al., 2022)**.** Faba beans act as atmospheric nitrogen fixers in the soil, reducing the need for synthetic fertilisers. They can also help in increasing soil fertility and lowering weeds, diseases and pests (López-Bellido et al., 2005). Recent studies have shown the use of faba bean as a promising ingredient for protein fractionation and also as a value-added food application (Badjona et al., 2024; Stone et al., 2024; Thomsen et al., 2025). It is a typical breakfast food in Middle Eastern countries, China, and Ethiopia. China, Ethiopia, the United Kingdom and Australia are the leading producers of the faba bean (Dhull et al., 2022).

In New Zealand, faba beans are the cool-season legume crop. Local varieties might differ in their seed size, protein and starch content and physicochemical properties which then influence extraction efficiency and functionality. A study investigated New Zealand grown faba bean varieties like Early Long Pod, Coles Dwarf, Evergreen and Janet conveying that local varieties can be considered when developing ingredients using faba bean protein (Navneet et al., 2025).

Table 1: Scientific classification of faba bean

Kingdom	Plantae
Family	Fabaceae
Subfamily	Faboideae
Tribe	Fabeae
Genus	*Vicia*
Species	*V. faba*

There are four botanical types of *Vicia faba* L.: *mayor, minor, puacijuga and equinoa.* They differ mainly in seed size. Environmental conditions significantly influence seed yield, as low European temperatures and limited rainfall in Mediterranean countries are key limiting factors for this legume. Water during flowering and plant density determine this plant's seed yield. Hence, both environmental and genetic factors can influence the composition and functionality of the faba bean which is important to consider when using it for protein extraction and in food formulation (Cormack et al., 2026; Navneet et al., 2025).

2.1.1. Nutritional value of faba bean:

- Vitamins and minerals:

Faba bean seeds are high in potassium and low in sodium, making them suitable for individuals on low-sodium diets and those with hypertension (Dhull et al., 2022). Faba beans contain folates, which are more abundant than in soybeans and peas, making them a valuable food source for pregnant women. A deficiency of folates can lead to spina bifida and anencephaly in newborn babies (Mutumbua et al., 2024). Therefore, faba bean should be considered as nutrient rich pulse with additional dietary value (Dhull et al., 2022; Navneet et al., 2025).

- Carbohydrates:

Faba bean seeds are a good source of carbohydrates, mainly consisting of starch, non-starch polysaccharides, and oligosaccharides. Faba bean starch is primarily composed of two

components: amylose and amylopectin. Hemicellulose, lignin, and cellulose are the predominant components of dietary fibre. Oligosaccharides of the Raffinose family: Raffinose, Verbascose, and Stachyose are the main soluble starches (Moussou et al., 2019). Carbohydrates such as starch and fibre can affect the water absorption, viscosity and gel structure which influences the functional properties of the faba bean ingredients. Therefore, the remaining carbohydrates after protein extraction can affect the final behaviour of the functional properties (Stone et al., 2024; Thomsen et al., 2025).

- Bioactive compounds:

Various bioactive phytochemicals in faba beans include flavonoids, phenolic compounds, terpenoids, and lignans (Dhull et al., 2022). Flavonoids in faba beans have anti-diabetic and anti-inflammatory properties. Faba bean is also an excellent source of polyphenols, having antioxidant properties. These bioactive compounds do provide some nutritional benefits, but they can interact with proteins hence influencing flavour, digestibility and functional properties which can create challenges during food formulation and protein extraction (Badjona et al., 2024; Thomsen et al., 2025).

- Antinutrients:

Faba beans have several antinutritional components, including phytic acid, protease inhibitors, tannins, hemagglutinins, trypsin inhibitors, and oligosaccharides, which can adversely affect human health (Samtiya et al., 2020). Pyrimidine glycosides, such as vicine and covicine, the precursors of divicine and isouramil, can lead to Favism in humans. Favism is a type of anaemia that occurs in people with a deficiency of glucose-6-phosphate dehydrogenase. It is a severe allergic reaction to consuming faba beans or inhaling their pollen. In some cases, it can lead to Hemolytic anaemia, which can further cause Hemoglobinuria, which can be life-threatening to children. Therefore, reducing the antinutritional factors in the final faba bean ingredients is essential which can be obtained through cultivar selection and processing methods (Badjona et al., 2024; Cormack et al., 2026; Samtiya et al., 2020)

- Proteins:

Proteins are peptide-bonded polymers made up of 20 distinct amino acids(Whitford, 2013). Amino acids are white crystalline solids with high melting and boiling points. Their melting points are -20.8 °C and 314 °C. Their high melting and boiling points are due to strong

charged interactions that hold them together. The faba bean, like the pea and the soybean, has a well-balanced amino acid profile. They have fewer sulfur-containing amino acids (cysteine and methionine), which can be explained by the fact that most proteins are globulins (Millar et al., 2019; Świątkiewicz et al., 2018).

Table 2: Amino acid profiles of Faba bean, Pea and Soy proteins (g/100g protein) (Martineau-Côté et al., 2022)

Amino acid	**Faba bean**		**Pea**	**Soybean**
	High Tannin variety (cultivar not mentioned; g/100g protein)	Low Tannin variety (cultivar not mentioned; g/100g protein)	g/100g protein	g/100g protein
Histidine	2.41	2.29	2.52	2.91
Isoleucine	3.94	3.91	3.33	4.6 0
Leucine	7.47	7.01	6.58	7.76
Lysine	7.08	6.71	6.84	7.08
Methionine	0.87	1.06	1.03	1.29
Cysteine	1.33	0.85	1.55	1.19
Phenylalanine	4.19	4.12	4.19	5.87
Tyrosine	2.78	2.59	3.16	3.65
Threonine	3.40	3.40	3.59	3.69
Tryptophan	0.87	0.85	0.94	1.38
Valine	4.31	4.12	3.89	4.64
Arginine	9.46	9.04	6.84	8.86
Alanine	4.15	4.03	4.27	4.39
Aspartic acid	10.74	10.4	10.68	11.98
Glutamic acid	16.51	16.26	16.92	17.88
Glycine	4.73	4.25	4.32	4.20
Proline	3.94	3.86	3.72	4.92
Serine	4.69	4.76	4.79	4.77

At neutral pH, amino acids exist in a zwitterionic state, characterised by charged carboxylic and amino groups attached to the central carbon atom. The charge of the amino acid changes with the pH. At low pH, a proton binds to the carboxylic acid; at high pH, a proton is extracted from the amino group.

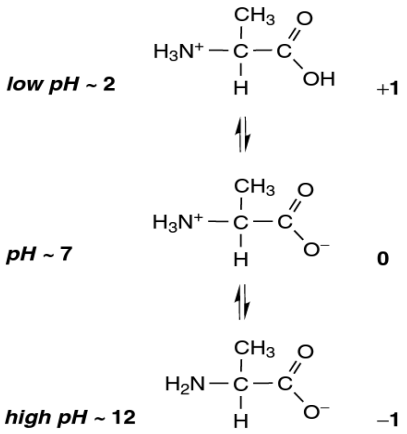


Figure 1. Different charged forms of the amino acid Alanine (Whitford, 2013)

Amino acids make peptide bonds when one amino acid interacts with another's carboxylic group. This is a condensation reaction as it leads to the release of a water molecule. When several amino acids are joined together, they form polypeptides. These polypeptide chains fold into a globular structure at physiological temperatures. Polypeptides that can create a stable tertiary structure can function as proteins.

- Primary structure: The linear order of amino acid residues along the polypeptide chain is the primary structure. This structure is crucial in determining the overall shape and function of the protein. Each amino acid in the chain is connected by peptide bonds, forming a long chain that eventually folds into a three-dimensional shape. Understanding the primary structure of a protein is essential in studying its properties and potential applications.

- Secondary structure: This structure has two basic subunits: α-helix and β-strands, made through the hydrogen bonding between amino and carboxylic groups of the different amino acids in the polypeptide chain.

- Tertiary Structure: Tertiary structure is made when α-helix and β-strands are looped or linked in a manner that the secondary structure is no longer present. Most of the globulins are tertiary-structured proteins (Petsko & Ringe, 2004).

- Quaternary structure: This structure is the folded chains formed by the association of more than one polypeptide.

Faba bean has high protein content ranging from 20-41% depending on varieties and source type (Yang et al., 2018). Factors such as fertilisation method, planting site, and growth season also significantly affect protein content. Faba bean flour's protein content is 29.7% on average (Gu et al., 2020).

Table 3: Types of major proteins found in Faba bean and their extraction relevance (Badjona et al., 2024; Sharan et al., 2021; R. Yu et al., 2024; Żmudziński et al., 2021)

Protein Type	**% of total protein content present (w/w)**	**Properties**	**Approximate isoelectric point / extraction relevance**
Globulins	60%	Soluble in low-salt solutions	Major protein fraction. Shows low solubility around pH 4.0 to 4.5. Individual globulins may behave differently depending on the extraction conditions.
Albumins	20%	Soluble in water	Exact pI information not clearly reported. But due to being water soluble, they may remain soluble over wider pH.
Glutelins	15%	Soluble in alkaline solutions	Exact pI information not clearly reported. They are soluble in alkaline solutions which points out that pH adjustments will strongly affect their extraction.
Prolamins	8%	Soluble in 70% alcohol	Exact pI information not clearly reported. Their solubility in alcohol makes them less relevant to aqueous isoelectric precipitation.

Faba bean proteins are mainly composed of globulins, albumins, glutenins and prolamins. These fractions have different structure, solubility behaviour and pH response. Understanding of the solubility and isoelectric points of these proteins is crucial during the protein extraction process. At isoelectric point (pI), the net charge on the protein comes closer to zero which reduces electrostatic repulsion which promotes precipitation and aggregation. Therefore, understanding of pI helps with understanding protein recovery and final functionality of the protein isolate.

The percentages of these protein types can vary with different genetic and environmental factors. As shown in Table 3, globulins are the major protein fractions which shows minimum solubility at pH between 4.0-4.5, this range is used for isoelectric precipitation. However, individual proteins may behave differently depending on their structure, composition and extraction conditions which can influence the final protein isolate (Badjona et al., 2024; Vogelsang-O'Dwyer et al., 2020; Żmudziński et al., 2021).

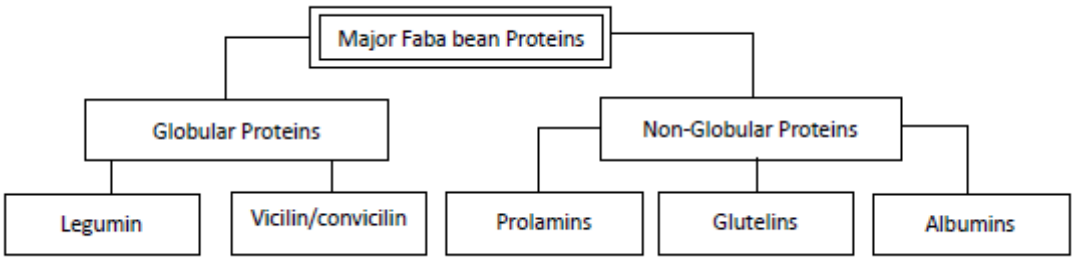


Figure 2. Faba bean proteins can be divided into two subunits (Dangi et al., 2022)

A. Globular proteins

Legumin and vicilin/convicilin are classified based on their sedimentation coefficients of 11S and 7S, respectively. Legumin is a major protein of mature faba bean seed. It has two significant subunits: A and B. A subunit contains a methionine residue, while the B subunit is free of it (Dangi et al., 2022). The other two subunits are called legumin minor, with molecular weights of 80 and 75 kDa. All of them are made up of α- and β-chains linked by a disulfide bridge. The molecular weights of vicilin and convicilin are 50 kDa and 70 kDa, respectively. Neither of these has disulfide bridges and is cysteine-free. Vicilin is the second most dominant protein in mature faba bean seeds.

The ratio of legumin and vicilin is a critical factor in determining faba bean characteristics and significantly affects the protein's functional properties. Faba bean legumins are more thermostable than vicilin, with denaturing temperatures of 95.4 °C and 83.8 °C, respectively contributing to gel formation, on the other hand, vicilin has been reported to have better emulsifying properties. Therefore, the proportions of these fractions may influence the interactions between FPI and WPI (Martineau-Côté et al., 2022; Oluwajuyitan & Aluko, 2024). The denaturing temperatures of these globulins are higher than those of globulins from peas and soybeans.

B. Non-globular proteins

Albumins are comprised of higher amounts of sulfur-containing amino acids than any other protein (El Fiel et al., 2002). Prolamins are rich in glutamic acid, proline, and leucine but lack tryptophan and lysine. Glutelins have a similar profile to prolamins but have higher amounts of histidine, methionine, and glycine.

2.1.2. Extraction of protein from faba bean

Producing protein isolates in the food industry is crucial for concentrating proteins and enhancing their functional and nutritional properties. Faba bean proteins can be isolated using two main methods: wet extraction/aqueous fractionation and dry fractionation.

A. Wet extraction/Aqueous fractionation:

The characteristics and yield of the protein fractions depend on the extraction parameters. The type of solvent, pH, and temperature determine the extraction fractions. Wet extraction is of two kinds: Alkaline and Acidic. The alkaline method exhibits high extraction efficiency, resulting in the degradation of vicine and convicine. Another patent method, WO2021116703, can also be used to extract protein and employs acid extraction. The acidic extraction method reduces trypsin inhibitor activity and removes vicine and convicine. This method gives high-purity protein.

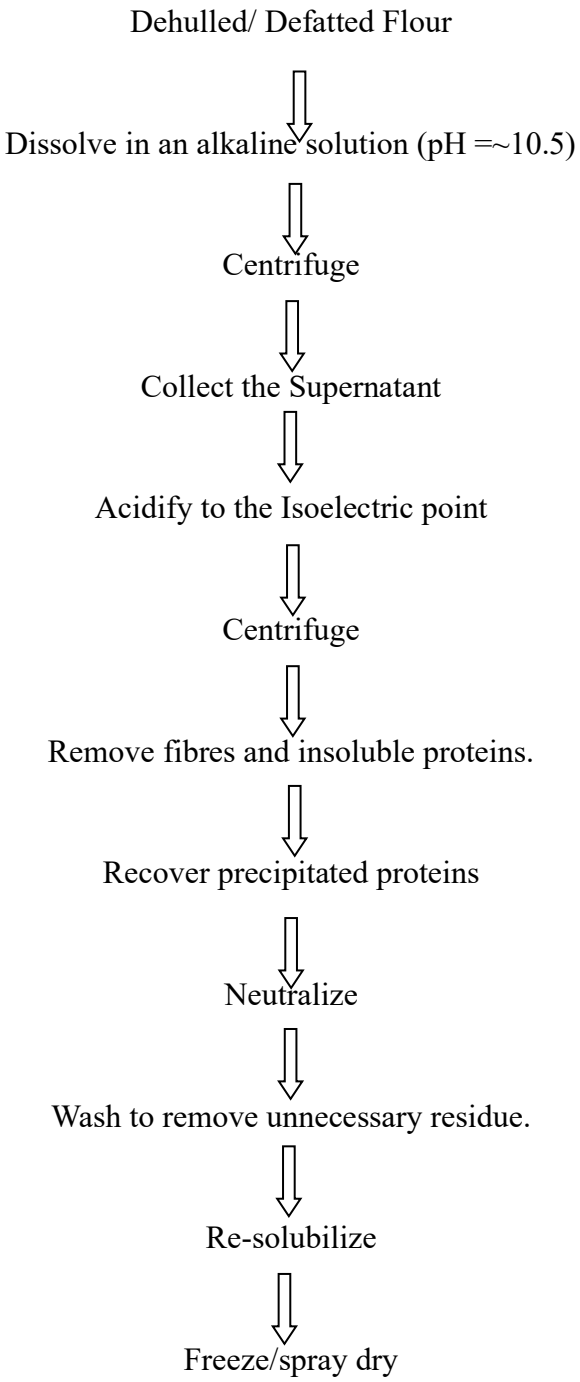


Figure 3. Wet extraction (alkaline) process of faba bean proteins of the globulin-rich protein fraction (Boukid & Castellari, 2022)

Figure 3 shows the wet alkaline extraction process. It starts with dehulled or defatted faba bean flour, which is dissolved in an alkaline solution at pH ~10.5. This solution is then centrifuged, and the supernatant is collected and acidified to its isoelectric point. In this step, globulin-rich proteins are acidified to the isoelectric point, which generally shows low

solubility at pH 4.0-4.5 (Badjona et al., 2024; Vogelsang-O'Dwyer et al., 2020; R. Yu et al., 2024; Żmudziński et al., 2021)**.** This supernatant is centrifuged again to remove fibres and insoluble proteins. The precipitated proteins are then recovered, neutralised and washed thoroughly to avoid removing any unwanted residues. The proteins are then re-solubilised and dried using freeze-drying or spray-drying techniques to obtain faba bean protein isolate.

The wet extraction using acidic conditions is shown in Figure 4. In this process, faba bean seeds are wet-milled under acidic conditions, resulting in a mixture that is then passed through centrifugal sieves to remove the fibres and insoluble proteins. The remaining slurry is then decanted to separate the starch and proteins. The collected proteins are then precipitated at pH 4.8. pH is gradually increased to 6.8, and precipitated proteins are then dried.

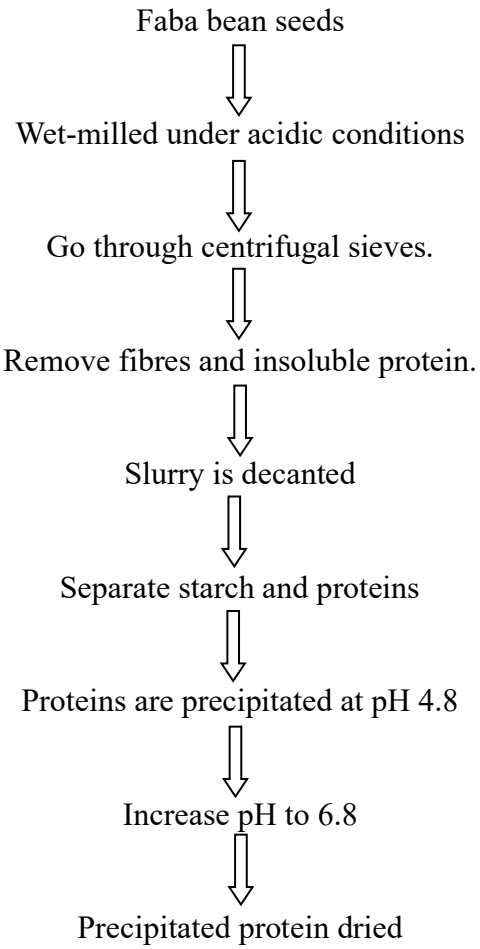


Figure 4. Wet extraction (acid) process of Faba bean proteins (Boukid & Castellari, 2022)

B. Dry fractionation:

Dry fractionation uses no water or chemicals, reducing waste. The functionality and native structure of a protein are preserved unaltered, but purity is low, and antinutrients are not entirely (Stone et al., 2024; Vogelsang-O'Dwyer et al., 2020)**.** Dry fractionation is composed of two steps: Milling and size separation. The process is shown in Figure 5. Faba bean seeds undergo dry milling, where dehulled beans are ground into a fine flour. The flour is then separated based on density, shape, and size using airflow or centrifugation. This divides the flour into two parts: the light fine fraction, which is rich in protein, and the heavy coarse fraction, which is rich in starch.

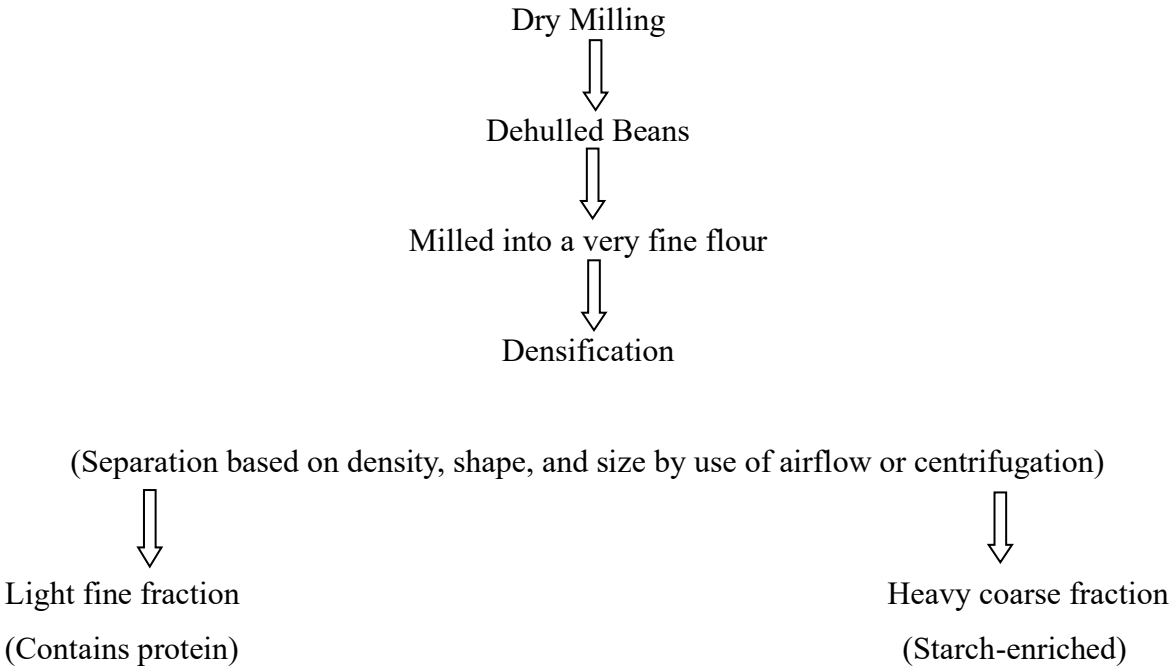


Figure 5. Dry fractionation process of faba bean proteins (Boukid & Castellari, 2022)

Table 4: Efficiency of the different treatments in obtaining the protein yield

Method	Protein content (%, dry matter)	Protein Yield (%)	Protein extraction efficiency (%)	Sources
Alkaline extraction	~88-94	~18–25	~55–77	(Eckert et al., 2019; Singhal et al., 2016)
Acid extraction	90.1	~43	~21	(Langton et al., 2020; Vogelsang-O'Dwyer et al., 2020)
Dry extraction	51–66	~26–49	~16–18	(Coda et al., 2015; do Carmo et al., 2020; Vogelsang-O'Dwyer et al., 2020)

2.1.3. Properties of faba bean

A. Solubility:

Solubility plays an integral role in determining a protein's functional properties. pH is a very significant factor in determining solubility. It affects hydrophobicity, influencing protein-protein (hydrophobic) interactions and protein-solvent (hydrophilic) interactions. It also controls the charge of the protein, which influences electrostatic repulsion. pH and ionic strength determine the solubility of globulin proteins. Recent studies have shown that the solubility of FPI is strongly influenced by temperature, pH and water hardness which can further affect particle size (R. Yu et al., 2024) Low solubility of the FPI is a major limitation as it reduces their ability to make a stable emulsions and gels (Badjona et al., 2024; Vogelsang-O'Dwyer et al., 2020).

B. Surface characteristics:

Some physicochemical properties of the protein, including surface charge, surface hydrophobicity, molecular size, and surface tension, significantly affect the functional

properties. These surface characteristics judge how proteins interact with water, oil or other proteins in a food system. Changes in particle size, surface charge and hydrophobicity strongly affect the emulsion stability and gel network formation of FPI (Hu, Cheng, Gilbert, Lee, et al., 2024; Hu et al., 2023).

C. Water and oil absorption capacity:

The water holding capacity of faba bean increases with increasing pH. Faba bean proteins obtained by dry fractionation show high solubility but low water-holding capacity. Faba bean protein has a fat absorption capacity of 87g/100g. The protein's conformational change and increased molecular size increase its fat absorption. Water and oil absorption is important in food applications as they can influence mouthfeel, viscosity and texture in products like spreads, sauces, bakery products and meat analogues (Stone et al., 2024; Thomsen et al., 2025).

D. Emulsifying properties:

The emulsifying property of faba bean protein is influenced by the following factors:

- Protein solubility
- Surface-hydrophobicity
- Surface charge
- Protein composition
- Conformation site
- Molecular flexibility

The smaller the protein size, the better the emulsifying property (Dangi et al., 2022). 7S globulin proteins are not N-glycosylated, leading to a larger emulsion particle size as the carbohydrate moiety contributes to this property. Vicilin exhibits a faster adsorption rate at the oil-water interface at pH 7, contributing to its emulsifying properties, and it is a better emulsifier than legumins. The emulsifying performance of FPI strongly depends on protein concentration, particle size, pH and processing conditions (Hu, Cheng, Gilbert, Lee, et al., 2024; Hu et al., 2023). Reducing particle size can result in better emulsification of systems with higher FPI content.

E. Gelling properties:

The gelling property of protein depends on

- Patterns of interaction
- Electrostatic and hydrophobic interactions
- Bonding: hydrogen and covalent bonds

Faba bean protein gel quality is affected by several factors, including cell wall integrity, protein interactions, a particle size, and antinutrients. Recent studies show that pH, protein composition and interactions with other constituents like polysaccharides can affect the gelation of FPI (Thomsen et al., 2025).

Various processing techniques can enhance the functionality of protein isolates as explained below.

Table 5: Processing Techniques to increase protein functionality

Processing Techniques	**Experimental Parameters**	**Findings**	**Conclusion**	**Sources**
Extrusion	Temperature: 100-130℃ The screw speed of 150-160 rpm Moisture: 30-70%	Destruction of antinutrients	Improved solubility, Emulsifying, gelation properties, texture, and digestibility	(Akharume et al., 2021; Alfaro-Diaz et al., 2021; Nasrabadi et al., 2021)
pH shifting	pH range: 4-8	Unfolding of proteins into a molten globule	Increase in protein reactivity Increase in Emulsion quality	(Muneer et al., 2018)
Enzymatic hydrolysis	Transglutaminase	Cross-linking of protein increased Peptide bond breakage	Improved protein nutritional value Improved texture	(Aguilar et al., 2019; Akharume et al., 2021; Villamil et al., 2017)

2.2. Whey proteins

Whey proteins are the soluble milk proteins, accounting for approximately 20% of the total milk protein (Davoodi et al., 2016; Kilara & Vaghela, 2018) Whey proteins are ideal ingredients in the formulation across the food industry due to their solubility across a wider pH range, a desirable nutritional profile, the presence of essential amino acids, favourable functionality, and a low manufacturing cost (Kilara & Vaghela, 2018). Whey proteins are obtained from two main processes: casein production and cheese manufacturing (Bansal & Bhandari, 2016). Two types of whey can be obtained during these processes: acid and sweet whey. Acidification of milk to obtain casein or acid coagulation during cheese formation leads to the formation of acid whey. However, the manufacture of cheese and casein using rennet results in sweet whey. The pH of acid whey ranges between 4.6 and 5.0, but for sweet whey, it varies between 6 and 6.5 (Bansal & Bhandari, 2016).

The most common types of whey protein concentrates are WPC 34, WPC 60, and WPC 80, which are named after their protein concentration (Guo & Wang, 2019). Whey protein concentrates are obtained by ultrafiltration technology (Bansal & Bhandari, 2016; Kilara & Vaghela, 2018). This technology uses low temperatures and avoids the use of chemicals or enzymes, enabling the protein to be obtained in its natural conformation (Kilara & Vaghela, 2018). With an additional microfiltration step, a 90% concentration can be achieved, removing excess fat. This whey concentrate with 90% protein is called WPI (Kilara & Vaghela, 2018). WPI has extraordinary functionalities, which include gelling, foaming, and emulsifying properties (Kilara & Vaghela, 2018).

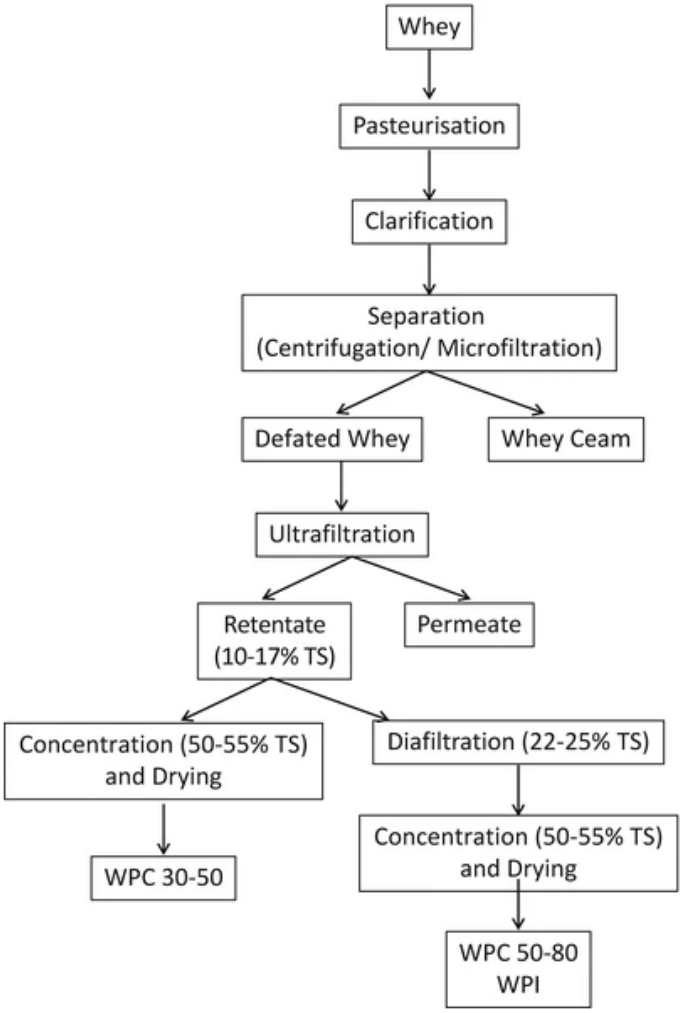


Figure 6. A flowchart explaining the production of whey protein concentrates and whey protein isolates, where TS stands for total solids (Bansal & Bhandari, 2016)

Table 6: Composition of major whey protein (Guo & Wang, 2019)

	Protein	**Lactose**	**Fat**	**Ash**	**Moisture**
WPC 34	34.0-36.0	48.0-52.0	3.0-4.5	6.5-8.0	3.0-4.5
WPC 60	60.0-62.0	25.0-30.0	1.0-7.0	4.0-6.0	3.0-5.0
WPC 80	80.0-82.0	4.0-8.0	4.0-8.0	3.0-4.0	3.5-4.5
WPI	90.0-92.0	0.5-1.0	0.5-1.0	2.0-3.0	4.5

Major whey proteins are:

- β-lactoglobulins- 50-60% in bovine milk. It is a small globular water-soluble protein of 18.20-18.46 kDa (Madureira et al., 2007).
- α-lactoglobulins- small compact globule of 14 kDa (Kilara & Vaghela, 2018; Madureira et al., 2007).
- Bovine serum albumin- single-chain globular non-glycoprotein. It has a molecular weight of approximately 64 kDa (Madureira et al., 2007).
- Lactoferrin- 80 kDa iron-binding glycoprotein (Madureira et al., 2007).
- Immunoglobulins- globular proteins with immunological activity with a molecular weight varying depending on the immunoglobin class (Madureira et al., 2007).
- Minor proteins- low quantity but essential functions like lactoperoxidase, growth factors, and vitamin-binding proteins (Madureira et al., 2007).

Whey protein is soluble in a wide range of pH, but its solubility does depend on factors such as the presence of ions and temperature (Kilara & Vaghela, 2018; Wit, 1989) . WPI leads to an increase in the viscosity of a solution, and this increase begins at 65 °C because of the denaturation of the proteins above this temperature (Wit, 1989).

Table 7: Denaturation temperature and approximate isoelectric points of the whey protein components (Bramaud et al., 1997; Jenness et al., 1988; Kilara & Vaghela, 2018; Wit, 1989).

Whey protein components	**Denaturation temperature**	**Approximate isoelectric points**
β-lactoglobulin	62°C	5.1-5.3
α-lactalbumin	78°C	4.2-4.8
Bovine serum albumin	64°C	4.7-4.9
Immunoglobulin	72°C	Depending on immunoglobulin type

Whey protein solubility, aggregation and charge is highly influenced by the pH which makes isoelectric point crucial in a protein system. Emulsion and gel formation through

protein unfolding, adsorption and network formation is affected by both temperature and pH (Bramaud et al., 1997; Kilara & Vaghela, 2018; Wit, 1989).

2.3. Development of protein hybrids

Whey proteins have desirable functional properties, which are the main reason for their incorporation into food systems. Plant proteins, on the other hand, generally have poorer functionalities, limiting their use. However, amid growing concerns about sustainability, partially replacing milk proteins with plant proteins to leverage their superior functionalities and eco-friendliness seems to be a practical option. Because these proteins have different natures, forming a hybrid can be challenging; however, various processing techniques can be used to achieve it. Below is a process for explaining how hybrids can form.

2.3.1. Fibrilization

Proteins and peptides can self-assemble under conditions such as high temperatures, prolonged heating, low pH, and low ionic strength through various interactions (Meng et al., 2021). These self-assemblies are known as Fibrils. Protein fibrils are unbranched and highly organised macromolecular structures ranging from nanometres to micrometres (Wang et al., 2020). Most protein fibrils have a diameter of less than 100 nm. Protein fibrils are non-toxic and biocompatible and have a variety of functional properties. Protein fibrils influence viscosity, emulsifying behaviour, gelation and foaming in a food system (Herneke et al., 2023; Meng et al., 2021; Wang et al., 2020).

Self-assembly to form fibrils:

Protein aggregates with varying structures, such as fractal clusters, flexible chains, and fibrils, can be formed under various external conditions. These structures are extensively studied for potential applications in the food industry.

Various models of fibril formation have been proposed, of which the nucleated conformation conversion (NCC) model is most widely accepted. The NCC model is divided into three phases:

• Lag phase: This phase has a slow spontaneous growth as it is a preparation step for forming the newly ordered structures. The nucleus is the smallest unit of these structures, and its formation is known as the primary nucleation stage.

• Growth phase: Protofilaments are made, which form fibrils with continuous growth. The conversion rate is the highest at this stage.

• Maturity stage: This is a relatively stable stage in which either equilibrium is achieved or all proteins are exhausted.

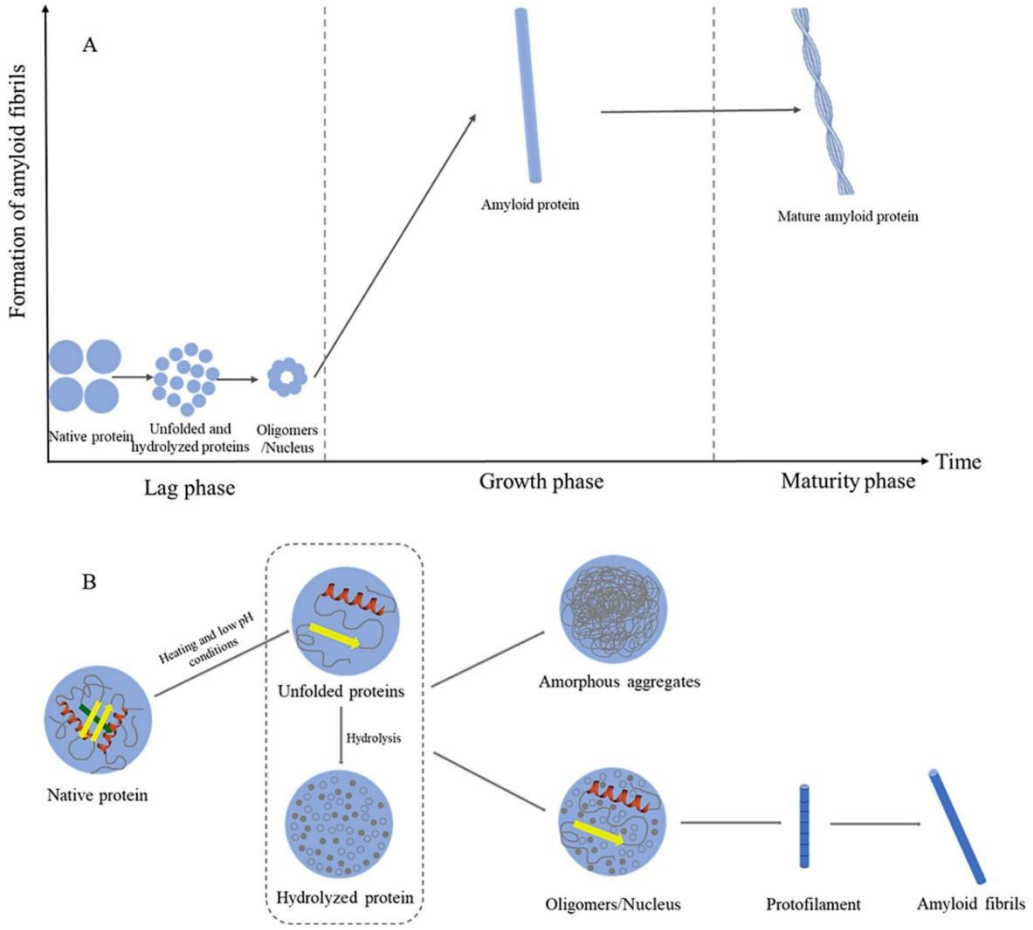


Figure 7. A. Phases during the formation of protein fibrils. B. Schematic representation of the protein amyloid fibrils (Z. Yu et al., 2024). Licence documentation provided in Appendix A.

Two types of nuclei are formed during fibrillization:

a. Homogeneous nuclei: These are unstable structures with high energy formed in the lag phase

b. Secondary nuclei: As the process of protein fibrillation progresses, the initial primary nuclear structures elongate and come together to create fully formed protein fibrillar structures.

Protein fibrils typically have similar structures, characterised by β-sheet and cross-β formations. The individual β-strands are spaced approximately 4.8 Å apart within the fibril and are arranged perpendicular to its axis. Additionally, β-sheet structures form parallel to the fibril axis and are spaced approximately 10 Å apart due to β-strand stacking. Even though they are structurally similar, the fibrils' heterogeneity offers them polymorphism.

Factors affecting fibrillization:

Three types of factors can affect the fibrillization of the proteins:

A. Physical factors:

Heat is used to induce fibrillization alongside changes in pH. It has been confirmed that prolonged heating at high temperatures at pH 2 leads to the opposite result (Xia et al., 2017). Analysis of the protein's secondary structure revealed that the β-sheet had decreased and was replaced by a disordered structure, mainly composed of β-turns and irregular coiling. Stirring has been proven to have positive effects, as it can help produce mature fibrils by breaking down immature fibrils, thereby accelerating the process (Wei & Huang, 2020). The effect of pressure during fibrillization is not fully understood and might be affected by type of protein and processing conditions.

For WPI and FPI, heat is an important factor as it can unfold globular proteins hence exposing the hydrophobic groups. Heat treatment can influence protein aggregation, structural changes, particle size and fibril formation in FPI, while in WPI, it promotes protein unfolding that contributes towards gel formation (Hu, Cheng, Gilbert, Lee, et al., 2024; Hu, Cheng, Gilbert, Loo, et al., 2024; Wit, 1989)

B. Chemical factors:

When the pH level of a protein dispersion drops below its isoelectric point, the number of positive charges on the protein surface continues to increase until it reaches a point where they cannot be effectively shielded. This leads to changes in their spatial orientation, the formation of protein fibril aggregates, and fibrillation. Ionic strength and protein concentration play critical roles in the self-assembly of protein fibrils. Seeding is a process in which performed protein fibrils are added to the protein dispersion to further promote fibril formation. The purpose of promoting fibrillation is to reduce the lag phase of fibrillogenesis by utilising preformed protein fibrils. Protein fibrils from other proteins can also be added, a process known as cross-seeding. To accelerate fibril formation, it is essential that the seed protein shares some degree of peptide sequence with the dispersion protein (Janssens et al., 2019). Recent studies have shown that environmental conditions such as temperature, pH, protein composition and ionic strength affect fibril formation (Herneke et al., 2023; Hu, Cheng, Gilbert, Loo, et al., 2024)

Exogenous macromolecules like polysaccharides, dyes, polyphenols, and chaotropic agents like ethanol, dithiothreitol, and urea affect fibrillization by interfering with protein-protein interactions, protein unfolding and fibril assembly (Rostamabadi et al., 2026; Ruan et al., 2022; Wang et al., 2025)

C. Biological factors:

Biological factors mainly include enzymes which can modify protein structure through cross linking and hydrolysis. Proteolytic enzymes can break proteins intro smaller peptides initiating unfolding and assembling into fibrillar structures. It has been found that adding trypsin can increase fibril formation and accelerate the process (Gao et al., 2017). Transglutaminase, a cross-linking enzyme can promote covalent bonding between proteins hence improving gel strength and water-holding capacity (Aguilar et al., 2019; Villamil et al., 2017).

Whey protein:

Whey protein isolates possess exceptional nutritional and functional properties that enable them to self-assemble and create whey protein isolate nanofibrils (WPNFs). These WPNFs can revolutionise the industry with their economic viability as commercial products.

Legume protein fibrils:

Most legume proteins fibrillize at low pH and high temperatures, leading to denaturation and disruption of protein structure. This leads to a decrease in protein particle size. During further heating, these peptides assemble into oligomers that elongate, associate further, and form fibrils. The fibrils formed can be semi-flexible or rigid, depending on the legume. The 7S unit of faba bean protein forms fibrils easily at pH 2 when heated at 85 °C, compared to the 11S unit. In both faba bean proteins, fibril formation increased at pH 7.

Table 8: Overview of protein fibrils from different proteins

Protein	Fibrillization conditions	Shape	Size	Source
Whey Protein Isolate	80ºC, pH2, 24 hours	Straight semi-flexible fibrils	Length: 500-1000nm Diameter: 10nm	(Hu et al., 2019)
Faba bean-7S globulin	85ºC, pH2, 24 hours	Straight semi-flexible	Length ~100-200nm	(Herneke et al., 2023)

2.3.2. Protein-protein interactions

Protein's techno-functional properties can be explained as its behaviour in a food system, which is based on the protein's physicochemical properties. Colloidal systems, such as dispersions, gels, and emulsions, are influenced by physicochemical interactions, including hydrogen bonds, disulfide bonds, and electrostatic and hydrophobic interactions (Lima Nascimento et al., 2023). A colloidal system of dispersion where solid material is dispersed in a liquid medium must be made before gels or emulsions. The interaction potential in a mixed protein system is also modulated by ionic strength, temperature, pH and solvent composition (Hinderink et al., 2021). When the electrostatic attraction between protein molecules is strong, precipitates are favoured, as this leads to a frozen state with reduced molecular flexibility and fewer structural rearrangements. On the contrary, when the forces

are weak, they coacervate, forming a viscoelastic liquid solution. Solubility is another factor affecting protein interactions. Plant proteins have low solubility, which can hinder their commercial use. A plant protein-dairy protein hybrid can enhance the solubility and other functionalities of plant protein. Different methods, such as pH-shifting (Wang et al., 2019), low-temperature homogenization (Krentz et al., 2022), and ultrasonication (X. Du et al., 2023), have been used to increase the solubility of the mixed system dispersions. Particle size of the protein in a dispersion is also another main factor which impacts the techno-functional properties. Physical treatments such as ultrafiltration, homogenization, and ultrasound can be applied to plant proteins to reduce particle size, thereby enhancing solubility (Yousefi & Abbasi, 2022).

Table 9: Studies on Protein-Protein hybrid preparation

Name and concentration of protein used	**Treatment/method use**	**Characteristics studied**	**Conclusion**	**Sources**
Casein Micelle (CM) and Pea protein (PP) Hybrid	• Pre-treatment of CM with sodium citrate • three homogenization cycles with low temperatures (4 °C) at two stages. Stage 1-24,130 kPa; Stage 2-3,447 kPa • Pasteurised in a water bath for 30 minutes at 63 °C. • Stored at 20 °C	• Solubility • Particle size	All blends remain stable at 4 °C for at least four weeks. Conformational changes in CM pre-treated with PP led to its dispersion with PP, resulting in increased colloidal stability.	(Krentz et al., 2022)

Pea Protein isolate (PPI) and Sodium Caseinate (NaCas) hybrid at different concentrations	• PPI and NaCas dissolved in water at 3% concentration (w/w) • pH adjusted to 12 • Stirred for 1 hour, pH adjusted to 7 • Samples were placed in an ice-water bath, temperature- 25± 2 °C • Ultrasonication probe placed below the liquid level • Ultrasonicated for 5 minutes at 600W (probe working for 2 seconds and stopped for 2 seconds) • Samples stored in the fridge at 4 °C	• Turbidity and Solubility • Hydrodynamics and Zeta potential • Acid coagulation behaviour • Water holding capacity and textural properties	The solubility of all samples increased after ultrasonication. Samples with more PPI were turbid. Showed great colloidal activity, even after one week Samples showed better water-holding capacity.	(X. Du et al., 2023)
Rice Glutelins (RGs) and Soy β-Conglycinin(7S) /Glycinin (11S)	• RGs hydrated with deionised water at 1% concentration (w/v) • After complete hydration, Soy protein is added • pH adjusted to 12 with NaOH for 2 hours • pH neutralised to 7 by 1M HCl • Dialysis was conducted against deionised water for 24 hours with a molecular cut-off weight of 3400 Da.	• Emulsifying Capacity • Foaming Capacity	The RGs/11S hybrid demonstrated strong emulsifying capacity, even exceeding that of 11S alone. More RGs/11S and RGs/7S showed similar emulsion stability. The foaming capacity of both hybrids increased. 7S and 11S interacted with RGs to form binary structures after pH cycling.	(Zhu et al., 2021)

2.3.3. Plant-dairy protein emulsion

Emulsions are multiphase systems that often contain proteins and amphiphilic molecules, which play crucial roles in their formation and stability. During homogenization, protein adsorption at the oil-water interface reduces surface free energy, leading to the formation of small droplets. Proteins then form an interfacial layer, which protects these droplets from physical destabilisation. This occurs either through the formation of viscoelastic layers or through electrostatic repulsion, which prevents coalescence (Hinderink et al., 2020). Electrostatic repulsion and hydrophobic interactions play a significant role in determining the emulsion's elasticity. A previous study showed that a WPI and pea protein isolate (PPI) blend forms a stronger interfacial layer than PPI alone (Hinderink et al., 2020). Physical methods, such as electrostatic association, heating, high-pressure homogenization, ultrasonication, and pH shifting; chemical processes, including acetylation reactions and the Maillard reaction; and enzymatic methods, including hydrolysis reactions and cross-linking reactions, can help enhance the emulsion stability of the FPI (Liu et al., 2022). Various studies have been conducted, as shown in Table 9, to modify FPI to improve its emulsifying properties.

Table 10: Methods to improve FPI's emulsifying property

Treatment	**Property modified**	**Source**
pH shifting	Increase in adsorption rate and strength of the interfacial layer	(Felix et al., 2019)
Heating	Increase in surface hydrophobicity	(Gürbüz et al., 2018)
High-pressure homogenization	Increase in solubility and surface hydrophobicity.	(Yang et al., 2018)
Alkaline shifting and ultrasound	Decrease in particle size and improvement in emulsion stability	(Alavi et al., 2021)

pH shifting along with heating (Lam & Nickerson, 2015) and high-pressure homogenization with ultrasound (Shi et al., 2020) has also been shown to increase the hydrophobicity and emulsion stability index of WPI.

2.3.4. Plant-dairy protein gelation

A gel is a colloidal system in which long, thread-like molecules are cross-linked to form a continuous three-dimensional network. Gelation of the protein systems depends on intrinsic and extrinsic factors like amino acid composition, disulfide bond, electrostatic and hydrophobic interactions, protein concentration, temperature, ionic strength, pH and pressure (Zhu, 2024). Gelation can be induced using heat, acid, enzymes and ultrasound (Lima Nascimento et al., 2023).

Acid-induced gelation is induced by using acidulants such as gluconic-δ-lactone (GDL). Acid-induced gelation involves three sequential events: denaturation, aggregation, and gelation (Xia et al., 2024). During this type of gelation, pH is modified towards the isoelectric point of the proteins. During the reduction in pH, electrostatic repulsions between the protein molecules decrease, which allows them to come close to each other, allowing the formation of new interactions and finally a three-dimensional network (Totosaus et al., 2002). The pH at which gelation begins varies among proteins, thereby affecting the gel network structure.

Pre-heating proteins can be beneficial for modifying their properties and interactions, thereby improving gelation. It has been observed that heating the proteins together can lead to a more organised structure with higher water holding capacity (WHC) (Chihi et al., 2018). On the other hand, some researchers have observed that heating the proteins separately led to more elastic gels (Mession et al., 2017).

Cold-set gelation is a two-step procedure. Firstly, a native protein solution is heated at a pH away from its isoelectric point; upon the addition of acid, the pH is reduced, inducing gelation.

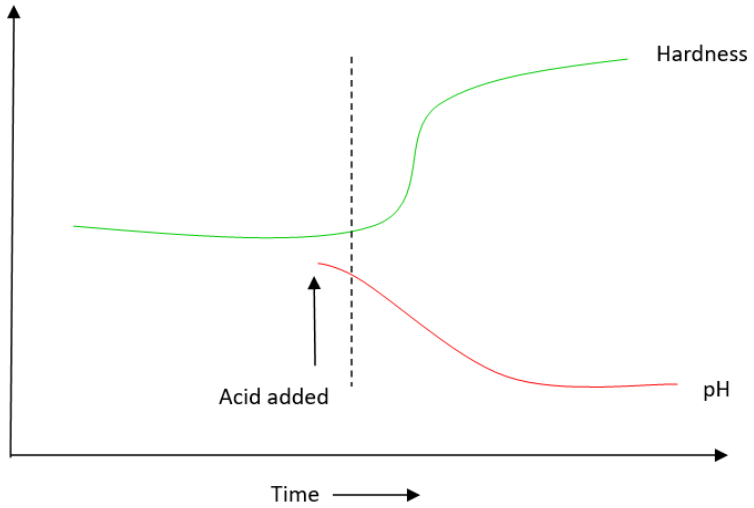


Figure 8. Cold-gelation procedure of the proteins

Some research has been conducted on interactions between plants and dairy proteins (Table 11), but the mechanisms underlying these interactions remain under investigation. Further research is needed to understand the process of acid-induced gelation in mixed systems.

Table 11: Acid-induced gelation in a mixed system

Proteins	**Properties**	**Source**
Pea/Whey	Decrease in gel stiffness and gelation pH; no interactions	(Chihi et al., 2018)
Soy/Milk	No interactions	(Grygorczyk et al., 2013)
Pea/Milk	Faster gelation, decrease in firmness	(Yousseef et al., 2016)
Lentil/Milk	Properties similar to milk gelation	(Zare et al., 2011)

2.3.4.1. Effect of pH on gelation

pH plays a vital role in gel formation. A fibrillar gel network forms for whey protein, soy protein, and pea protein at acidic pH. It has been observed that 4% pea protein isolate (PPI)

forms a gel at pH 6.6 after 24 minutes, and β-lactoglobulin forms a gel at pH 5.7 after 58 minutes; therefore, an increase in β-lactoglobulin in a mixed system decreases the gelation pH (Chihi et al., 2018). Similar observations have been made in soy protein isolate (SPI) and cow's milk. SPI gelation occurs around a pH of 6, but cow's milk does not gel below 5.6; thus, the gel network that forms above a pH of 5.6 only has the SPI network. It has also been observed that cow's milk interferes with this network, leading to a weak structure (Grygorczyk et al., 2013).

2.3.4.2. Effect of temperature

Temperature also plays a significant role in the formation of gel. It has been observed that when heated above 50 °C, proteins unfold, leading to an irreversible accumulation due to hydrogen, disulfide and hydrophobic interactions (Chen et al., 2016). The SPI gelation temperature also decreases with increasing temperature. It has been noticed that the G' of the gel when the SPI solution is heated at 65 °C is less than 85 °C.

2.3.4.3. Effect of protein concentration

Gel strength increases with increasing protein concentration. When the concentration of the SPI was increased from 0.3g/L to 90g/L, the storage modulus increased to ~1000 Pa as well (Chen et al., 2016).

2.3.4.4. Covalent and non-covalent interactions in gel formation

Electrostatic interactions:

Electrostatic interactions in protein solutions occur due to the presence of charged amino acids. These charged molecules depend on their charge, repel or attract each other, resulting in the formation of the gel. Changes in pH and ionic strength influence the charges on the amino acids. When pH is below or above the isoelectric point, the protein molecules are positively or negatively charged (Patole, 2022). At the isoelectric point, the net charge of proteins becomes zero. Electrostatic interactions play a crucial role in the folding and unfolding of proteins, which in turn dictate the structure and network of the gel.

Hydrogen bonds:

Between polar groups, there are short-range attractive interactions called hydrogen bonds. The interaction between a hydrogen atom and an electronegative atom, such as nitrogen, oxygen, sulfur, or fluorine, forms a hydrogen bond. These bonds are also responsible for the folding and unfolding of the protein molecules (Semenova & Dickinson, 2010). Hydrogen bonds mediate significant interactions between protein molecules, leading to the formation of secondary and tertiary protein structures.

Hydrophobic interactions:

Hydrophobic interactions are formed by the non-polar side chains of the amino acids, which are hydrophobic in nature (Matthews, 2001). In globular proteins, hydrophobic groups are embedded within, whereas hydrophilic groups are on the surface. When the temperature rises, these hydrophobic groups are exposed, leading to the formation of the hydrophobic interactions.

Disulfide bonds

Disulfide bonds are formed between the thiol groups of the cysteine residues. This occurs through oxidative folding. During gelation, these bonds improve molecular entanglements by facilitating polypeptide chain extension, preventing thermal motion, and stabilising the gel network.

Van Der Waals interactions:

Nearby non-bonded and uncharged atoms form Van der Waals interactions, which generate forces between permanent and/or induced dipoles. Van der Waals interactions do not influence gel formation (Semenova & Dickinson, 2010), but can support aggregation to form the gel, especially when the polymer molecules are large.

2.4. Analysis techniques:

2.4.1. SDS-PAGE

Polyacrylamide gel electrophoresis is a critical technique for separating proteins primarily based on their molecular size.

In this technique, proteins are solubilised in a strong cationic detergent, sodium dodecyl sulfate (SDS), and then electrophoresed on an SDS-polyacrylamide gel. This detergent denatures proteins by attaching to them, thereby providing a negative charge-to-mass ratio. This treatment, by masking the original charges and shapes, allows separation based on molecular weight (Muhammad & Massey University, 2024). A discontinuous gel system consisting of a stacking and a resolving gel is applied to enhance separation. The stacking gel helps concentrate the protein before it enters the resolving gel. In the resolving gel, proteins are separated according to their size while travelling through the gel under the influence of an electric field. The binding of SDS to proteins ensures proper separation, with smaller proteins migrating farther in the gel than the larger ones. All three components—the stacking gel, the resolving gel, and the electrophoresis buffer—play a crucial role in achieving proper separation. SDS-PAGE is a well-accepted technique with an accuracy rate of within ±10%. The larger deviation can occur when the protein shows abnormality due to post-translational modifications, such as glycosylation and phosphorylation (Matsumoto et al., 2018). SDS-PAGE has a wide range of applications, including the estimation of relative molecular weights of proteins and protein hydrolysates, and the quantification of molecules and identification of impurities in samples.

2.4.2. Rheology

Rheology is the study of understanding the deformation and flow under the influence of external forces. The name Rheology has been derived from the Greek word “rheo”, which means “flow”. The molecules or particles in liquid dispersions move by sliding past one another; any resistance to this flow results in internal friction, a key aspect of rheology.

Rheology plays a crucial role in various industries, including food manufacturing, personal care products, and pharmaceuticals.

2.4.2.1. Oscillation rheology:

Oscillation rheology is used to understand the viscoelastic properties of a material, in which a sinusoidal stress is applied, and the material's response is observed. This method provides valuable insight into the material behaviour when the resulting strain or stress is measured. The material behaves in two ways: elastically, exhibiting solid-like behaviour, or viscously, exhibiting liquid-like behaviour. When the phase difference calculated by phase angle (δ) between the stress and strain wave is 0°, at that time the material behaves like elastic, but when this difference is 90°, it exhibits viscous behaviour as shown in Figure 9. Some parameters used to understand oscillation rheology are outlined in Table 12.

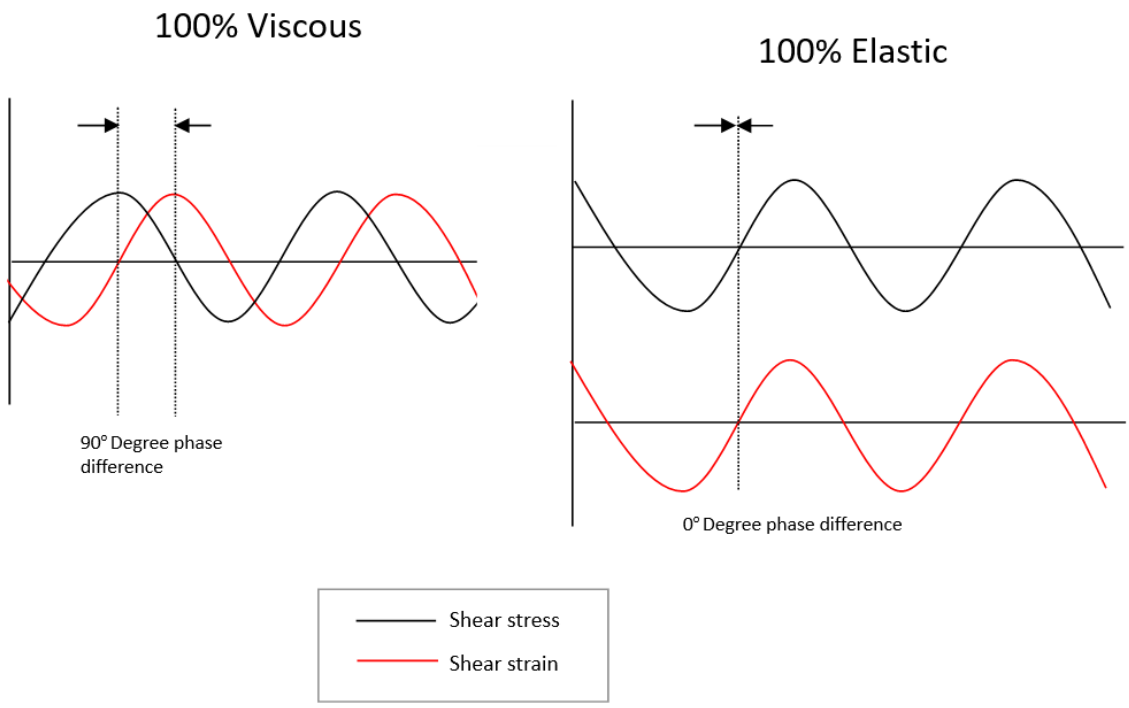


Figure 9. Oscillation stress and strain waves for elastic and viscous materials.

Table 12: Parameters used in oscillation rheology (Tabilo-Munizaga & Barbosa-Cánovas, 2005; Tunick, 2011)

Property	**Description**
Storage modulus/elastic modulus (*G′*)	This represents the elastic behaviour of a sample. It is a measurement of deformation energy stored in the sample.
Loss modulus/viscous modulus (*G″*)	This represents the sample's viscous behaviour. It is a measurement of energy dissipated in the sample.
Complex modulus (*G**)	It is a measurement of the sample's resistance to deformation.
Loss Tangent/ Tan δ (*G″/G′*)	It is the ratio of the loss modulus to the storage modulus. If Tan δ is more than 1, the sample shows viscous behaviour; if less than 1, the sample shows elastic behaviour.

Types of measurement in oscillation rheology:

Oscillation rheology provides valuable insights into the material's behaviour. The key techniques of oscillation rheology are:

- Time sweep: During the time sweep test, a constant stress is applied over time, and then this stress is removed to understand the recovery behaviour of the material. This helps to understand the stability and viscoelastic properties (Stojkov et al., 2021).
- Frequency sweep: Frequency sweep is measured at a fixed oscillation amplitude and temperature. It determines the material's viscoelasticity. If the material shows a higher *G′* than *G″* over a wide range of frequencies, it shows elastic behaviour of the gel, but if *G″* is higher, it means that the material has viscous behaviour (Franck, 2004).
- Amplitude sweep (strain sweep): This test involves applying oscillating strains to the sample with increasing magnitude while the frequency remains the same. This helps characterise the material's integrity and rigidity. The material's internal structure is destroyed as the strain amplitude continues to increase, thereby allowing the breaking stress

and strain to be determined. It can offer insights into the material's structural attributes (Stojkov et al., 2021).

2.4.3. Confocal Laser Scanning Microscopy (CLSM)

In CLSM, confocal refers to images taken only from the focal plane, and laser scanning means images are captured only where the light is illuminated. It reduces out-of-focus light, providing more precise, detailed pictures of the layers within the sample. This is an advanced imaging method that is useful in specialised areas of food science and nanotechnology. During this process, different wavelength of the laser is projected onto the sample and the three-dimensional distribution of the food constituents is identified using the detector (Pawley, 2006). CLSM uses multicolour fluorescence staining and labelling, enabling the visualisation of detailed, complex structures and their interactions, thereby helping differentiate between nano-encapsulated food ingredients.

Compared to Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM), preparation of CLSM samples is relatively easy. It is a one-step process that involves adding a dye to the sample, thoroughly mixing it, and loading it onto a glass slide. In CLSM, magnification is adjusted electronically, allowing the visualisation on both horizontal and vertical planes for a detailed examination of the sample's structure (Falsafi et al., 2020). Dynamic processes such as aggregation, coagulation, and phase separation can also be identified using CLSM without distributing the sample, which offers insights into the changes that occur in a food system (Sharif et al., 2020).

CLSM has some limitations, among which the major one is the lack of suitable lasers for efficient fluorophore excitation. CLSM uses high-intensity laser illumination, which can damage the sample. CLSM also has a limited resolution of 100 μm as compared to SEM (0.5-4 μm) and TEM (below 50 picometers).

2.5. Research gap

FPI has a huge potential as a sustainable plant protein ingredient in a food system. However, due to its aggregation tendency and low solubility affects its techno-functionality which limits its direct use. Previous studies have studied faba bean protein modifications, whey protein functionality and some dairy-plant combinations but there is still a limited understanding on how FPI and WPI would interact in a hybrid system. This is important as both WPI and FPI has different protein fractions which may respond differently to different treatments like thermosonication, heat treatment and cold sonication (Herneke et al., 2023; Hinderink et al., 2021; Hu, Cheng, Gilbert, Lee, et al., 2024; R. Yu et al., 2024).

There is also limited research on how thermosonication will impact WPI-FPI hybrids and influencing emulsification and gelation. Most of the studies have been focused on either individual proteins or other plant-dairy hybrids. Therefore, this study addresses that gap by preparing WPI-FPI hybrids by using heat treatment, cold sonication and thermosonication.

3. Preparation of hybrid nano assemblies from plant and dairy proteins and characterisation of their emulsifying properties

3.1. Introduction

In today's world, plant proteins have been recognised as a replacement for meat and dairy proteins. They are being used as functional agents or meat substitutes due to their various benefits and the growing awareness of the need for a sustainable world.

Despite its high protein concentration, the functional properties of faba bean, such as emulsification and gelation, remain poorly understood. Physical stability is essential for an emulsion, as emulsions are susceptible to creaming, flocculation and coalescence (Liu et al., 2022). Previous studies have shown that modifications to FPI can improve its emulsifying activity and emulsion stability by altering its physicochemical properties.

Various physical, chemical and enzymatic methods have been adopted previously to modify FPI to increase its emulsifying properties. These treatments mainly act by changing particle size, surface hydrophobicity, interfacial tension, surface charge and solubility as summarized in Table 13.

Table 13. Types of treatments that can enhance FPI emulsion stability (Alavi et al., 2021; Felix et al., 2019; Gürbüz et al., 2018; Liu et al., 2022; Yang et al., 2018)

Type of Treatment	Treatment	Results
Physical	Electrostatic association	Decrease in Interfacial tension and surface hydrophobicity
	Heating (e.g., oven heating at 170 °C for 30 min)	Improved Surface hydrophobicity (Gürbüz et al., 2018)
	High pressure/ultrasonication (e.g., HPH at 15 kpsi)	Improved Surface hydrophobicity, solubility (Yang et al., 2018)
	pH shifting (pH 3.0, 5.0 and 8.0; alkaline shifting with ultrasound)	Improved interfacial adsorption while alkaline shifting with ultrasound led to smaller particle size

		(Alavi et al., 2021; Felix et al., 2019)
Chemical	Acetylation reaction	Decrease in Interfacial tension and improved surface hydrophobicity
	Maillard Reaction	Decrease in Interfacial tension and improved surface hydrophobicity
	Functional groups	Decrease in Interfacial tension and improved surface hydrophobicity
Enzymatic	Hydrolysis reaction	Increased surface charge and solubility
	Cross-linking reaction	Increased surface charge and solubility

One study was conducted on pea protein isolate and Sodium caseinate co-dispersions using ultrasonication, and it was noticed that the co-dispersions had good solubility and colloidal stability (X. Du et al., 2023). A stable colloidal solution of pea protein and casein micelles has been prepared using low-temperature homogenization. This solution also had higher solubility than the Pea protein solution by itself (Krentz et al., 2022).

Whey protein isolate emulsions also show a smaller particle size and better stability than the FPI (Gumus et al., 2017). Various treatments have been done to increase the emulsifying properties of the WPI:

1. pH/heating: It has been observed that heating WPI at 85 °C at pH 5 leads to an increase in hydrophobicity and emulsion activity index (Lam & Nickerson, 2015).

2. High-Pressure Homogenization (HPH)/Ultrasonication (US): A combined treatment of HPH/US on WPI led to improvement in the Emulsion Stability Index (Shi et al., 2020).

This research focuses on developing a nano-hybrid of whey protein isolate and faba bean protein isolate to leverage WPI's functionalities and FPI's environmentally friendly nature. Although interactions between plant and dairy proteins have been explored, there is a limited understanding of how WPI and FPI interact. In this research, thermosonication is used as

one of the processing treatments to understand its thermal and mechanical effects on proteins, for which limited studies have been conducted to date.

3.2. Materials and Methods

3.2.1 Materials

Faba bean protein isolate was purchased from NZ Protein, New Zealand. According to the supplier, it had 85 g of protein, 5.4 g of fat, and 3.8g of carbohydrates per 100g of powder. Whey protein isolates 895 were provided by Fonterra, New Zealand, and manufactured using ion-exchange and proprietary ultrafiltration techniques. It contains 92g of protein, 0.9 g of fat, and 0.3 g of carbohydrate per 100g of powder. All chemicals, including sodium dodecyl sulfate, sodium azide, isopropanol, acetone, HCl, and NaOH, were of analytical grade and purchased from Sigma-Aldrich (USA). Mini-PROTEAN® Precast Gels, 10x Tris/glycine/SDS Protein Electrophoresis buffer, β-mercaptoethanol, Coomassie Brilliant Blue R-250 staining solution, Dual Xtra Standards and 4× Laemmli sample buffer were purchased from Bio-Rad (USA). Soybean oil was purchased from Pak'n Save, New Zealand. The samples were prepared using reverse osmosis (RO) water or Milli-Q water provided by Massey University (Auckland, New Zealand).

3.2.2. Treatment

A 3% (w/w) protein solution for FPI and WPI was made separately using a 0.04% sodium azide solution. These solutions were hydrated for 48 hours at room temperature, with continuous stirring. After 24 hours of hydration, the pH of these solutions was measured, and NaOH/HCl was used to adjust the pH to 7. The pH was checked every 5 hours, and the final pH was measured at 48 hours. Five ratios of these solutions were made in 22mL glass vials. Three treatments were applied to each ratio in duplicates. Two sets (duplicates) were

kept aside as untreated. All sample emulsions were prepared with 70% (w/w) soybean oil using an IKA® T10 basic homogeniser.

Table 14: Sample number with their corresponding WPI and FPI ratios in 3% protein solutions.

Sample Number	WPI (g)	FPI (g)
1	10	0
2	8	2
3	5	5
4	2	8
5	0	10

1. Thermosonication (TS)—Each vial was sonicated for 2 seconds on and 2 seconds off at 50% power for 60 minutes using a 20 kHz ultrasonic processor equipped with 6mm horn (JY92-IIN, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China). During the treatment, these vials were put in a small 90 °C water bath.

2. Cold-Sonicated (CS): Each vial was put into a 4 °C water bath and ultra-sonicated for 60 minutes using the same ultrasonic processor (JY92-IIN, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) with 2 seconds on and 2 seconds off at 50% power.

3. Heat Treatment (HT): All vials were in a 90 °C oven (Labserve, Australia). After the sample inside each vial reached 90 °C, they were kept inside for an additional 30 minutes.

3.2.3. Determination of particle size distribution

Particle size distribution was determined using dynamic light scattering (Zetasizer Nano Series, Malvern Instruments, UK). The protein solutions were diluted 100 times, and the solutions were gently shaken before taking the readings to avoid sedimentation. All the

readings were taken at room temperature in 2.5mL cuvettes. The dispersant (water) 's refractive index of 1.33 and viscosity of 0.89 mPa s was used for the measurement (Luo et al., 2022). The laser's scattering angle is 173°, and its wavelength is 633 nm.

The polydispersity index (PdI) was obtained using Zetasizer software through cumulants analysis of the dynamic light scattering data.

3.2.4. Zeta potential

The zeta potential of the samples was determined using the Zetasizer Nano Series (Zetasizer Nano Series, Malvern Instruments, UK). Samples were diluted 100 times, and readings were taken using Zetasizer Nano Series disposable folded capillary cells (Hu et al., 2023). Zeta potential helped describing the surface charge of the protein particles in the solution.

3.2.5. Sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

For all treatments and untreated samples, both reducing and non-reducing SDS-PAGE were conducted for Samples 1, 3, 4, and 5, these corresponded to WPI: FPI ratios of 10:0, 5:5, 2:8 and 10:0, respectively; Sample 2 with WPI: FPI (8:2) was not analyzed using SDS-PAGE. This method was performed as described in a previous study by Hu et al. (2023).

For SDS-PAGE, 8.75 µL of Laemmli sample buffer was mixed with 25 µL of 0.1% sample in 2-mL Eppendorf tubes. 1.25 µL of β-mercaptoethanol was added. The Eppendorf tubes were then heated in hot water for 10 minutes. For non-reducing SDS-PAGE, 1.25 µL of β-mercaptoethanol was replaced with 1.25 µL RO water. 7.5 µL of the sample was loaded into the second well of the Mini-PROTEAN® Precast Gels. Initially, 5µL of Dual Xtra Standard was loaded. Gels were then set up in an electrophoresis tank. Tris/Glycine/SDS running buffer was added till it covered the gels properly. This running buffer was prepared by diluting the Tris/glycine/SDS solution 10 times. The electrophoresis was then run at 130 V for 60 minutes using a PowerPac Basic (Bio-Rad, USA). After 60 minutes, the gels were removed from the tank. The gels were stained using Coomassie Brilliant Blue R-250 for 40 minutes. After staining, the dye was carefully removed, and destaining was performed with

10% isopropanol and 10% acetic acid by mild shaking. The destaining solution was changed every 3 hours until the background became clear. The gels were then visualised using the Bio-Rad ChemiDoc™ MP imaging system.

3.2.6. Turbidity

The turbidity of all samples was measured using a PharmaSpec UV-1700 UV-Visible Spectrophotometer. The blank was a 0.04% sodium azide solution. The absorbance of all samples was measured at 600nm (Ryan & Foegeding, 2015). Samples were diluted to obtain a reading below 1 milli-absorbance unit. The absorbance was then multiplied by the dilution times to get the turbidity.

3.2.7. Preparation of emulsion

The emulsions were made using an Ultra-Turrax rotor-stator mixer (T10 Basic, IKA Corp., Staufen, Germany). The equipment had a 10 mm probe (S10N-10 G, IKA, Germany). 1 ml of the sample was homogenised with 70% (w/w) soybean oil to make an oil-water mixture for 2 mins at 20,000 rpm. The method was taken from Hu et al (2024).

3.2.8. Determination of oil droplet size using static light scattering

The oil droplet size in the emulsion was measured using a Malvern Instruments Masterizer 2000. This instrument works on the principle of static light scattering to determine the droplet size. Samples with and without SDS were determined. For SDS samples, one droplet of each emulsion was added to a separate 1mL SDS solution and left overnight. The samples without SDS were determined after 30 minutes of emulsion formation. The refractive index chosen for samples without SDS was soybean oil (1.47) and protein solution (1.45). The refractive index of the samples with SDS was water (1.33) and protein solution (1.45). The

mean particle size was determined using the volume mean diameter (D[4,3]). All the readings were taken at the room temperature (approximately 20°C).

3.2.9. Rheological Properties

All emulsion deformation viscoelasticity was characterised at room temperature (20 °C) using a stainless-steel parallel plate geometry (diameter 40mm, gap 1000μm) on a rotational stress-controlled rheometer, as described in Hu et al., 2023. Time, frequency, and amplitude sweeps were measured. Storage Modulus and Loss Modulus of the samples were analysed. All Measurements were done in duplicate.

3.2.10. Statistical Analysis

All measurements were conducted in duplicate, except for zeta potential which was analysed in triplicate. The mean ± standard deviation was reported for all samples. IBM SPSS Statistics software was used to analyze variance (ANOVA) to evaluate the mean differences according to Tukey's Test (Hu, Cheng, Gilbert, Lee, et al., 2024).

3.3. Results and discussion

As most measurements were conducted in duplicate, the statistical analysis provides an indication of treatment-related differences; however, the limited number of replicates may reduce robustness of the statistical interpretation. Therefore, the statistical outcomes should be viewed as supporting the experimental trends rather than as the sole basis of interpretation.

3.3.1. Particle size

The Z-average particle size was measured using dynamic light scattering. This technique provides insight into the aggregation state and the structural organisation of the proteins in a dispersed state.

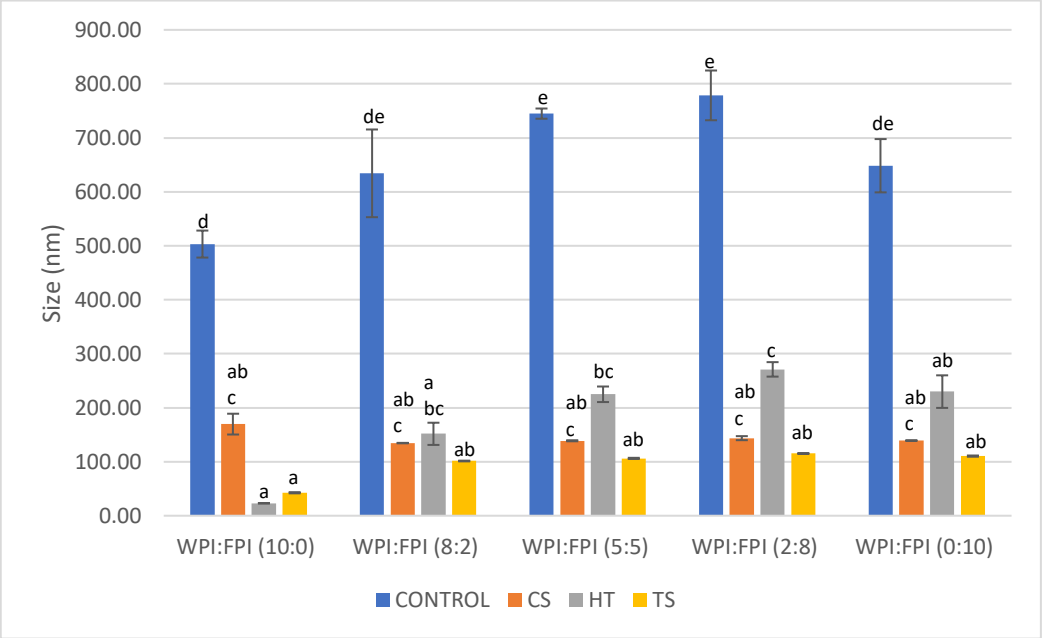


Figure 10. Comparison of particle size of the 3% protein solutions of WPI: FPI with different ratios when treated with cold-sonication (CS), heat-treatment (HT) and thermosonication (TS). Control represents the untreated sample. Different letters above the columns indicate significant differences ($p<0.05$).

As shown in Figure 10, Untreated samples showed the largest particle sizes, ranging from 503.2 nm to 778.65 nm. Samples with higher FPI ratios, especially WPI: FPI (2:8 and 0:10) had larger particles. A similar trend has been seen in quinoa protein isolate (Luo et al., 2021). This explains the protein's large hydrophobic diameter, which leads to protein aggregation. The cold-sonication treatment shows a significant decrease in the particle size of the protein

molecules. Z-average of pure WPI decreases to 169.68nm from 503.2nm, and for pure FPI, it decreases from 648.35nm to 139.2nm. Mechanical shear and the cavitation forces were effective in disrupting the intermolecular bonds, such as hydrogen bonds, hydrophobic interactions, and van der Waals forces. The previous study reported that the ultra-sonication's cavitation effect has been proven helpful to particle size in soy protein isolate (SPI) by disrupting the non-covalent bonds between the SPI molecules (Ren et al., 2020). Heat treatment further reduces the particle size of pure WPI. Similar findings have been reported previously, in which heating WPI to 90 °C results in complete protein denaturation (Nicorescu et al., 2009). Heat-treatment was less effective as FPI concentration increased; similar results were reported for FPI (Hu, Cheng, Gilbert, Lee, et al., 2024). This might be due to partial dissociation of the protein particle at high temperatures. Thermosonication has been shown to reduce particle size in solutions with higher FPI, such as WPI: FPI (2:8 and 0:10) (~106-115nm), demonstrating disruption of aggregates through synergistic efficacy. Some studies have suggested that a combination of sonication and heating can disrupt hydrogen bonds and hydrophobic interactions, leading to smaller particle sizes (Zhu et al., 2023).

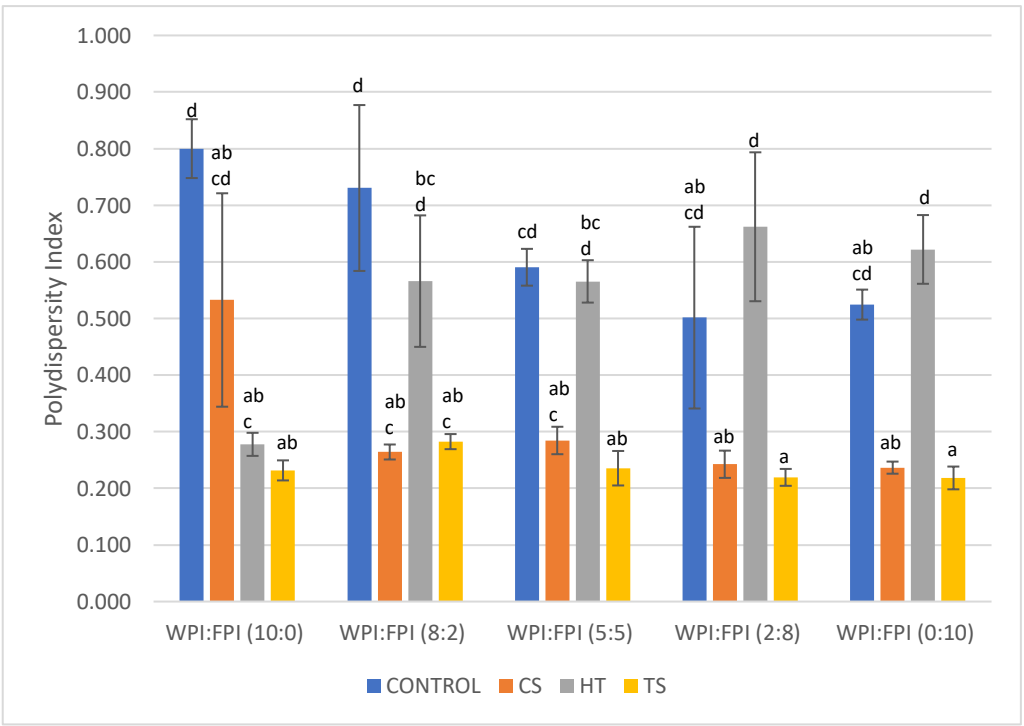


Figure 11. Polydispersity Index of 3% protein solutions of WPI: FPI with different ratios when treated with cold-sonication (CS), heat-treatment (HT) and thermosonication (TS). Control represents the untreated sample. Different letters above the columns indicate significant differences ($p<0.05$).

Polydispersity Index (PdI) is a measurement of the distribution of particle sizes. Higher PdI shows non-uniformity, and lower PdI shows uniformity in particle size distribution. Thermosonicated samples show the lowest PdI (~0.232-0.283), indicating that it is most effective to have a uniform particle size distribution in a solution. Heat treatment is effective for pure WPI but loses its effect as the FPI concentration increases. As with plant proteins, it only leads to partial dissociation of the protein, as observed previously in FPI (Hu, Cheng, Gilbert, Lee, et al., 2024). The heat-treatment effect decreases, which is also why cold-sonicated samples have lower PdI due to mechanical and cavitation effects.

3.3.2. Determining the surface charge by Zeta potential

The Zeta potential of a protein particle is the net electrical charge at its outer surface when dispersed in an aqueous solution. It is used to evaluate the electrostatic stability of a colloidal system. Higher absolute value of zeta potential, either negative or positive, reflects increased electrostatic repulsion and reduction in aggregation tendency (Li et al., 2020). Zeta potential is influenced by both the processing method and the sample's protein concentration.

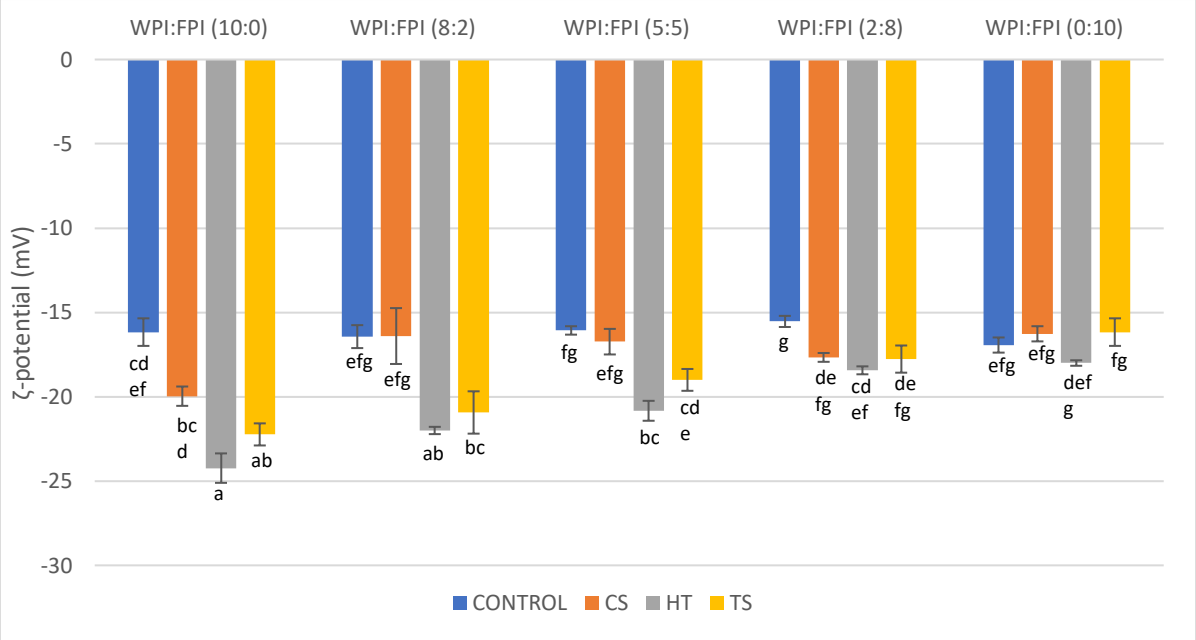


Figure 12. Zeta-potential of 3% protein solutions of WPI: FPI with different ratios when treated with cold-sonication (CS), heat-treatment (HT) and Thermosonication (TS). Control represents the untreated sample. Different letters above the columns indicate significant differences ($p<0.05$).

At neutral pH, both WPI and FPI exhibit negative zeta potential values due to the deprotonation of the carboxyl groups from the residues of the amino acids (Zhang et al., 2022). As shown in Figure 12, the group with higher WPI (WPI: FPI (10:0 and 8:2)) exhibits higher negative values, which explains the higher density of carboxyl groups that can be ionised. This indicates higher electrostatic stability. Heat treatment is most effective in this case, as it can induce conformational changes in the protein's structure, exposing its polar groups. Thermosonication is not very effective, which can be attributed to the re-aggregation of protein molecules, thereby hiding the polar groups. A similar finding has been observed in pea protein isolate when treated with higher ultrasound powers (Zhang et al., 2022).

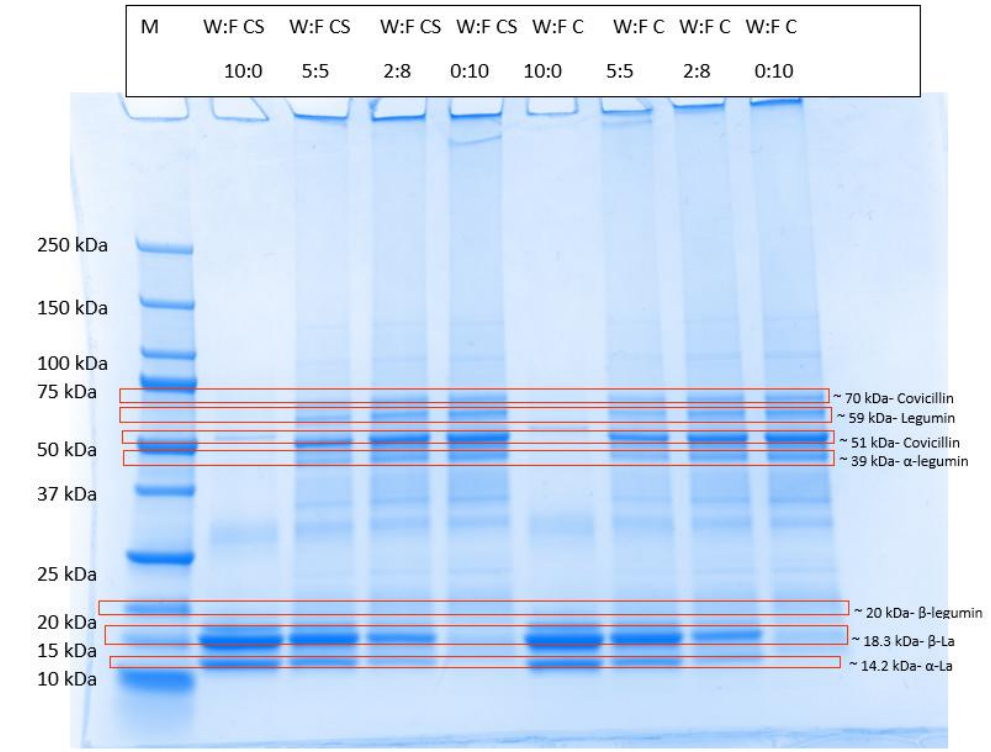
3.3.3. Protein profiles as per sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to determine protein composition and identify protein interactions at varying concentrations using protein banding patterns. Only four ratios were used to conduct this analysis: WPI: FPI (10:0), WPI: FPI (5:5), WPI: FPI (2:8), and WPI: FPI (0:10). SDS-PAGE was performed under both non-reducing (Figure 13. 1A and 1B) and reducing conditions (Figure 13. 1C and 1D) to help understand the protein-protein interactions. Non-reducing conditions in SDS-PAGE confer a negative charge to protein molecules while preserving their denaturing properties. This allows proteins to separate based on their size, while maintaining their undivided single units by preventing them from disassembling into monomers. In reducing conditions, a negative charge is imparted to the protein, thereby disrupting disulfide bonds. This leads to the formation of -SH bonds, which separate the protein into its monomers. The protein can then either stay in this disassociated form or become more compact.

The major WPI proteins are β-lactoglobulin and α-lactalbumin, which have molecular weights of ~18.3 kDa and ~14.2 kDa, respectively (Hosseinpour et al., 2011). SDS-PAGE Profiles show that as the concentration of WPI decreases, the bands of β-La and α-La become lighter, as these are not the dominant proteins in FPI. For FPI, dominant proteins are Vicilin (~51 kDa), Covicillin (~70 kDa), Legumin (~59 kDa), α-legumin (~37 kDa) and β-legumin (~20 kDa) (Hu, Cheng, Gilbert, Lee, et al., 2024). These protein bands are visible in the SDS-PAGE.

In FPI, untreated, and CS samples, darker bands are observed around 70 kDa and 50 kDa, but they are lighter at 37 kDa and 20 kDa in non-reducing gels. Under the non-reducing conditions, a smeared and profound protein band was observed on the top of the loading wells, suggesting the presence of aggregates larger than ~250 kDa, which has been observed previously in quinoa protein isolate (Luo, 2022). These aggregates were observed on top of all loading wells, without any clear pattern, for the Thermosonicated and heat-treated gels. However, in the untreated and cold-sonicated samples, aggregate size increased with FPI concentration, suggesting the presence of larger aggregates.

A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W:F CS 5:5
W:F CS 2:8
W:F CS 0:10
W:F C 10:0
W:F C 5:5
W:F C 2:8
W:F C 0:10
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa
~ 70 kDa- Covicillin
~ 59 kDa- Legumin
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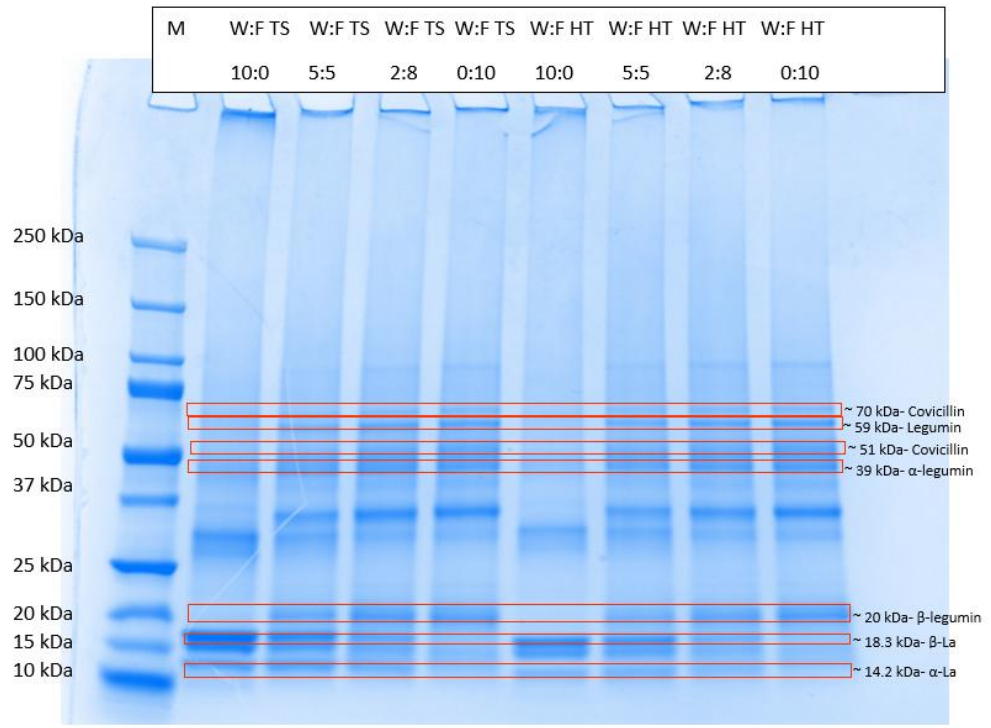
~ 39 kDa- α-legumin
~ 20 kDa- β-legumin
~ 18.3 kDa- β-La
~ 14.2 kDa- α-La

B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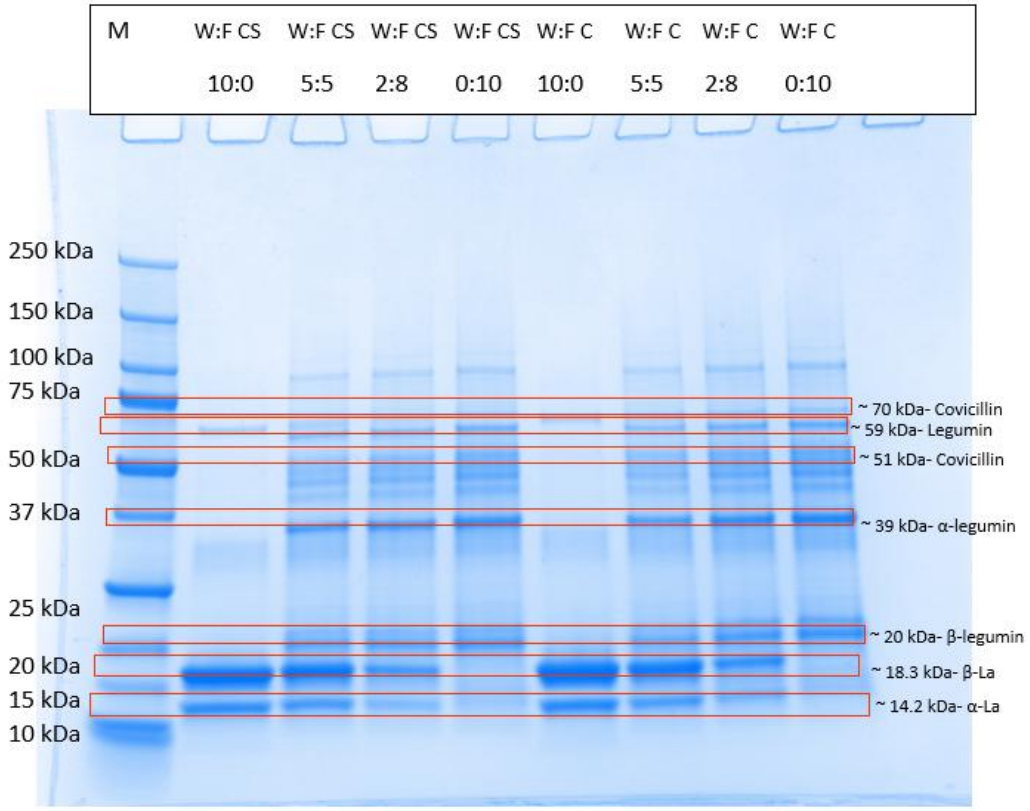
W:F HT 0:10
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa
~ 70 kDa- Covicillin
~ 59 kDa- Legumin
~ 51 kDa- Covicillin
~ 39 kDa- α-legumin
~ 20 kDa- β-legumin
~ 18.3 kDa- β-La
~ 14.2 kDa- α-La

C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W:F C 5:5
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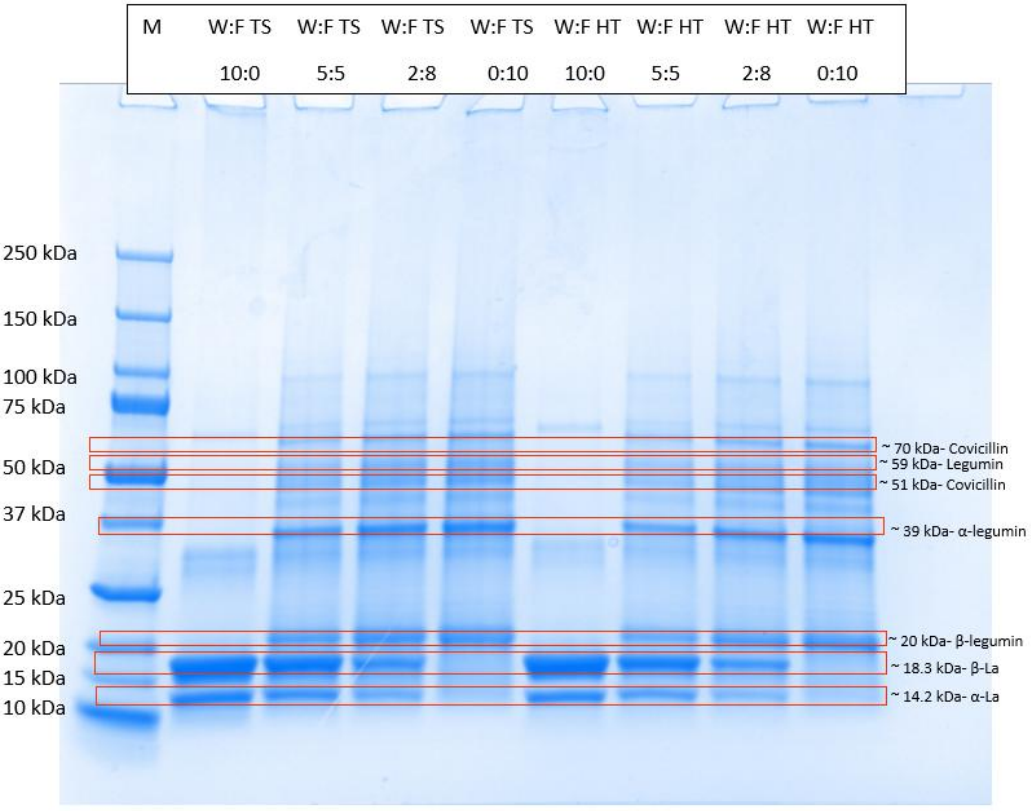
W:F C 0:10
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa
~ 70 kDa- Covicillin
~ 59 kDa- Legumin
~ 51 kDa- Covicillin
~ 39 kDa- α-legumin
~ 20 kDa- β-legumin
~ 18.3 kDa- β-La
~ 14.2 kDa- α-La

D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W:F TS 5:5
W:F TS 2:8
W:F TS 0:10
W:F HT 10:0
W:F HT 5:5
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W:F HT 0:10
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
25 kDa
20 kDa
15 kDa
10 kDa
~ 70 kDa- Covicillin
~ 59 kDa- Legumin
~ 51 kDa- Covicillin
~ 39 kDa- α-legumin
~ 20 kDa- β-legumin
~ 18.3 kDa- β-La
~ 14.2 kDa- α-La

Figure 13. Sodium dodecyl sulphate polyacrylamide gel electrophoresis, Non-reducing for Cold-sonicated (CS) and Untreated (C) samples (A); Non-reducing for Thermosonicated (TS) & Heat-Treated (HT) (B); Reducing for CS & C (C) and Reducing for TS & HT (D).

In reducing gels, the bands for α-legumin and β-legumin become stronger for FPI, explaining that a disulfide bond cross-links these subunits to form legumin (Herneke et al., 2023), which is not seen under non-reducing conditions. β-mercaptoethanol, which is used as a reducing agent, helps in breaking down the aggregates, thus resulting in more precise identification of the distinct bands (Muhammad & Massey University, 2024). For reducing conditions, the large aggregates also disappeared from the top of the loading cells, indicating the disulfide bond stabilisation (Hu, Cheng, Gilbert, Lee, et al., 2024). For WPI, no change in bands was observed across the different treatments. A similar finding was observed when WPI was treated with Ultrasonication at a power of 600 W (Meng et al., 2021). In heat-treated and thermosonicated gels, bands have been noticed between 25 and 37 kDa. This can be due to structural rearrangements of the protein molecules during intensive treatment. Similar changes have been seen when WPI undergoes ultrasonication (Chandrapala et al., 2011).

3.3.4. Turbidity

Turbidity measurements were performed to evaluate the degree of light scattering, which reflects protein aggregation and dispersion stability in protein solutions. Turbidity is essential for maintaining protein stability because increased protein aggregation reduces solubility. Turbidity is linked with the particle size; the smaller the turbidity, the smaller the particle size (Malik et al., 2017).

As shown in Figure 14, both thermosonicated and cold-sonicated samples exhibit lower turbidity than untreated samples. Ultrasound has shown a similar effect on the soy protein concentrate (Yildiz et al., 2017). In contrast, the heat-treated samples showed the highest turbidity. This happens as heating at 90 °C induces protein aggregation by exposing hydrophobic regions, leading to the formation of the intermolecular networking, which leads

to a thicker and more opaque solution (Shimada & Matsushita, 1980). Sonication, on the other hand, uses mechanical shear forces leading to cavitation, which reduces particle size, hence enhancing light transmission (Alavi et al., 2021). Recent study has shown that ultrasound can alter the structural and physicochemical properties of FPI by improving solubility (Adal, 2024). As the concentration of FPI increases, turbidity increases, explaining its low solubility and its tendency to form larger aggregates. Thermosonication can lead to the formation of hydrogen bonds between the protein (after TS) and water, which then replaces the intramolecular hydrogen bonds (Hu, Cheng, Gilbert, Lee, et al., 2024). Table 15 shows the appearance of the samples after different treatments. The photos clearly show that thermosonicated WPI: FPI (10:0) exhibits the most excellent transparency among all the samples, which aligns with its turbidity. After thermosonication, FPI-rich samples become translucent. Similar findings have been noticed before in FPI (Hu, Cheng, Gilbert, Lee, et al., 2024).

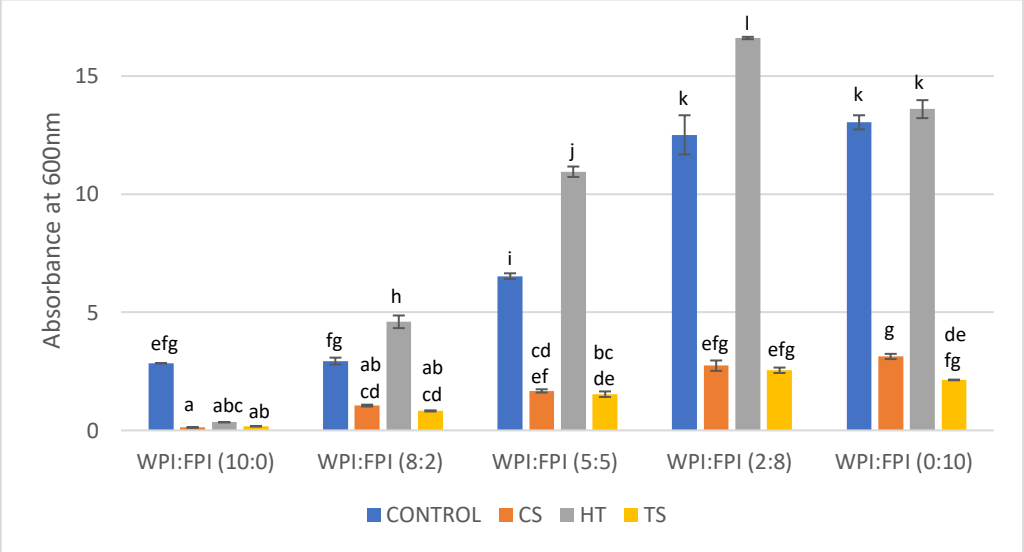


Figure 14. Turbidity of the 3% protein solutions of WPI: FPI with different ratios when treated with cold-sonication (CS), heat-treatment (HT) and thermosonication (TS) at 600nm. Control represents the untreated samples. Different letters above columns indicate significant differences ($p < 0.05$).

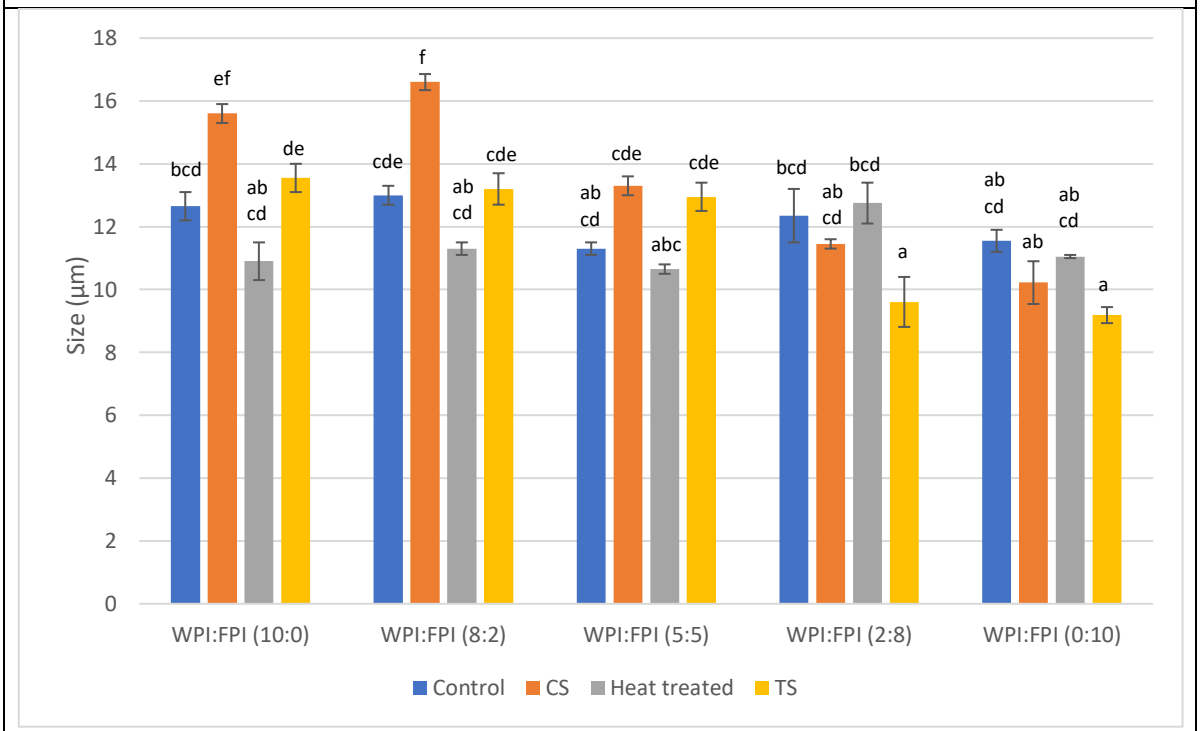
Table 15: A visual appearance of 10g protein solutions in sample vials.

Sample	Untreated	Cold-sonicated	Heat-treated	Thermosonicated
WPI: FPI (10:0)				
WPI: FPI (8:2)				
WPI: FPI (5:5)				
WPI: FPI (2:8)				
WPI: FPI (0:10)				

3.3.5. Emulsion oil droplet size using Mastersizer

Oil droplet size analysis in emulsions is an important parameter to understand emulsion stability, as it influences the functional performance of the protein-based emulsions. As shown in Figure 15, the evaluation of D [4,3], the volume-weighted mean diameter, and D [3,2], the surface-area-weighted mean diameter, was conducted to better understand the emulsions.

A


18
16
14
12
10
8
6
4
2
0
Size (µm)
bcd
ef
ab cd
de
cde
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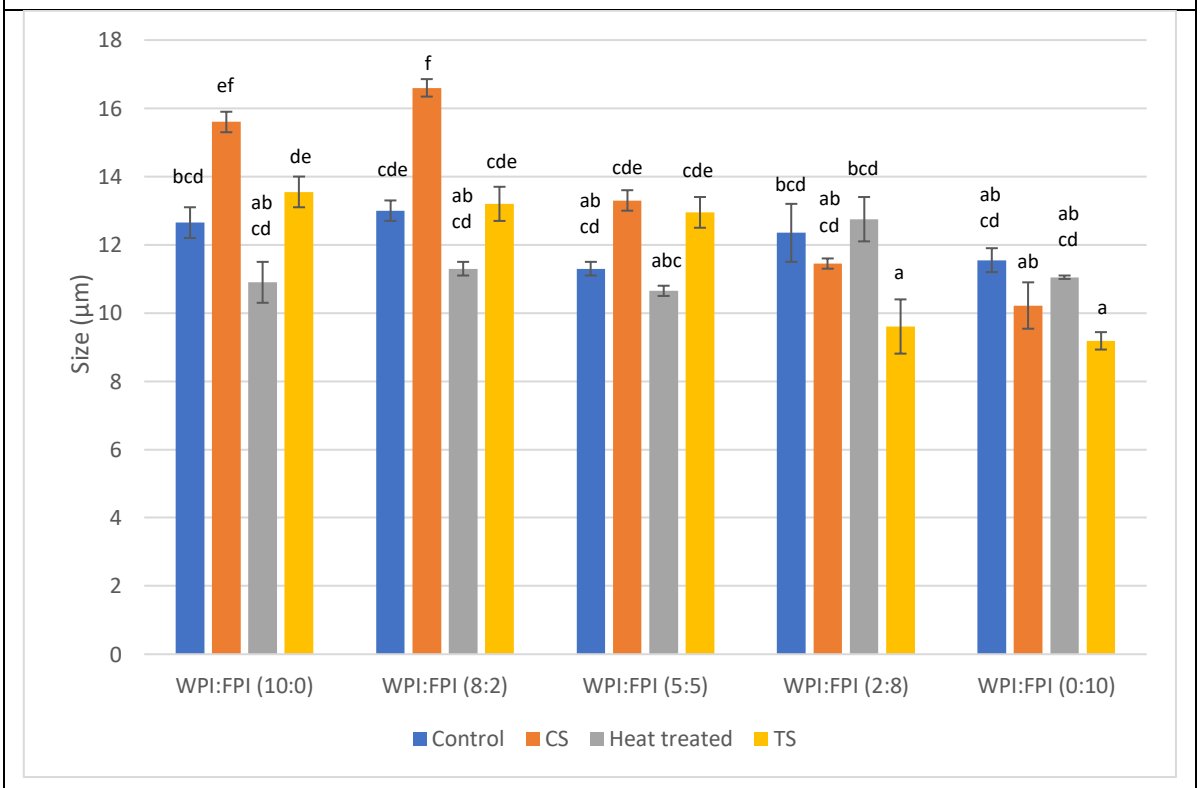
WPI:FPI (10:0)
WPI:FPI (8:2)
WPI:FPI (5:5)
WPI:FPI (2:8)
WPI:FPI (0:10)
Control
CS
Heat treated
TS

B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WPI:FPI (10:0)
WPI:FPI (8:2)
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Heat treated
TS

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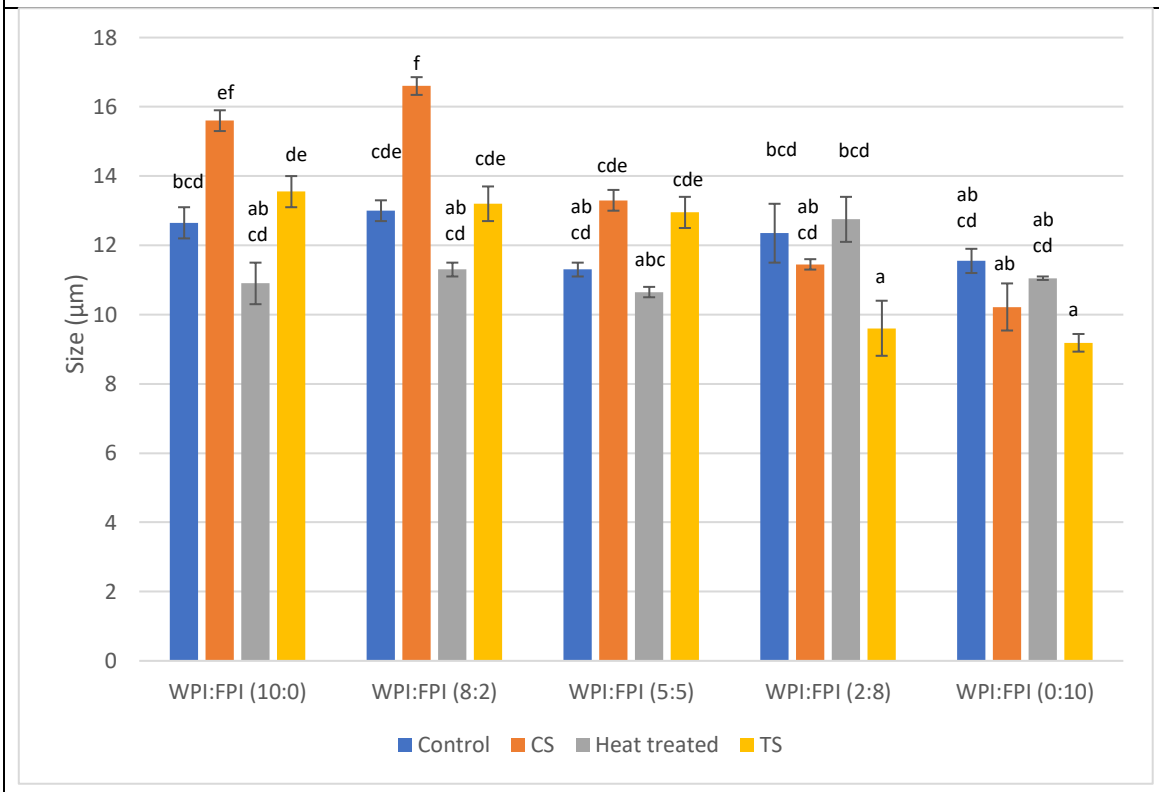
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WPI:FPI (8:2)
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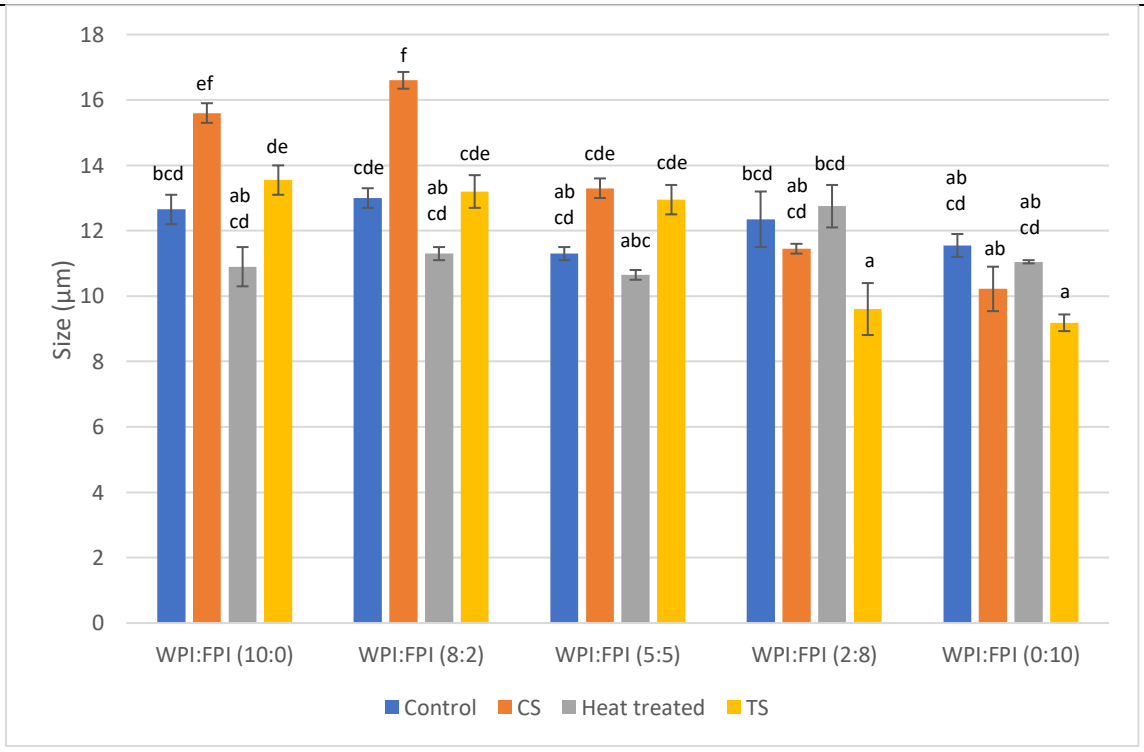
WPI:FPI (0:10)
Control
CS
Heat treated
TS

D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WPI:FPI (10:0)
WPI:FPI (8:2)
WPI:FPI (5:5)
WPI:FPI (2:8)
WPI:FPI (0:10)
Control
CS
Heat treated
TS

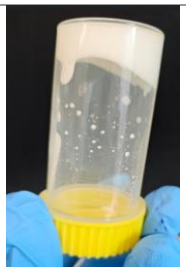
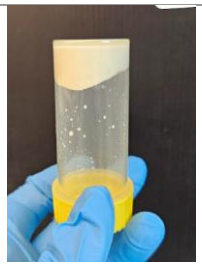
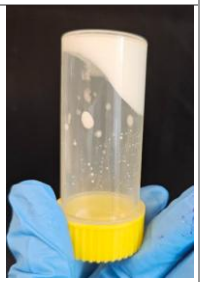
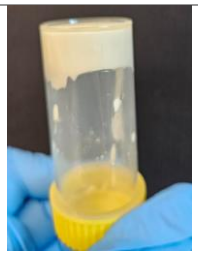
Figure 15. A. D[3,2] oil droplet size of emulsion with 70% (w/w) oil when measured in mastersizer, B. D[3,2] emulsion oil droplet size with SDS added overnight, C. D[4,3] oil droplet size of the 70% (w/w) emulsion and D. D[4,3] oil droplet size with SDS added overnight of different ratios when treated with cold-sonication (CS), heat-treatment (HT) and thermosonication (TS). Control represents the untreated sample. Different letters above the columns indicate significant differences ($p<0.05$).

Cold-sonication and thermosonication are less effective in decreasing the size as compared to the heat-treatment in the WPI dominant solutions, as it has been noticed in the past studies that heating WPI can lead to complete denaturation of the WPI, which leads to hydrocarbons solubilising in an aqueous phase, leading to inter-droplet oil exchange (McClements et al., 1993).

It has been observed that cold-sonication and thermosonication decrease oil droplet size as faba bean concentration increases, due to their mechanical effects; however, thermosonication exhibits a more significant effect, due to the additional thermal effect. It has been found in soy protein that alkaline shifting alongside ultrasonication led to the exposure of some hydrophobic residues and some of the primary protein aggregates and molecules rearrangement, which might have led to the smaller particle sizes, as such increased hydrophobicity led to better absorption at the air/water interface (Lee et al., 2016). It has been observed previously that thermosonication has led to a decrease in interfacial tension, enhancing emulsification capability, which helps stabilise larger surface areas and therefore reduces the oil-droplet size (Hu, Cheng, Gilbert, Lee, et al., 2024).

Table 16 shows the flowability of the emulsions. Heat treatment improved the flowability of emulsions made from pure WPI more than thermosonication did. As the FPI concentration increases, the emulsion forms a gel-like structure, which explains its smaller oil droplet size. Heat treatment also leads to gel-like emulsions in FPI-rich samples, which can be attributed to protein and droplet aggregation, as observed in canola protein isolate (Tang & Ghosh, 2021). Sonicated emulsions exhibit gel stability and strength due to the formation of a network and interactions between protein aggregates and oil droplets. This has been observed previously when FPI emulsions are made at pH 7 (Hu et al., 2023)

Table 16: Flowability of the emulsions made with 70% (w/w) soybean oil.

SAMPLE	Untreated	Cold-sonicated	Heat-treated	Thermosonicated
WPI: FPI (10:0)				
WPI: FPI (8:2)				
WPI: FPI (5:5)				
WPI: FPI (2:8)				
WPI: FPI (0:10)				

3.3.6. Storage modulus of the emulsion using rheology

The storage modulus (G′) was measured to identify the elastic component of the emulsions of 3% (w/w) protein solutions made with 70% (w/w) soybean oil. During deformation, G′ indicates the energy stored in the system; therefore, higher values mean a stronger and more intense network to resist the breakdown.

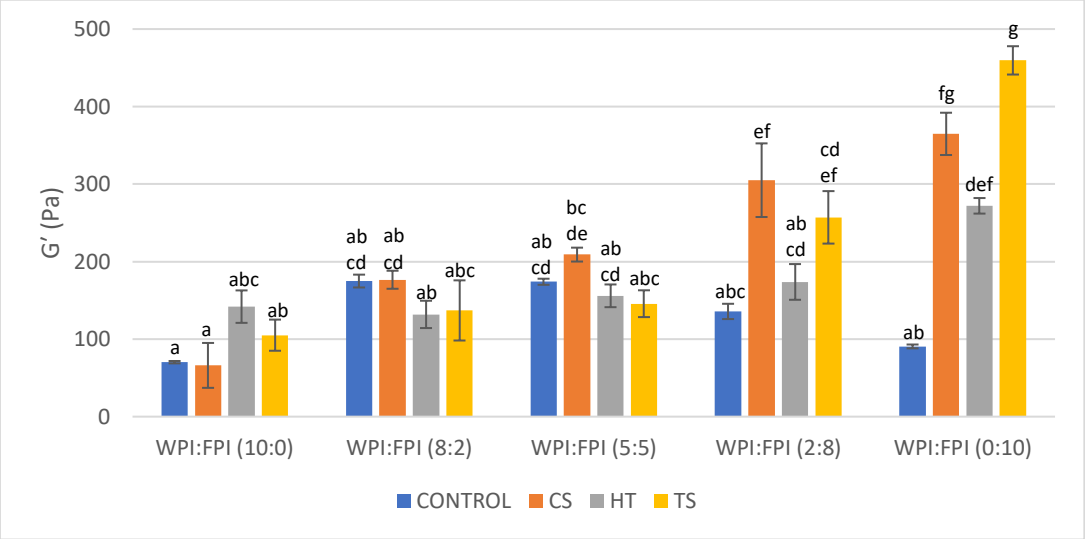
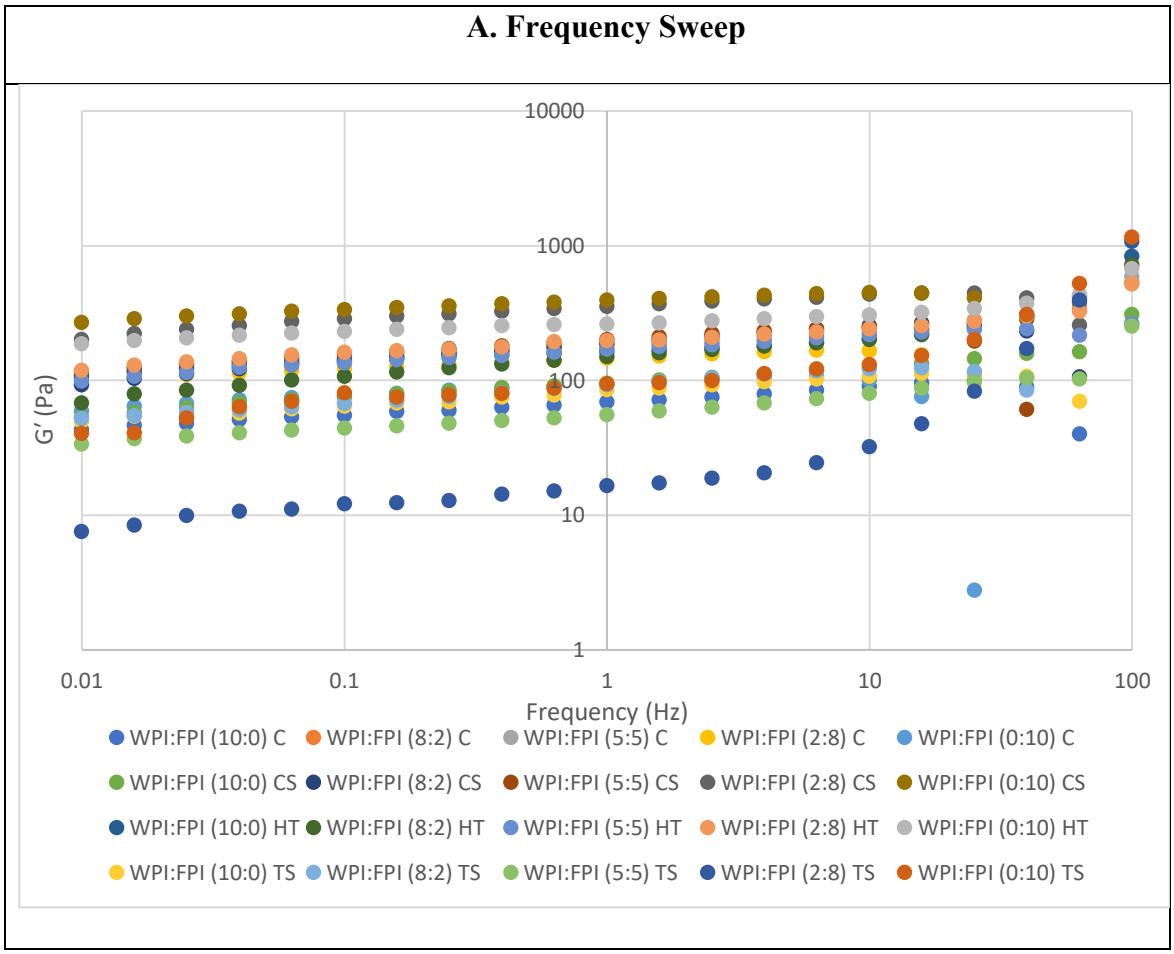


Figure 16. Storage modulus of emulsion before and after treatment 3% (w/w) protein solutions of different ratios when treated with cold-sonication (CS), heat-treatment (HT) and thermosonication (TS). Control represents the untreated sample. Different letters above the columns indicate significant differences ($p<0.05$).

The storage modulus of untreated samples showed composition dependence. Pure WPI and FPI show the lowest G′ as shown in Figure 16, explaining the inelastic nature of these in their pure form. On the contrary, the blended samples showed higher G′ between 135 Pa and 174 Pa, suggesting a stronger network. Heat-treatment was most effective in increasing G′ for the pure WPI to 141.93 Pa. Previously, heat-treatment has been shown to increase the hydrophobicity of WPI to some extent, leading to better adsorption at the Oil-water interface (Ye, 2010). G′ of heat-treated pure FPI was also increased to 271.97 Pa. This explains that the thermal nature of the treatment helps unfold the protein's structure, thereby enhancing network binding. Cold-sonicated and thermosonicated samples exhibit a higher storage

modulus as the FPI concentration increases, indicating that these treatments facilitate the unfolding of this protein, resulting in a more robust gel-like structure. Thermosonication of the FPI solution has been proven to be the most effective method for increasing the storage modulus of FPI-dominant solutions. Thermosonication increased the G′ of pure FPI to 459.62 Pa, which is 5 times more than its untreated form. A similar finding was observed for SPI after thermosonication, which increased its emulsion stability and its ability (Yildiz et al., 2017). Acoustic cavitation during thermosonication alters the protein's globular structure, increasing the number of charged molecules. This further increases the electrostatic forces and interactions between the molecules.

Figure 17A shows the frequency dependence of the storage modulus of WPI-FPI emulsions across the frequency range of 0.01 to 100 Hz. Thermosonication of pure FPI maintains its structure even at 100Hz, explaining the robust, elastic nature of the emulsion under rapid oscillatory deformation. This explains the synergistic effect of thermal treatment with ultrasonication, resulting in complete denaturation and molecular rearrangement.



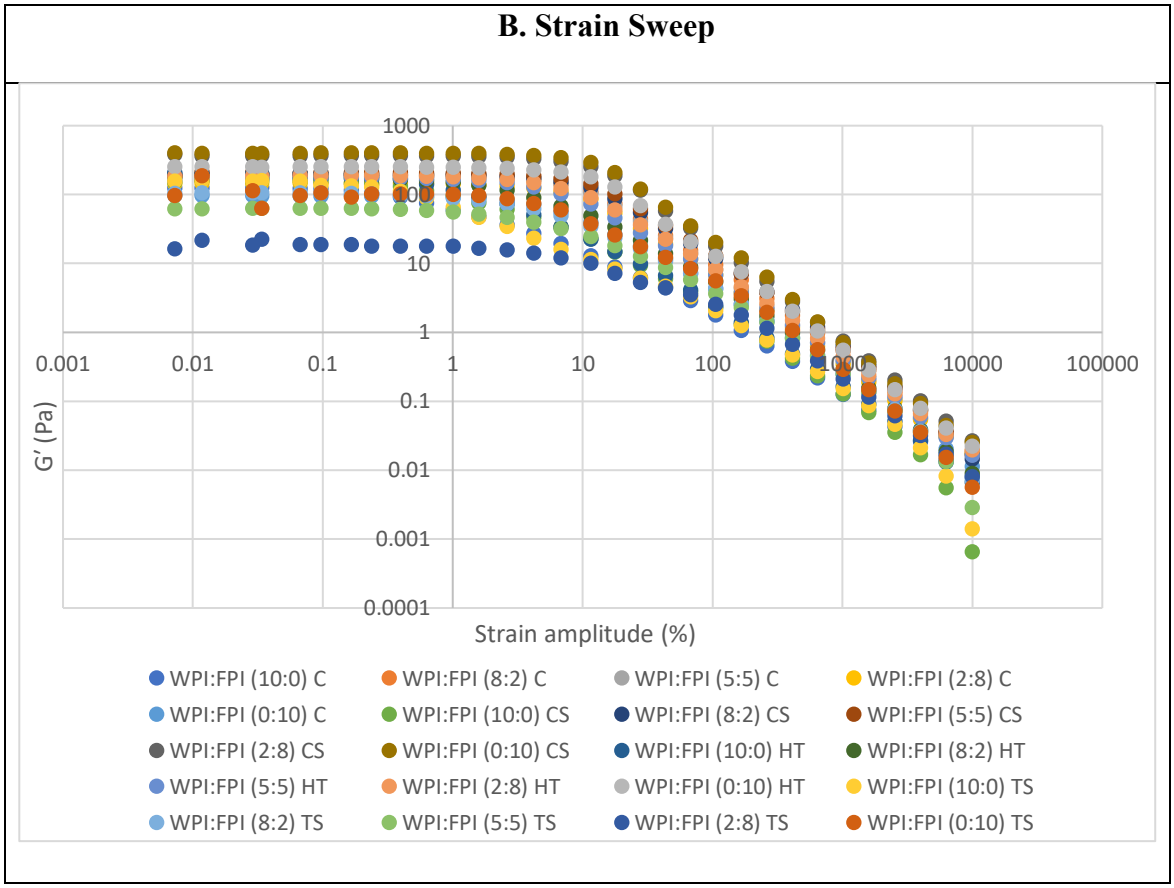


Figure 17. Dependence of the Storage modulus (G′) on the Frequency (A) and strain amplitude (B).

The strain sweep graph (Figure 17B) shows that as the Oscillatory strain % increases, WPI: FPI (10:0) cold-sonicated emulsions exhibit the greatest decrease in storage modulus, indicating that they lose their elasticity the most. The elasticity of most samples gradually decreases after the strain exceeds 10%. Thermosonicated samples exhibit high resistance, which explains the reduction in particle size and protein-protein interactions.

3.4. Conclusion

The effects of different methods—untreated, cold sonication, heat treatment, and thermosonication—on whey protein isolate and faba bean protein isolate solutions, separately and in various mixed ratios, were analysed.

Particle size data showed that heat treatment was most effective in reducing WPI particle size, whereas an increase in FPI led to smaller particle sizes via thermosonication. Thermosonication of WPI: FPI (5:5) blends resulted in a decrease in particle size from ~700nm in an untreated sample to ~100nm. On the contrary, heat treatment was most effective in reducing the zeta potential of pure WPI, FPI, and their blends.

SDS-PAGE showed that the concentrations of WPI and FPI influenced band visibility in the sample. Non-reducing gels with FPI-rich samples showed the presence of large aggregates (>250 kDa).

Heat-treated samples exhibited the highest turbidity, likely due to the formation of protein aggregates. In contrast, thermosonicated samples exhibited the highest decrease in turbidity among those with the highest FPI ratio. Thermosonication reduced the absorbance at 600nm of WPI: FPI (2:8) by up to 5 times, and for WPI: FPI (5:5) by more than 4 times.

Thermosonicated samples with higher FPI have exhibited smaller oil droplets, indicating improved emulsifying properties. Heat treatment also reduces oil-droplet size; however, this effect is lost at higher FPI concentrations. These results reflect in the storage modulus and elasticity of the samples as well.

Overall, the functionalities of the WPI: FPI hybrids cannot be understood by examining WPI and FPI alone. In a mixed system, thermosonication is a powerful tool that can combine the desired properties of WPI, such as solubility and stability, with the properties of FPI, like firmness, by reducing particle size.

The ability to enhance FPI performance through blending and thermosonication opens the door to living more sustainably by incorporating plant proteins into food products without compromising on quality. This blend can be used in high-protein yoghurts and beverages where both texture and stability are desired.

4. Effect of thermosonication on gels produced from hybrid nano-assemblies of plant and dairy proteins

4.1. Introduction

As the world's population grows, the demand for protein rises, highlighting the need for sustainable plant-based options (Augustin & Cole, 2022). This involves transitioning to a diet higher in plant-based foods and reducing reliance on animal products.

Faba bean, scientifically known as *Vicia faba,* is gaining popularity for its benefits to human nutrition and crop rotation. However, faba bean protein isolate (FPI) generally exhibits lower solubility and other functional properties compared to those of animal proteins.

Conventional heating is a popular method in which the protein solution is heated above its denaturation point, typically between 75-95 °C, to induce protein unfolding and aggregation, resulting in changes in their techno-functional properties, such as emulsification and gelation (H. Du et al., 2023). However, this treatment usually takes longer to achieve the desired results with plant protein (Jo et al., 2020), which is neither favourable nor sustainable for the food industry. Ultrasonication is another popular method that employs physical force to alter the physicochemical and techno-functional properties of plant proteins. During this treatment, high-shear forces and cavitation induce the microbubbles in the liquid to collapse, generating high energy that leads to the breakdown of large protein particles and an increase in solubility. Still, it alone is not very successful in enhancing the techno-functional properties of the proteins (Zhong & Xiong, 2020). Therefore, ultrasonication is often combined with heat treatment (Zhong & Xiong, 2020) or pH shifting to achieve better results (Yildiz et al., 2017). Ultrasonication combined with heat treatment is known as thermosonication. There have been studies on plant proteins, but fewer on their techno-functional characteristics (Hu, Cheng, Gilbert, Lee, et al., 2024).

This research addresses the limitations of plant-based proteins and the sustainability concerns associated with dairy proteins by developing a hybrid protein. It focuses on the gelation properties using different processing methods, including thermosonication, heat treatment, and cold-sonication.

4.2. Materials and Methods

4.2.1. Materials

The FPI used in the experiments was sourced from NZ Protein Inc. (New Zealand). According to the ingredient specification, 100 g of the protein powder contains 85% protein, 5.4% fat, 3.8% carbohydrates, and dietary fibre. The whey protein isolate 895 was provided by Fonterra, New Zealand, which consists of 92% protein, 0.9% fat, and 0.3% carbohydrate per 100 grams of powder. All chemicals, e.g. HCl, NaOH, sodium azide, Fast Green and D-(+)-Gluconic acid δ-lactone (GDL) were purchased from Sigma-Aldrich (USA) in analytical grade. All samples were prepared using reverse osmosis (RO) water provided by Massey University (Auckland, New Zealand).

4.2.2. Preparation of protein solutions

To prepare a 3% FPI solution, FPI powder was dispersed in a 0.04% sodium azide solution for 24 hours using a magnetic stirrer at 300 rpm. The pH of this solution was adjusted to 7 using 1M HCl. 1 M HCl was added while stirring for 24 hours, and the pH was monitored and adjusted regularly until it became stable. 3% WPI solution was prepared using the same method. Three different ratios of these solutions were prepared in separate glass vials with a total volume of 22 mL, resulting in eight sets.

Table 17: Ratios of WPI: FPI added in different vials

Sample Number	3% WPI solution (g)	3% FPI solution
1	10	0
2	5	5
3	0	10

Two sets were set aside as controls. Each of the two sets was treated with the following treatment:

1. Thermosonication (TS): Each vial was placed inside the ultrasonicator in a small 90 °C water bath. The ultrasonication was then conducted using ultrasonic processor (JY92-IIN, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) for 2 seconds on and 2 seconds off at 50% power for 60 minutes. The samples were removed, sealed, and cooled immediately in cold water.

2. Cold-sonication (CS): Each vial was placed inside the ultrasonicator in a 4 °C ice water bath. The ultrasonication was then conducted using ultrasonic processor (JY92-IIN, Ningbo Scientz Biotechnology Co., Ltd., Ningbo, China) for 2 seconds on and 2 seconds off at 50% power for 60 minutes. The samples were removed, sealed, and cooled immediately in cold water.

3. Heat treatment (HT): All vials were placed in an oven (Labserve, Australia) at 90°C. The vials' temperature was monitored regularly with a sterile thermometer. After the sample in the vial reached 90 °C, it was maintained at 90 °C for 30 minutes in the oven. Samples were taken out and cooled immediately under running cold water.

4.2.3. Rheological characteristics

Rheological measurements were conducted on a stress-controlled rheometer (DHR-3, TA Instruments, New Castle, DE, USA) using a stainless-steel parallel geometry with a 40 mm diameter and a 1000µm gap. Two millilitres of each sample were pipetted and placed into separate vials. 0.05% GDL was added to this 2 mL sample. The samples were gently shaken 10 times, and then approximately 1 mL of sample was added to the rheometer plate using a 3 mL plastic dropper, with a geometry gap of 4000 µm. This step was done just before the rheological measurement. Geometry was used to create a 1000 µm gap, and the sample edges were trimmed. Then, mineral oil was added to the sides to prevent the sample from evaporating. The following protocols were conducted:

1. Temperature sweep: This was performed while maintaining the temperature at 37 ºC. During this time, G′ and G″ were recorded at a fixed frequency for 4 hours.

2. **Frequency sweep:** This was performed while keeping the strain constant at 1% and changing the frequency from 0.01 to 100 Hz at 37 ºC.

3. **Strain sweep:** During this protocol, the frequency was fixed at 1 Hz, and the strain changed from 0.01% to 1000% at 37 ºC.

4.2.4. pH profile of the samples during gelation

A temperature-controlled water bath was set to 37 °C (Thermo Fisher Scientific, USA). A sample of 5 mL was added to a 28 mL vial with 0.02% GDL. The sample was gently mixed. The sample was placed in the water bath using a polywire rack, and a temperature probe (Hanna Instruments, Romania) was kept in the container to measure the temperature and pH of the sample. Once the temperature inside the vial reached 37 °C, the pH of the sample was monitored for 4 hours using the Hanna Lab app (Hanna Instruments, Romania).

4.2.5. Confocal Laser Scanning Microscopy (CLSM)

CLSM was conducted to check the microstructures of the gels. A few drops of 1% fast green (ratio to sample 1:500 (v/v)) were added to a sample of 1 mL, and 0.05% GDL was added. One drop of this solution was added to the slide and covered with a cover slip sealed with nail polish at the edges to avoid evaporation. The slides were incubated for 4 hours at 37 ºC. The confocal laser scanning microscope from Leica DM 6000 B (Leica Microsystems, Wetzlar, Germany) with a 100X oil-immersed objective lens, was used to view the gel structures at a 633 nm laser wavelength. The micrographs were then analysed with the assistance of ImageJ software (NIH, MD, USA).

4.2.6. Water holding capacity (WHC)

Only samples that can form gels were tested for water-holding capacity. A 10 mL sample was added to a 15 mL centrifuge tube, and 0.05% GDL was added for 4 hours at 37ºC. The total weight of the tubes with the sample (W1) and without the sample (W0) was measured.

After 4 hours, the tubes were centrifuged in a Thermo Fisher Scientific 3000 centrifuge (Thermo Fisher Scientific, Waltham, MA, USA) for 10 minutes at 10,000 RPM. The water was drained from the tube, and the tube was weighed again (W3). The following formula was used to measure the water-holding capacity:

$$\text{WHC (\%)} = \frac{(W_3 - W_0)}{(W_1 - W_0)} \times 100$$

This method was adopted from Patole (2022).

4.2.7. Statistical data analysis

All the experiments were carried out in duplicate or triplicate. Microsoft Excel presented the mean ± standard deviation (SD) of the findings. An analysis of variance (ANOVA) was performed using IBM SPSS Statistics software to compare the means.

4.3. Results and Discussion

As most measurements were conducted in duplicate, the statistical analysis provides an indication of treatment-related differences; however, the limited number of replicates may reduce robustness of the statistical interpretation. Therefore, the statistical outcomes should be viewed as supporting the experimental trends rather than as the sole basis of interpretation

4.3.1. Effect of gelation on the rheological properties

4.3.1.1. Frequency sweep

The viscoelastic response of all samples as a frequency-dependent property is shown in Figure 18.

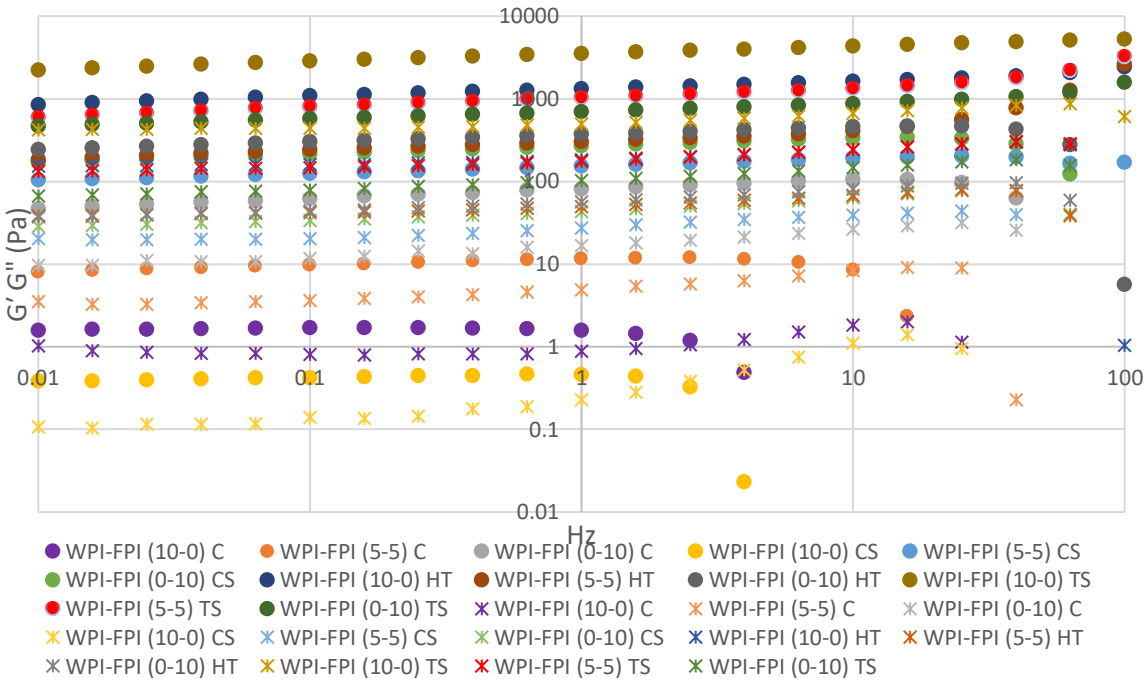


Figure 18. Storage modulus (G′) and Loss modulus (G″) as a function of frequency

A constant strain of 1% was applied to all frequency sweep measurements to ensure that the strain of 1% was in the linear viscoelastic region. The presence of GDL reduces electrostatic repulsion between protein molecules, thereby bringing them closer together (Liu et al., 2019). Most of the control and cold-sonicated gels of all ratios were dependent on the frequency, indicating a weak gel structure (Sun & Arntfield, 2012; Yang et al., 2022). Both G′ and G″ of all samples were plotted in Figure 18 for better comparison of gel strength among all samples. All control gels show the lowest G′ compared to others. After the frequency of 63.06 Hz, G″ becomes less than G′, which explains the weak structure of the gels. Similar findings were noticed in the case of soy protein isolate (Tang et al., 2011). Cold-sonicated gels with more WPI concentration also showed low G′, and G′ increased with an increase in FPI concentration. It has been observed that heat is required to break the cross-linking of inter- and intra-molecular bonding in the case of WPI, allowing bonds to form (Errington & Foegeding, 1998; Havea et al., 2009). It has been previously observed during the cold gelation of soy protein that plant protein forms an independent network regulating the gel network (Schmitt et al., 2019). Cold-sonicated WPI: FPI (5:5) has a lower G′ (157.132 Pa) at 1 Hz than WPI: FPI (0:10), which has a G′ of 266.02 Pa at the same

frequency, indicating the gel network between the plant proteins, as shown in Figure 19. The ratio of G″/G′ is not high, indicating that the gels formed are still weak.

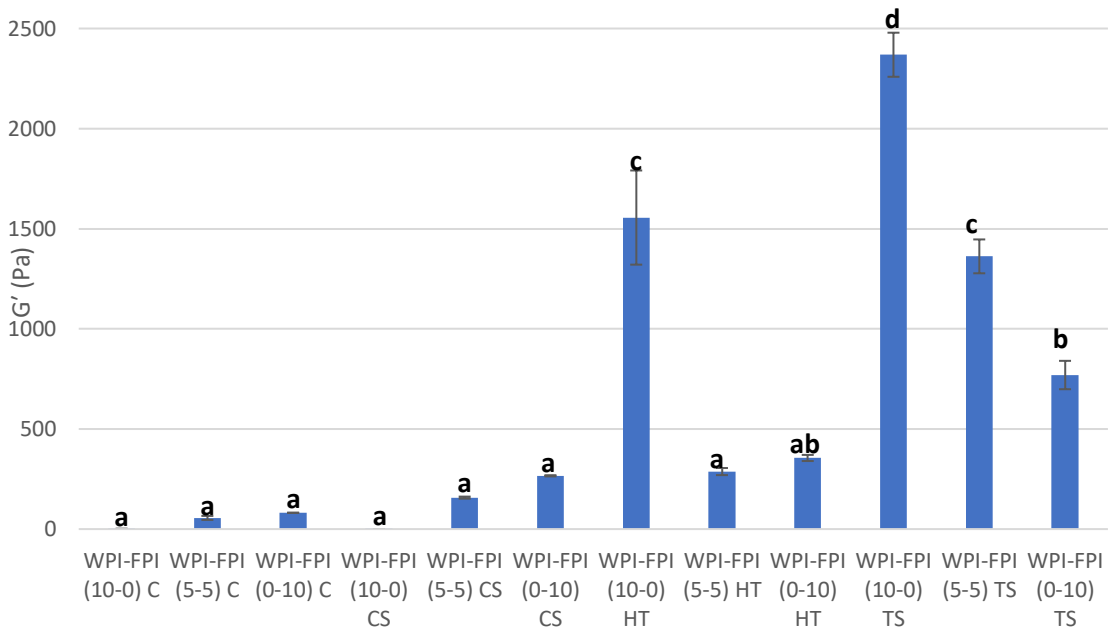


Figure 19. Changes in G′ (Storage modulus) for the different ratios and treatments. The letters in the columns above indicate significant differences in storage modulus G′.

Heat-treated gels with higher WPI concentrations form stronger gels, with G′ increasing and G″ decreasing with increasing frequency. This increase can be due to cross-linking of inter- and intra-molecules by covalent and non-covalent bonds (Errington & Foegeding, 1998; Havea et al., 2009). Heat treatment had little effect on gels with higher FPI concentration. A similar trend has been observed for the quinoa protein isolate (Patole, 2022). It has been suggested that covalent bonds, such as disulfide bonds, do not play a significant role in plant protein linkage, which might lead to the weaker gels (Ruiz et al., 2016; Yang et al., 2022).

Thermosonicated gels showed the strongest storage modulus among all treated samples. Gels with higher WPI concentrations showed greater elasticity. Thermosonication shows a higher effect due to its cavitation process (Zhong & Xiong, 2020). It has been observed that FPI gels were relatively weak. It has been reported that acoustic cavitation may have over-

exposed the molecules, leading to the breakdown of protein chains, which in turn disrupts protein association (Hu et al., 2013; Khatkar et al., 2020).

4.3.1.2. Strain Sweep

Strain-sweep measurements were used to study the large-deformation properties of all samples. It was observed that at the slight strain, there was a linear viscoelastic region showing that G′ and G″ were constant with increasing strain, except for WPI: FPI (10:0) cold-sonicated gel, as shown in Figure 20. Increasing the strain amplitude further leads to a decrease in both G′ and G″, suggesting that the gel shows thinning behaviour (Hamedi et al., 2022).

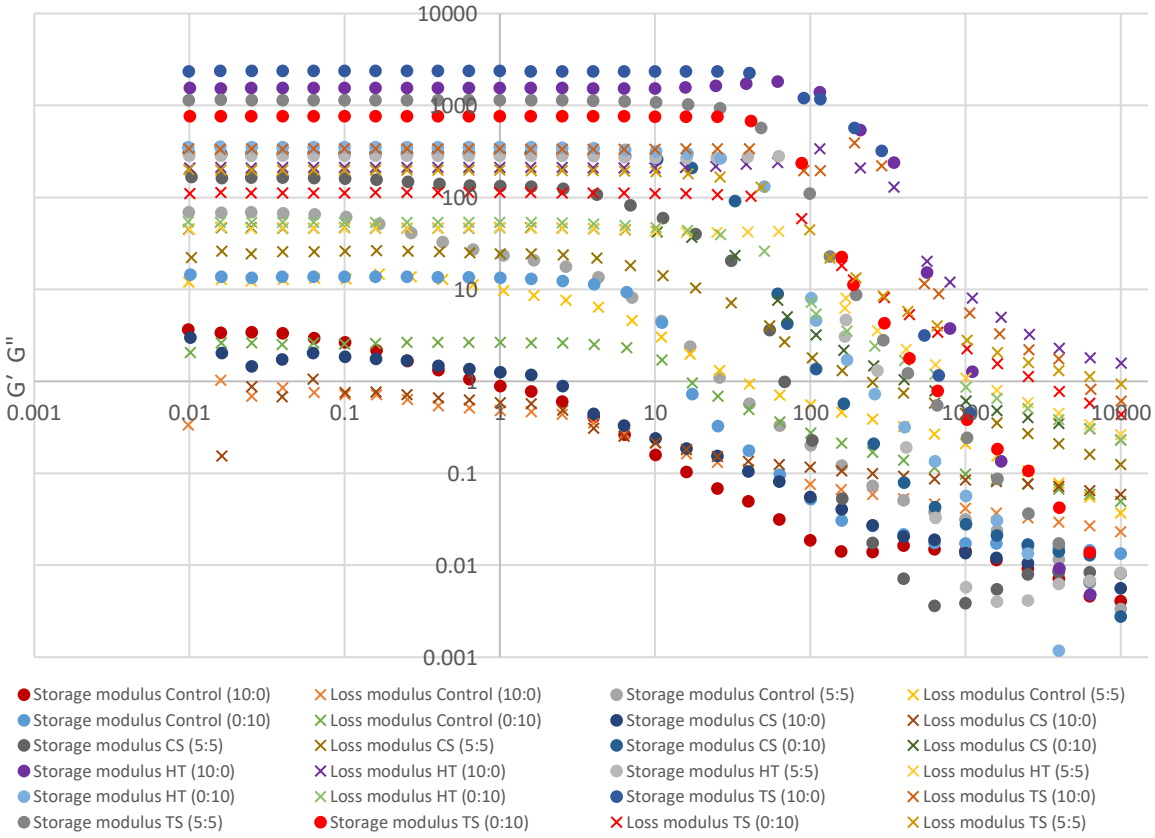


Figure 20. Loss modulus (G″) and storage modulus (G′) as the function of strain amplitude

A crossover point, where G′=G″ was observed, is called breaking stress. After this point, G″ becomes greater than G′, leading to the flowing of the samples because of the structural disintegration of the gels (Yang et al., 2016). Even for the strongest gels, this behaviour was observed. Breaking stress was plotted against breaking strain in Figure 21, which showed that the breaking stress of heat-treated WPI: FPI (10:0) was 14396.89 Pa, and for thermosonicated WPI: FPI (10:0) was 6490.91 Pa, thus making the heat-treated WPI: FPI (10:0) the strongest gel among all samples.

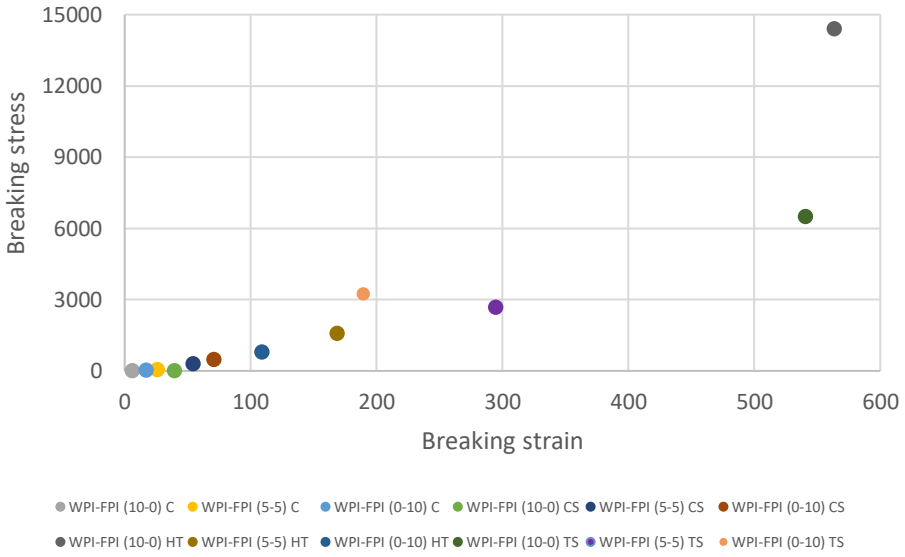


Figure 21. Breaking stress and breaking strain as a function of the protein concentrations of the WPI and FPI.

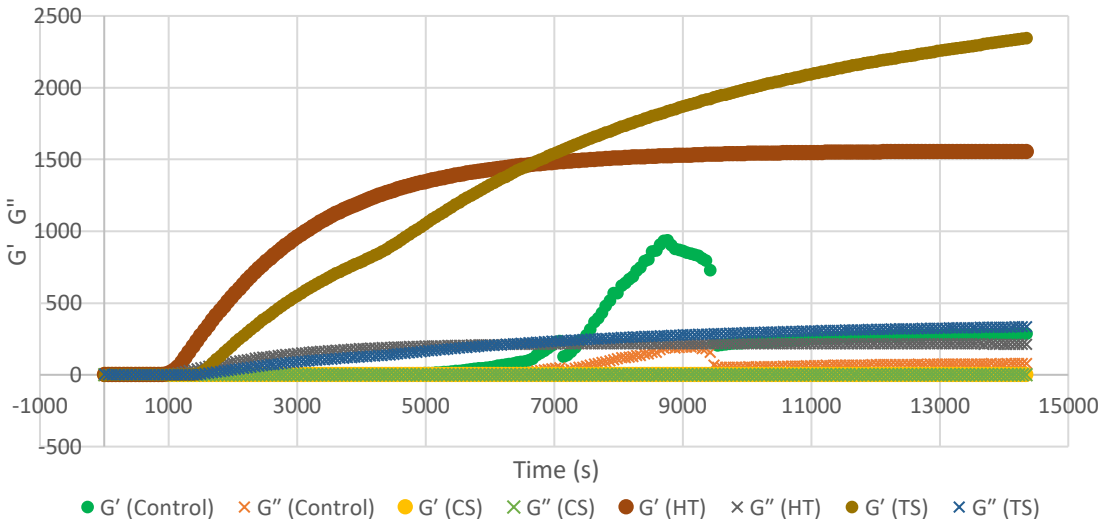
4.3.1.3. Time Sweep

The viscoelastic properties of all samples were measured by using small-deformation rheological measurements. Figure 22 shows the behaviour of storage modulus and loss modulus as a function of time at a temperature of 20 °C for 4 hours. The time-sweep results are presented according to WPI: FPI ratio as Figure 22A-C, representing 10:0, 5:5 and 0:10.

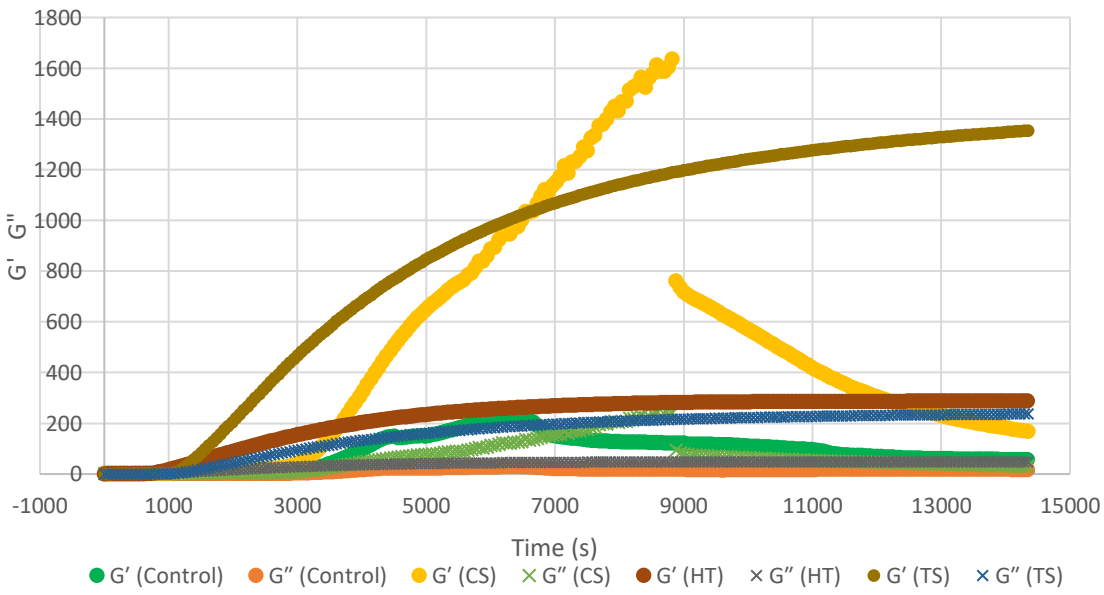
It has been noticed that with time, G′ of WPI: FPI (10:0) TS kept on increasing, explaining the continuous inter- and intra-linking using the covalent and non-covalent bonds, while G″ remains low with a very slow growth. For WPI: FPI (10:0) HT, G′ with time remains steady, which could be due to the possible hydrogen bonds and van der Waals interactions already

in place (He et al., 2021). A sudden increase in G′ was seen between ~15 and 30 minutes, suggesting a strong electrostatic repulsion between the molecules, leading to the increase in solubility. G′ of WPI: FPI (5:5) and WPI: FPI (0:10) TS also showed a gradual increase, suggesting that the cavitation process of thermosonication has helped molecules to open up for bonding, but this has less effect on the sample with a higher FPI.

WPI: FPI (10:0)



WPI: FPI (5:5)



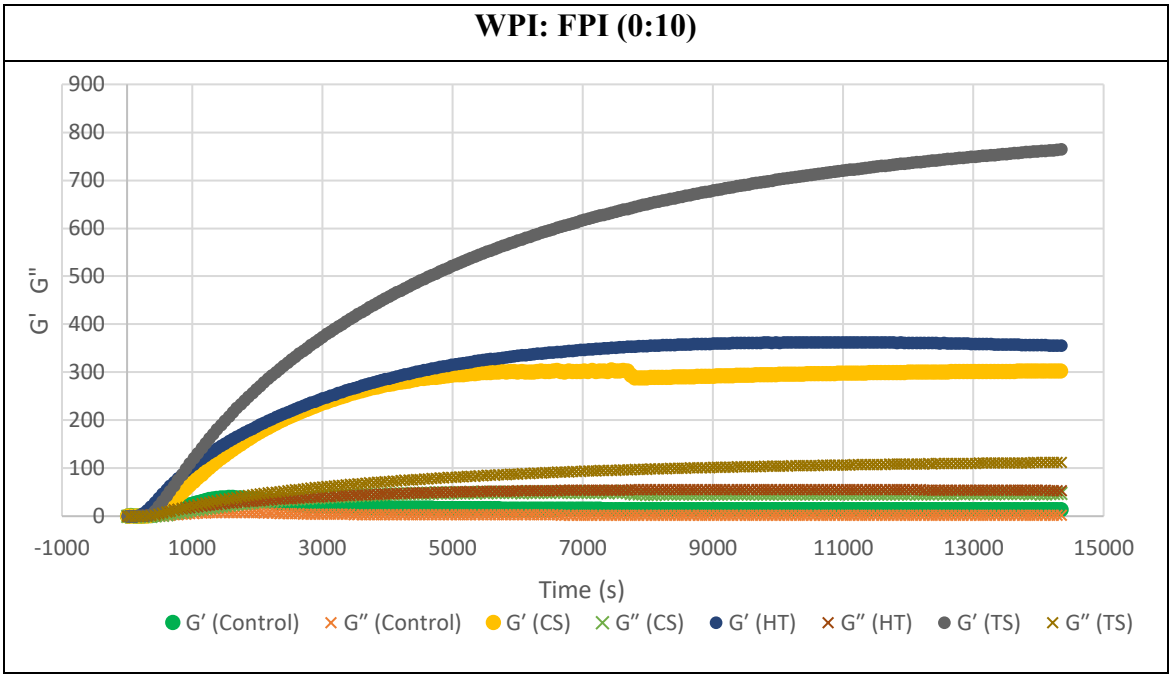


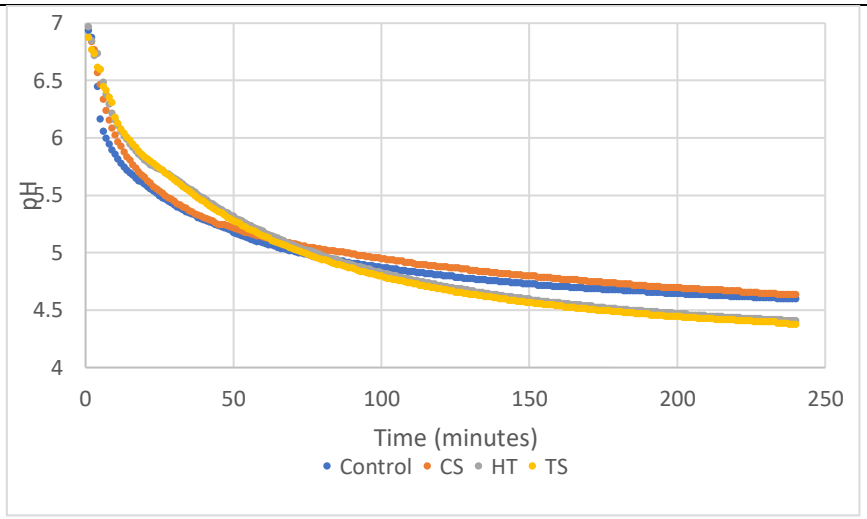
Figure 22. Time-dependent changes in storage modulus (G′) and loss modulus (G″) during GDL-induced gelation of WPI: FPI systems at different ratios: (A) WPI: FPI (10:0), (B) WPI: FPI (5:5), and (C) WPI: FPI (0:10). Solid lines represent G′ and dashed lines represent G″. Measurements were conducted at 20 °C for 4 h.

4.3.2. pH profile after the addition of GDL into the samples

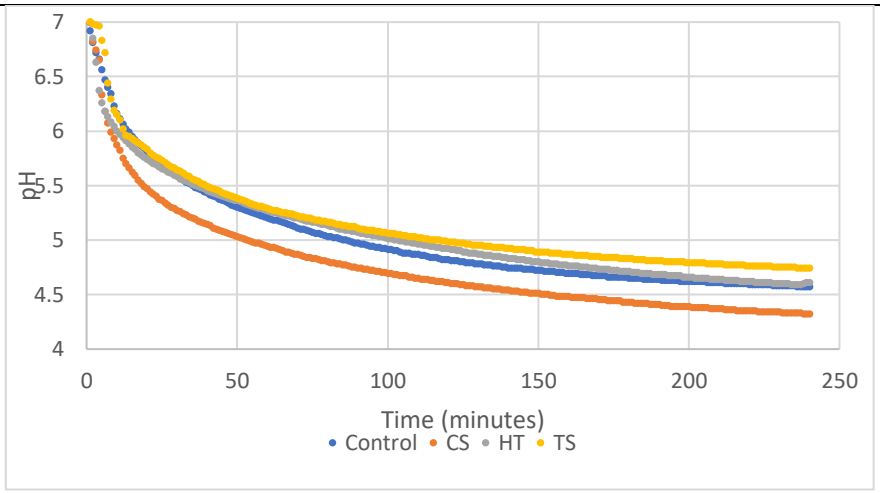
Figure 23 shows the effect of the GDL on the pH of the samples. The pH of all samples decreases sharply for 2 hours and stabilises thereafter.

GDL has been used to make acid-induced gels in plant protein and has shown promising gel characteristics in soy protein (Wan et al., 2021). The presence of GDL lowers pH, reducing repulsions between protein molecules and helping them form bonds.

WPI: FPI (10:0)



WPI: FPI (5:5)



WPI: FPI (0:10)

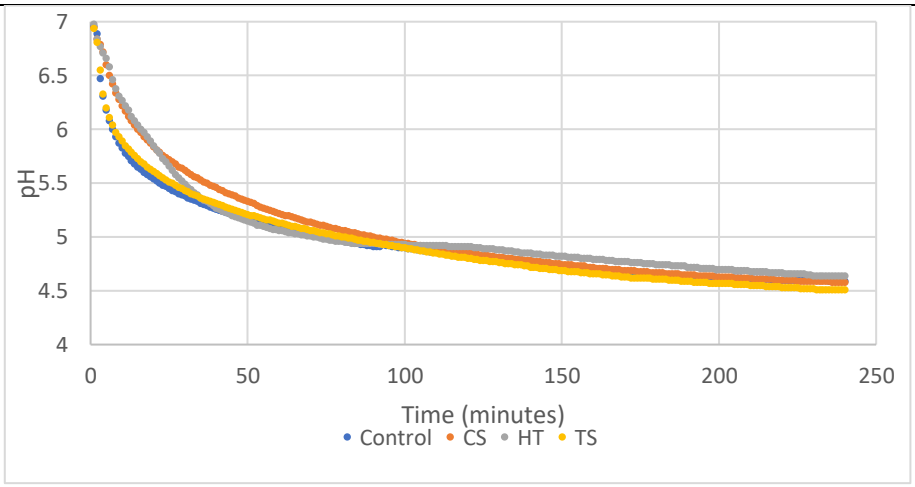


Figure 23. Changes in pH during GDL-induced gelation of WPI: FPI systems at different ratios: (A) 10:0, (B) 5:5, and (C) 0:10, over 4 h at 37 °C.

4.3.3. Confocal laser scanning microscopy observations

CLSM was conducted to understand the microstructure of the gels. The proteins were stained green by fast green, while the voids were black. CLSM photos from Figure 24 show no gel network for the control and cold-sonicated samples. Some large protein structures show a deformed gel for the cold-sonicated pure WPI, aligning with this sample's low G′ value.

The heat-treated WPI gel exhibits the strongest network, which weakens as the FPI concentration increases, leading to a deformable gel structure. It has been observed previously that heating promotes protein-protein interactions, resulting in stronger gels (Molina & Ledward, 2003). The thermosonicated gels, overall, have a stronger gel network than the others; however, the structure weakens again with increasing FPI concentration. Similar trends have been observed where large porosity has been observed when the FPI solutions undergo thermosonicated treatment (Hu, Cheng, Gilbert, Loo, et al., 2024).

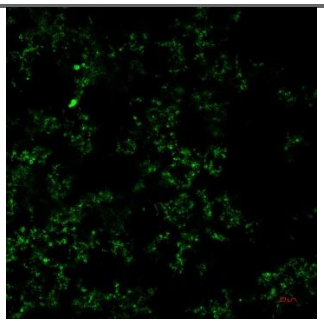
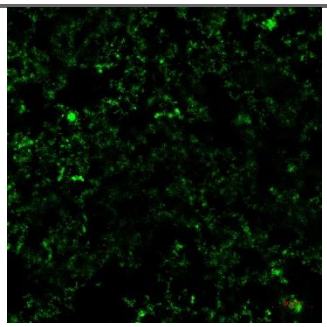
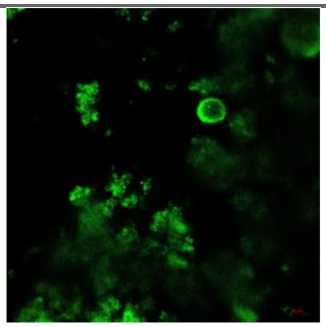
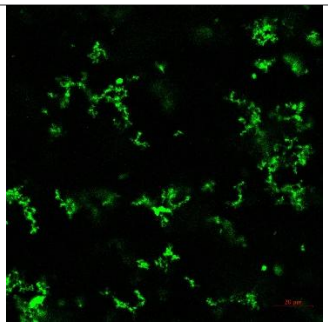
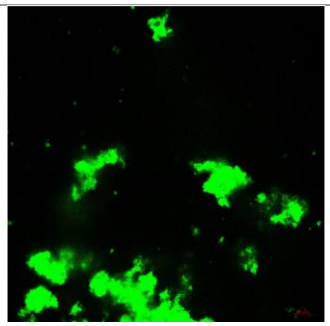
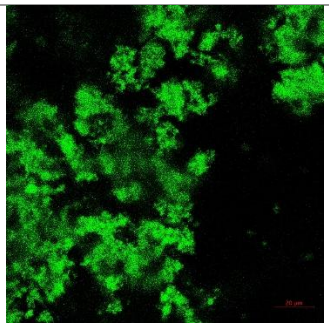
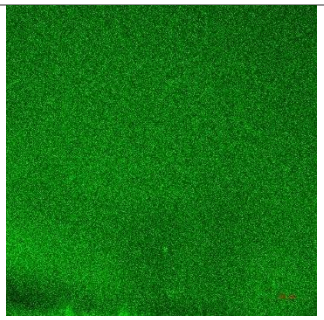
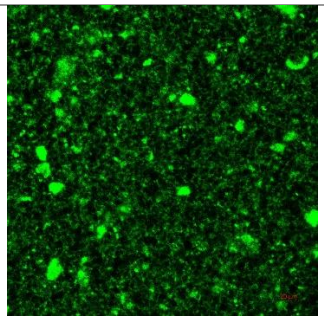
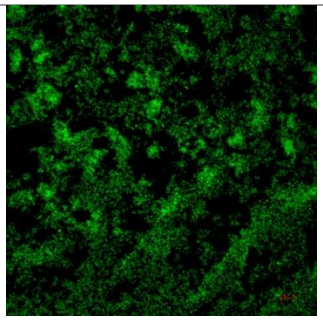
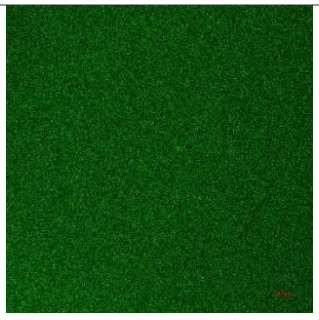
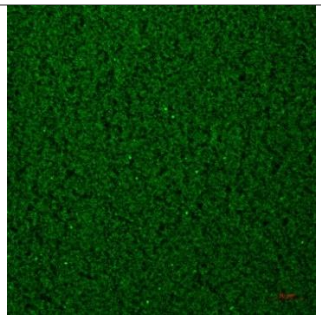
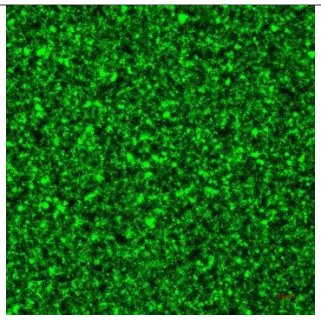
	WPI: FPI (10:0)	WPI: FPI (5:5)	WPI: FPI (0:10)
Control			
Cold-sonicated			
Heat-treated			
Thermosonicated			

Figure 24. Microstructure of the gels formed by different treatments

4.3.4. Water holding capacity (WHC)

The water-holding capacity was measured to assess the strength of the gels. The higher the percentage of water retained, the higher the water holding capacity, which means stronger gels, as WHC is related to the compactness of the gel's structural arrangements (Cortez-Trejo et al., 2021; Zhuang et al., 2020) . Only the samples that formed gels were used to conduct this analysis, as shown in Figure 25.

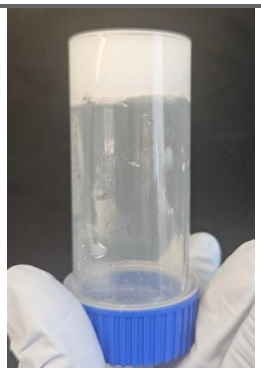
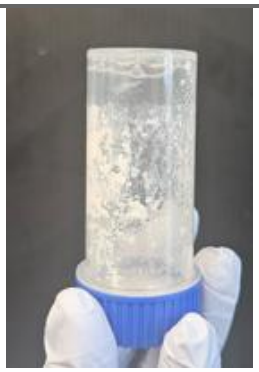
Sample	WPI: FPI (10:0)	WPI: FPI (5:5)	WPI: FPI (0:10)
Heat-Treatment			
Thermosonication			

Figure 25: The heat-treated and thermosonicated gels with different WPI:FPI ratios (10:0, 5:5, and 0:10)

The pure WPI gels, both heat-treated and thermosonicated, exhibit the highest water retention of ~97%, which aligns with their high storage modulus, as shown in Figure 26. GDL, when added to the WPI: FPI (10:0), both heat-treated and thermosonicated, entraps

water tightly, which is not easily disrupted by centrifugal forces. This enhances the formation of a protein gel network, resulting in a firmer texture and improved structural integrity.

WPI: FPI (5:5) and WPI: FPI (0:10) heat-treated gels exhibit lower water-retention percentages than thermosonicated gels. Heat-treated gels also show a lower storage modulus than thermosonicated gels, indicating that they are weaker.

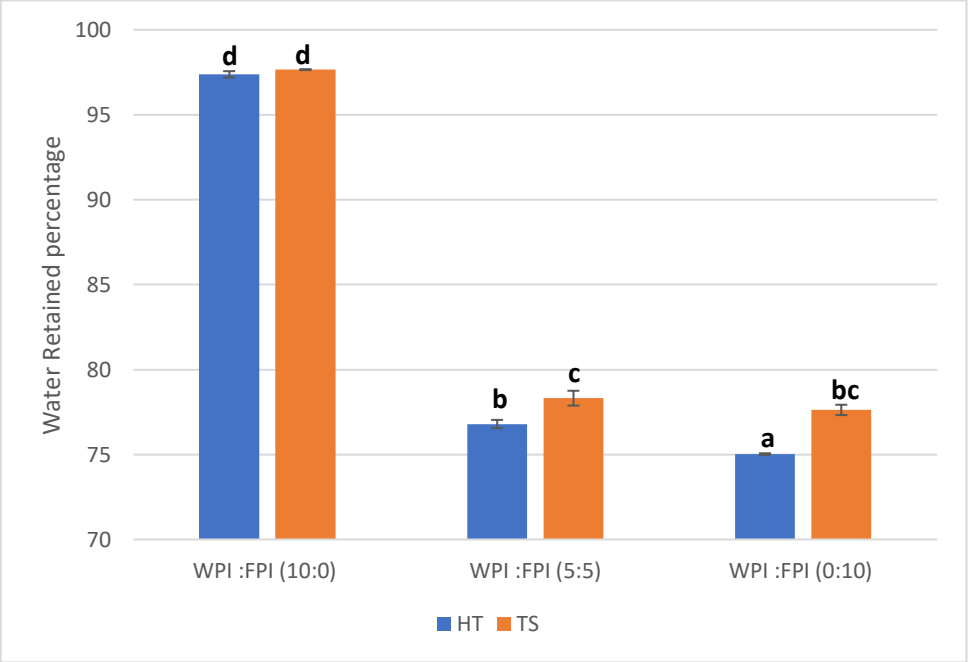


Figure 26. Water-holding capacity is shown as the water retained percentage for the gel samples. The letters in the columns above indicate significant differences in water-holding capacity.

4.4. Conclusion

This study confirmed that Thermosonication had a significant effect on the gelation behaviour of FPI, WPI, and their blends, as compared to cold sonication and heat treatment. Compared with heat treatment, thermosonication resulted in a higher storage modulus, a denser gel network, and improved water-holding capacity. These improvements result from

the combined effects of heating and cavitation during thermosonication, which lead to protein unfolding and the exposure of hydrophobic sites, thereby facilitating the formation of a stronger gel network. The addition of GDL promoted acid-induced gelation, lowering the solution pH and reducing repulsions between protein molecules. This decrease helped the protein molecules bond with each other.

Rheological properties showed that thermosonicated pure WPI had the highest storage modulus (2369 Pa), followed by heat-treated pure WPI (1555 Pa). Thermosonication increased the storage modulus of pure FPI (769 Pa) compared with the other treatments, which can be attributed to the limited number of disulfide bonds in plant proteins.

CLSM images showed a delicate, interconnected stranded gel network in pure WPI gels, but this weakens as the FPI concentration increases, indicating reduced connectivity. All samples exhibited high water-holding capacity, with thermosonicated WPI: FPI (10:0) having the highest value of 97.65%, indicating strong water binding that also resisted centrifugal disruption. WHC is vital to maintain the gel structure, as high WHC helps prevent syneresis and promotes shelf stability.

Overall, thermosonication has been shown to significantly enhance the gelation behaviour of WPI, WPI: FPI blend and pure FPI. This understanding can guide the formulation of hybrid protein gels that can partially replace WPI, providing an eco-friendly solution.

Different gelation pHs for both proteins can pose a challenge during gelation, but other treatments before gelation would be of interest in the future. Future studies can also explore different ultrasound powers, temperatures and times to optimise the gelation behaviour of FPI-rich solutions.

5. Conclusion and future work recommendations

5.1. Conclusion

The study aimed to address sustainability concerns among consumers and utilise the exceptional functional properties of milk proteins and the eco-friendly nature of plant protein to reduce the environmental impact of food production. The development of hybrids made from whey protein isolate (WPI) and faba bean protein isolate (FPI) were explored. It examined the emulsifying and gelation properties of these proteins, both individually and as blends, to understand how they can be combined to mimic the functional properties of WPI. Three treatments were applied: thermosonication, heat treatment, and cold sonication, which were compared with each other and with the untreated samples.

The study was divided into two parts, and its findings are summarised below:

The first part of the study investigated the proteins and their emulsifying properties at different WPI: FPI ratios (0:10, 8:2, 5:5, 2:8, and 0:10) by examining their rheological and microstructural properties. The particle size of the protein molecules was analysed using a Zetasizer, which showed that thermosonication effectively reduced the particle size of samples rich in FPI. However, heat treatment was the most effective for WPI. Heat treatment effectively decreased the zeta potential of all samples; however, thermosonication and cold sonication were less effective. Both heat treatment and thermosonication resulted in the structural rearrangements observed by SDS-PAGE analysis. In the details, SDS-PAGE showed that heat-treated WPI samples had clearer bands at lower molecular weights and reduced intensity in the higher-molecular-weight region, explaining partial unfolding and protein rearrangement. On the other hand, for FPI-rich samples, legumin dissociated into its smaller subunits during thermosonication and heat-treatment, confirming that these treatments disrupted covalent bonds, thereby improving emulsification behaviour.

Pure WPI showed the lowest turbidity, with thermosonicated WPI being the most transparent. With increasing FPI concentration, thermosonication was very effective at decreasing the sample's absorbance at 600nm. Low turbidity is crucial because it indicates reduced aggregation and smaller particles, thereby allowing proteins to be more soluble. This leads to improved emulsification, which is very important for the products which

require smoother texture and clarity, such as beverages. As FPI concentration increased, turbidity also increased, but thermosonication reduced the turbidity of the FPI-rich samples. Reduction in absorbance at 600nm means fewer larger aggregates, which leads to finer droplet size, which is an essential property for products like dressings, sauces and dips.

An emulsion containing 70% (w/w) soybean oil was designed to test the emulsification efficiency of the individual protein and their blends. The oil droplet size showed the most reduction by heat treatment in WPI-dominant samples; however, as the FPI concentration increased, thermosonication became more effective. Storage modulus (G′) of the pure FPI emulsion was the highest, explaining its elasticity. Cold sonication also showed promising results in samples with high FPI. A frequency sweep of the thermosonicated pure FPI showed that the samples retained their structure even at 100 Hz. Similar results were observed during the strain sweep: the thermosonicated samples showed the most significant resistance with increasing oscillatory strain %.

Based on all the results, WPI showed strengths such as low turbidity and smaller particle size, thereby enabling the formation of finer, more stable emulsions. FPI contributes towards the thickness, while WPI's contribution is less, but FPI has poor solubility, high turbidity and aggregations, which become its limitation. The blends solved this problem to some extent, but with FPI-rich blends, reduced clarity and increased turbidity limit their suitability for beverage applications.

From a product development perspective, WPI-rich ratios are suitable for products that prioritise clarity and low viscosity, such as protein drinks. Blends are best suited for medium-viscosity systems such as sauces and glazes. FPI-rich emulsions are more suitable where high viscosity is preferred, such as dips and thick sauces like mayonnaise. Heat treatment is the most effective treatment for WPI-rich solutions, but as the FPI concentration increases, thermosonication becomes increasingly effective.

The second part focused on the gelation properties of three WPI: FPI ratios (10:0, 5:5, and 0:10). It was observed that heat-treated and thermosonicated gels with higher WPI concentration showed higher storage modulus, indicating more elastic gels. Thermosonication has been demonstrated to be the most effective method for enhancing the storage modulus of FPI-rich gels. Breaking stress of the gels showed that the heat-treated WPI: FPI (10:0) is the most potent gel among all samples. Time-sweep analysis showed that the storage modulus of thermosonicated WPI: FPI (10:0) increased over time, whereas that

of heat-treated WPI: FPI (10:0) remained constant. Confocal laser scanning microscopy, in alignment with the other results, showed that the heat-treated WPI: FPI (10:0) exhibited the strongest gel network, followed by the thermosonicated WPI: FPI (10:0) gel. The water-holding capacity was measured to assess the strength of the gels, which showed values above 95% for the heat-treated and thermosonicated WPI: FPI (10:0) gels.

These results helped identify the suitability of the WPI, FPI and blend gels on the food systems. Heat-treated pure WPI formed an elastic, firm gel, suitable for applications requiring structure and firmness, such as custards, mousse, and high-protein set yoghurts. Whereas pure FPI gel was a bit weaker, making it unsuitable for the products where shape retention and firmness are desirable; however, it will be suitable for spreads and sauces. Blended gels show intermediate behaviour, which may be useful for some semi-solid products, such as spoonable dressings. Heat-treatment was most effective on WPI and WPI, whereas thermosonication is recommended when the FPI concentration increases.

5.2. Future work recommendation

Based on the findings above, the future work recommendations are:

- Different settings for thermosonication, like power, time and temperature, should be explored to get a better understanding of which setting is the most suitable in increasing the functionality of the hybrids.

- Other treatment methods, like pH shifting, can be incorporated with the thermosonication to get better unfolding of the FPI. It would be beneficial to understand the effects of other treatments, such as high-pressure homogenization, on the formation of these hybrids.

- It would be good to understand how different treatments affect the solubility of the hybrids and compare it with the pure samples.

- It has been noticed that pH plays an essential role in the formation of the gel. In this study, only GDL was used to form gels at pH 7. It would be of great interest to study the effects of salts such as NaCl and CaCl2 on the gel formation of the hybrids at pH 3, 5, and 9. Gelation can also be induced using polysaccharides such as locust bean gum, xanthan

gum, guar gum, starch, gelatin, kappa-carrageenan, and iota-carrageenan. It would be interesting to see the effects of these polysaccharides on gel formation. Differential scanning calorimetry or Fourier-transform infrared spectroscopy was conducted to fully understand the unfolding and denaturation of the protein's structure.

- Techniques like TEM and SEM could be used to understand the microstructures of the hybrid gels.

- The molecular forces, including covalent and non-covalent forces, interactions during the gel formation can be understood better by measuring rheological properties or the protein solubility in the presence of SDS, urea, 2-mercaptoethanol and thiourea.

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7. Appendix

Appendix A

Table 1: Nutritional profile of the faba bean protein isolate

Name	Ave Quantity Per Serving	Ave Quantity Per 100g
Energy	522kJ	1670kJ
Calories	125	399
Protein	26.5g	85g
- Gluten	0g	0g
Fat - total	1.7g	5.4g
- Saturated	0.5g	1.2g
Carbohydrate	1.2g	3.8g
- Sugars	<0.5g	<1g
- Fibre	<0.5g	<0.5g
Sodium	91mg	290mg

Table 2: Nutritional information of the WPI used

NUTRITIONAL ANALYSIS	TYPICAL (per 100g of product)
Energy	1602 kJ
Calories	383 kcal
Fat	0.9 g
Energy from fat	33 kJ
Calories from fat	8 kcal
Cholesterol	18 mg
Saturated fatty acids	0.6 g
Trans fatty acids	0.04 g
Total Carbohydrate	0.3 g
Added sugars	0 g
Lactose	0.3 g
Dietary Fibre	0 g
Ash	1.6 g

MINERAL ANALYSIS	TYPICAL (per 100g of product)
Aluminium	0.03 mg
Cadmium	0.0005 mg
Calcium	79 mg
Chloride	16 mg
Copper	0.03 mg
Inorganic Phosphate	119 mg
Iodine	0.02 mg
Iron	0.2 mg
Magnesium	10 mg
Manganese	0.01 mg
Potassium	33 mg
Selenium	0.02 mg
Sodium	539 mg
Sulphur	1430 mg
Total Phosphorous	36 mg
Zinc	0.1 mg

VITAMIN ANALYSIS	TYPICAL (per 100g of product)	
Vitamin A	0	µg
Vitamin C	0	mg

Appendix B

Table 3: Amino acid profile of the FPI used for the study:

Typical Amino Acid Profile

(mg per serving - unflavoured)

Branched chain aminio acids:

Isoleucine	1010
Leucine	1750
Valine	1220

Other essential aminio acids:

Lysine	1460
Methionine	175
Phenylalanine	960
Threonine	880
Tryptophan	140

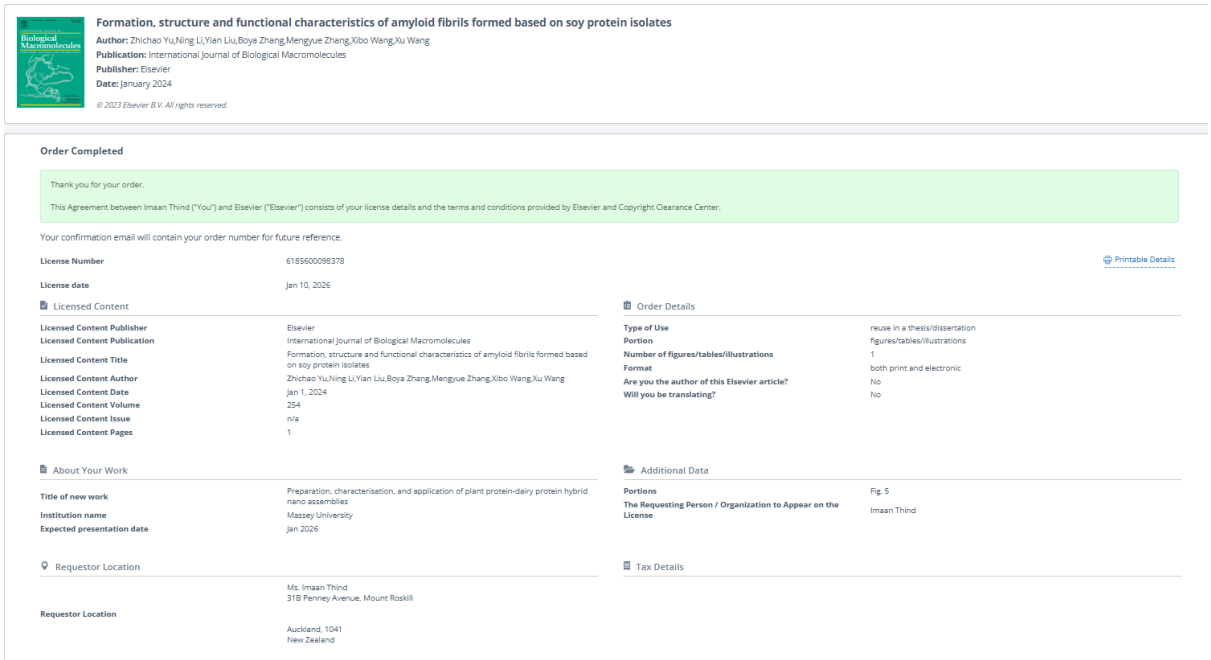
Non essential aminio acids:

Histidine	580
Alanine	1010
Arginine	2070
Aspartic Acid	2440
Cysteine/Cystine	180
Glutamic Acid	3720
Glycine	960
Proline	980
Serine	1190
Tyrosine	800

Table 4: Amino acid profile of the WPI used in the study

AMINO ACID PROFILE	TYPICAL (g/100g of protein)
Essential Amino Acids	
Isoleucine	6.3 g
Leucine	14.3 g
Lysine	11.2 g
Methionine	2.4 g
Phenylalanine	3.8 g
Threonine	5.3 g
Tryptophan	2.4 g
Valine	5.6 g
Non-Essential Amino Acids	
Histidine	2.0 g
Alanine	5.7 g
Arginine	3.0 g
Aspartic acid	12.5 g
Cysteine/Cystine	4.0 g
Glutamic acid	17.6 g
Glycine	1.8 g
Proline	4.5 g
Serine	4.5 g
Tyrosine	4.2 g

Appendix C



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