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Sensory and techno-functional properties of Alkaline solubilized-bacterial acidified pea protein isolate.

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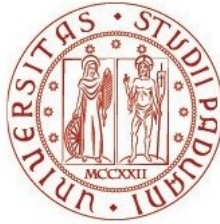
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Proprietà sensoriali e tecno-funzionali dell'isolato proteico di pisello solubilizzato alcalino e acidificato con batteri.

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Acknowledgments

Dedication

This thesis is dedicated to my parents, in-laws, sister Akansha and best friend (my better half) Aparna for supporting me every day of my life.

Abstract

Aim:

The use of plant proteins in food applications and meat alternatives is drastically increasing nowadays. Pea protein contributes a major proportion among them after soya protein. It has a good quality soluble protein profile, with one major limitation that is off-flavour compounds. The conventional alkaline solubilization and acidic precipitation method (AS-APM) is well-known among other methods for pea protein extraction with high protein recovery and yield. However, off-flavours still exist in the finished product. This problem could be potentially solved by fermentation, as it uses the natural power of bacteria for degradation or modification of off-flavour molecules.

Traditionally, a strong acid like HCl is used for protein precipitation in AS-APM. In this research we aimed to replace the chemical acidification step with fermentation by lactic acid bacteria (LAB) during processing and thereby reduce the off-flavours in the pea protein isolate.

Method:

Fermentations were performed by two starter cultures: 1) having a fast acidification rate and 2) having the ability to reduce the beany off-flavour. The target functional group for beany-ness is aldehyde (hexanal, nonanal). The volatile compounds were assessed by GCMS; 16s rRNA sequencing was used for detecting major contributing strains in fermentation; the degree of protein degradation was measured by HPLC-SEC; the sensory and techno-functional properties were also evaluated.

Results:

In the fermented samples, *Streptococcus thermophilus* or *Pediococcus pentosaceus* were the dominating LAB strains. The Pediococcus fermented sample had a stronger pea taste and odour reduction, and, interestingly, bitterness was also reduced significantly from 6.1 to 2.2 (sensory scale 0–9) compared to the conventional AS-APM sample. A greater amount of higher molecular mass protein fraction and slightly higher peptide content were observed

in the fermented samples. Fermentation enhanced water holding and foaming capacities and decreased the water solubility index. Unsubstantial changes were observed in other functional attributes.

Conclusion:

The study showed that LAB fermentation can be used for pea protein precipitation and can simultaneously greatly reduce typical off-flavours. The changes in off-flavour profile and techno-functional properties depend on the used starter culture.

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1 Introduction

The sustainability of food production has become a key topic in global discussions recently. With the world population expected to reach 10 billion by 2050, our current dietary habits and food production methods will not be able to support this population size (Willett et al., 2019). Producing animal-based protein presents several difficulties, including inefficient resource use, waste generation, and concerns about animal welfare. These problems can be significantly lessened by directly using plant proteins. Adopting a diet rich in plant-based foods is seen as a way to enhance human health and make supply chain more sustainable. Thus, the use of plant proteins in food applications and meat alternatives is drastically increasing nowadays. Substituting animal proteins with plant proteins in food products presents many technological challenges. Plant proteins typically have lower solubility, undesirable aromas, inferior colour and flavour, and reduced functionality compared to traditional milk proteins. Consequently, research has intensified to improve our understanding of the undesirable flavour molecules and functionality of plant-based proteins. Such research involves evaluating and comparing methods to reduce or eliminate compounds, like hexanal and nonanal, that cause undesirable flavours in these ingredients. Techniques used include direct steam injection, developing flavour maskers or modulators, and using lactic acid bacteria (LAB) fermentation to minimize off-flavours.

Pea protein contributes a major proportion among plant proteins after soya protein. It has a good quality soluble protein profile, with one major limitation that has off-flavour compounds. The conventional alkaline solubilization and acidic precipitation method (AS-APM) is well-known among other methods for pea protein extraction with high protein recovery and yield. However, off-flavours still exist in the finished product. This problem could be potentially solved by fermentation, as it uses the natural power of bacteria for degradation or modification of off-flavour molecules. There are two major stages in protein extraction method (AS-APM) where fermentation has been applied. First, before extraction

process where raw flour is fermented and second is at the end, where pea protein isolate is used as fermentation matrix. Both ways are well explored by researchers. Pei et al. (2022) reported a significant reduction in undesirable flavour in pea flour after fermentation with lactic acid bacteria. Shi et al. (2021) reported a reduction in off-flavour producing components after lactic acid fermentation of pea protein isolate. But both approaches are not economical options at commercial scale. There is still need of improvement for modification and optimizing the extraction process for effective removal of off-flavour molecules.

Traditionally, a strong acid like HCl is used for protein precipitation in AS-APM. This study aims to find an innovative way to modify precipitation step and reduce the off-flavour molecules. In this research we aimed to replace the chemical acidification step with fermentation by lactic acid bacteria (LAB) during processing and thereby reduce the off-flavours in the pea protein isolate. Fermentations were performed by various starter cultures and we selected the best two out of them: 1) having a fast acidification rate and 2) having the ability to strongly reduce the beany off-flavour. The target volatile group for beany-ness is aldehyde (hexanal, nonanal). The volatile compounds were assessed by GCMS; 16s rRNA sequencing was used for detecting major contributing strains in fermentation; the degree of protein degradation, the sensory and techno-functional properties were also evaluated.

2 Review of literature

Protein has become a major topic of discussion in the food industry in recent years. It is one of the main pillars of essential nutrients that our body requires. Plays a vital role in muscle development. In our diet, animal-based protein has major proportions, from livestock and seafood in the form of milk, yoghurt, cheese, eggs, and meat (chicken, beef, mutton, goat, pork, and fish). For the past 5-6 years, due to several sustainability reasons, protein intake from plant species has been recommended in the food industry. Plant-based proteins are sustainable as they require less water and other sources than livestock production, produce low methane emissions, thus have less carbon footprint on the environment and low contamination in food, thus reducing the risk of zoonosis. But plant protein has its limitations: the presence of antinutrients, off-notes in sensory, low PDCAAS score, and not being completely functional as an ingredient in the food industry. Nowadays, many industries produce protein concentrate or isolate from different plant sources commercially. However, there is still a need for optimisation, improvement, and modification in the extraction process, which will reduce the off-flavour notes and increase the protein yield and recovery to increase the market acceptability from an industry point of view.

2.1 Source of plant proteins

Plant-based proteins are derived from many plant species, such as soya, pea, rice, hemp, linseeds, legumes, seaweeds, etc. (Nadathur et al., 2016). These days, pulses and legumes like peas, lupins, lentils, and fava beans are most popular sources for protein extraction.

2.2 Peas

Peas are widely consumed legumes due to their high nutritional content, they contain carbohydrates, proteins, fibre, vitamins (folate and vitamin C), minerals (iron, magnesium, phosphorous and zinc), and lutein (a yellow colour carotenoid pigment that benefits vision).

Peas contain 59–70% (dry weight basis) carbohydrates (Arif et al., 2020), of which 39–46% are starch fractions (Raghunathan et al., 2017). They are rich in dietary fibres, ranging from

23–31%, out of which 4–8% are soluble fibres and 19–23% are insoluble fibres (Kan et al., 2018). The protein content varies between 20-25% (dry weight basis) (Shanthakumar et al., 2022), the lipid content ranges from 3–7% (Kan et al., 2018), and the total ash content is around 3% (Arif et al., 2020). The nutritional composition of crops varies depending on cultivar, environmental condition, and sowing year.

Pea protein can be classified majorly into 4 fractions based on its solubility: (1) salt-soluble globulin fraction; (2) water-soluble albumin fraction; (3) prolamins; they are soluble in a mixture of water and ethanol; (4) insoluble glutelin. Adebiyi et al. analysed different fractions of different solubility by SDS-PAGE. They observed that in non-reducing conditions pea protein isolates (PPI) had nine different polypeptides (68, 47, 35, 29, 25, 22,19,15, and 12 kDa), and the 35 kDa band had the largest proportion. Water soluble bands are observed at 21,15, and 11 kDa, while salt soluble fraction bands are observed at 43, 25, 21, 18, 12, and 10 kDa, with 25 kDa in the highest proportion. Alkali soluble fraction had seven different bands at 66, 45, 35, 28, 21, 18, and 12 kDa, and they had similar proportions as a polypeptide of pea protein isolates. Ethanol soluble fractions were not properly separated, but they were in the range between 50-75 kDa (Adebiyi & Aluko, 2011). The globulins are in the range 55–80%, and albumins are in the range 18–25%. Based on sedimentation coefficient globulin is classified majorly into 3 fractions: 11S (Legumin), 7S (vicilin), and con-vicilin. The hexameric protein fraction legumin (11S) comprises six pairs of subunits of molecular weight up to 60 kDa, which are linked non-covalently. Every pair consists of an acidic subunit of molecular wt. ~40 kDa and a basic subunit of ~20 kDa, linked via one or more disulfide bridges (Gatehouse et al., 1980). Vicilin consists of three subunits of 47–50 kDa. Some researchers marked con-vicilin as a tetrameric protein having molecular weight 290 kDa, other researchers mentioned it as a trimeric protein of molecular weight 210 kDa (Schmidt et al., 2022).

2.2.1 Protein health benefits

Pea protein is available in the market in different forms: pea protein concentrate (PPC), pea protein isolate (PPI), and pea protein hydrolysate (PPH). PPC has a protein content greater

than 50%, whereas PPI and PPH are purer and have high-quality protein greater than 80%. PPI and PPH differ in terms of processing and digestibility. For example, whey protein isolate is a milk protein that has been filtered, and most of the fat and lactose have been removed. Whereas hydrolysate is a more digestible protein than isolate. It has been pre-digested by enzymatic and other various treatments and made more biologically available to the human body (García Arteaga et al., 2020)(*Whey Concentrate vs Whey Isolate vs Hydrolyzed Whey*, n.d.). Pea protein isolates are good for people who are lactose intolerant.

Pea protein isolate is frequently used as a functional ingredient in the food and beverage market. It is commonly found in alternative meats, meal replacements, cereal bars, alternative milks, and other food products. There are no extensive findings on its health benefits to concur with its rising popularity. However, some existing research and evidence cover and connect known potential health benefits of PPI to the human body. Pea protein isolate showed anabolic properties, a glycemic-lowering effect, a high-satiety effect, a hypolipidemic effect, and a blood pressure-lowering effect (Stilling, 2020).

Pea protein fulfils the WHO's essential amino acid (EAA) content requirements, but methionine content is below recommendations (Gorissen et al., 2018). Specifically, considering branch chain amino acids, they have significant contributions to muscle anabolism; pea protein consists of a high amount of leucine, isoleucine, and valine, which are higher than in other plant-based proteins but in comparison to animal-based are still lower (Blomstrand et al., 2006; Gorissen et al., 2018). The protein digestibility corrected amino acid (PDCAAS) ratio of pea protein is 93% for adults, comparable to casein, eggs, and soya. In contrast, pea protein has an advantage over soy protein due to lower antinutrient content (phytates and saponins) and is thus preferred in the plant-based protein category (H. Yang et al., 2012).

2.2.2 Application in food products

Pea protein has gained vast interest in recent years as a functional ingredient in food applications. Its use primarily concentrates on bioactive molecule encapsulation, edible films, extruded products, addition in cereal flours for nutritional enhancement, substitution

for animal proteins. Nowadays, the use of pea protein isolates in high-moisture extrusion products is prevailing in research and industry as this application is applied in developing plant-based meat analogues due to its good functional properties (water-holding capacity and high water solubility). Industrial researchers are working on applying pea protein in the flour and dairy products without affecting the original texture and flavour of food products, and concrete research is still needed to implement the findings at the commercial level perfectly (Ge et al., 2020).

The function and stability of bioactive ingredients can be enhanced by encapsulating it with pea protein isolates. Aberkane et al. used pea protein isolate to microencapsulate the PUFA-rich-oil (rich in omega-3) through spray drying and showed improved oxidative stability of encapsulated oil (Aberkane et al., 2014). Another research group used commercially available pea protein isolates to encapsulate flaxseed oil (rich in α-linolenic acid (ALA)) by spray drying technique and reported high encapsulation efficiency at a 1:5 core-to-wall ratio (Bajaj et al., 2015). Shao and Tang et al. encapsulated β-carotene by PPI (at pH 3) using the Pickering emulsion technique and observed a controlled release of β-carotene with improved stability during the intestinal digestion process (Shao & Tang, 2016). Pea protein is also used to encapsulate vitamin D in nano-emulsions with improved cellular uptake efficiency (Walia & Chen, 2020).

In commercial food production, two types of extrusion techniques are widely used, which include low-moisture extrusion (LME, <35%) and high-moisture extrusion (HME, >40%). LME is generally used for production of extruded snacks and expanded low-moisture meat analogues and HME for production of fibrous high-moisture meat analogues. Pea protein usage in an extrusion process has extensively increased. The addition of it to snack recipes gives an advantage of higher nutritional content (good amino acid profile), benefit in digestion as an ingredient to develop low glycemic index food (López-Barón et al., 2018), and improved texture (Philipp et al., 2017) (Beck et al., 2018) in extruded products compared to products prepared from only starch (for example wheat, rice, and corn starch). In the case of HME, pea protein helps in the formation of a fibrous meat-like structure, but the fibrous structure also depends on different extrusion conditions (Osen et al., 2014, 2015).

2.2.3 Off-flavour issues with pea protein

Off-flavour compound issues are not only related to pea protein; they cover all plant protein sources. Off-flavour compounds in pulses can be volatile or non-volatile, but majorly, they are volatile. These compounds can be either inherited from pulses themselves or formed during processing. Legumes' most prevalent off-flavours are greeny, grassy, beany, bitter, and astringent (Viana & English, 2021). One of the key mechanisms and enzymes responsible is lipid oxidation by lipoxygenase. The lipoxygenase enzyme converts lipids to lipo-hydroperoxides (LHPs). On degradation, LHPs form volatile and non-volatile molecules during harvesting, processing, and storage (P. Shen, 2024). Another research group observed that in the case of fava beans, pea flavour was due to the presence of lipid-oxidation products like hexanal, heptanal, and nonanal. Bitterness and astringency were probably related to vicine, con-vicine, condensed tannins, and arginine (Wang et al., 2024). Non-volatile phenolic compounds like gallic acid, caffeic acid, and coumaric acid may be responsible for bitter taste. Xu et al. claimed that hydrophobic amino acids, tryptophan, isoleucine, and phenylalanine are responsible for bitter taste in protein hydrolysate (Q. Xu et al., 2019).

Benavides-Paz et al. studied aroma compound development during the alkaline extraction isoelectric precipitation method of pea protein isolates. They analysed the aroma compounds in raw pea flour and compared their concentration at each step in the extraction with that of the finished product (i.e., pea protein isolate). They concluded 3 major findings from their research work. First, they found 13 volatile compounds which probably contributed to aroma formation, and the results were also correlated with the sensory data. The second and third conclusion were that there was no new compound formation during the extraction process and no existing aroma compound was completely depleted, as concluded by olfactory analysis. The determined 13 compounds are listed in Table 1 with their respective odour description (Benavides-Paz et al., 2022). These compounds have their specific aromas which is acceptable in specific foods, but in the case of PPI, these compounds are off-flavours which are not acceptable by the consumers in the finished products.

Table 1. *Volatile compounds responsible for the flavuor of PPI (Benavides-Paz et al., 2022).*

No.	Compound	Description by panellists
1	(Z)-4-heptenal	oil, fatty, fishy, oxidized oil
2	2-octanol	grassy, musty, mouldy, earthy
3	(Z)-6-nonenal	raw cucumber, celery, beany
4	methional	raw potato
5	1-octen-3-ol	mushroom, brothy
6	2-isobutyl-3-methoxypyrazine (IBMP)	bell pepper, earthy, soil
7	(E)-2-nonenal	cucumber, nutty
8	butanoic acid	cheesy, spoiled milk, saliva
9	isovaleric acid	cheesy, sour, pungent
10	hexanoic acid	cheesy, pungent, rancid
11	maltol	sweet, caramel
12	unknown	woody, floral, sweet, toasty
13	(E)-2-octenoic acid	musty, mouldy, dirty

2.3 Off-flavour reduction strategies in pea protein

From an industry perspective, consumer acceptance decreases due to the off-flavour and off-taste of plant proteins. Widely used plant proteins like soy and peas have an off-flavour issue in the finished product.

There are many ways to reduce the off-flavours in plant protein. Based on the research database, off-flavour reduction strategies are divided into three ways: first, masking the off-flavour compounds present in the finished products by various masking agents. The second way is by degrading (by physical, chemical, or biological treatments) the off-flavour molecules during plant protein processing. The third approach is targeting the molecular source, or in common terms, by plant breeding methods. This approach is still confined in

research because only a few molecular targets are known as the source of origin of odorant molecules responsible for the pea's flavour profile (Trindler et al., 2022).

2.3.1 Masking agents in off-flavour reductions.

Various types of masking agents are used to mask off-flavour compounds in plant proteins, which cause beany, grassy, and bitter notes. Lan et al. tried to mask the beany notes in pea protein isolates using solid dispersion-based spray drying. Gum Arabic and maltodextrin were used as PPI carriers and cell wall encapsulating compounds or flavour masking agents. They observed that the amount of 1-pentanol and 1-octen-3-ol, which are beany flavour markers, were decreased by 3 folds and 2 folds compared to control PPI. This could be due to the unfolding of secondary structures of protein in PPI in combination with gum Arabic or maltodextrin (amorphous matrix carrier) in the spray drying process (Lan et al., 2019). Another research group, Wang et al., masked the off-flavours of fava bean protein with dextran, which was produced by fermentation by *Weissella confusa* or dextransucrase enzyme. They optimised the fermentation condition to produce low acid and adequate dextran to mask pea and bitter off-notes in end products. In the results they claimed that dextran has significantly masked the off-notes and bitterness in the end product and can be used as a potential masking agent (Wang et al., 2024). Sakai et al. used food-grade cyclodextrin glucanotransferase enzyme while preparing plant-based meat analogue patties, which produced cyclodextrin in patties. It was observed that enzyme-treated patties had significantly lower volatile compounds that are known as beany off-flavour-generating compounds compared to untreated patties and thus reported a potential clean label masking agent for plant protein (Sakai et al., 2022).

2.3.2 Off-flavour reduction during processing

Three types of treatment are used to degrade the off-flavour molecules during processing: physical, chemical, and biological. Physical methods involved thermal treatment, high-pressure treatment, and no-thermal methods, as well as ultrasonication, radio frequency, and pulsed electric field (PEF) treatment (Leonard et al., 2023). Xu et al. reported the decrease in off-flavour content at 95 °C observed the formation of large molecular weight

soluble aggregates in soya protein isolate. They also monitored that a temperature greater than 65 °C significantly enhanced the release of off-flavour molecules, favouring the Maillard reaction and completely depleting glycinin (J. Xu et al., 2024). The high-pressure treatment (HPT) has not been widely used for off-flavour reduction in plant protein, but a research study has observed that HPT has a significant effect on the reduction of lipoxygenase enzyme. In diced tomatoes, HPT at 400 MPa significantly reduced the lipoxygenase activity by 50%, reducing it to very low at 800 MPa pressure (Shook et al., 2001). HPT can enhance and retard the enzymatic and chemical reactions, which disturb the flavour composition and thus, HPT could result in unacceptable changes in flavour (Oey et al., 2008). In non-thermal methods, the application of ultrasonication and pulse electric field has been seen in the literature. Kong et al. reported the effect of ultrasonic treatment on soy protein isolate and revealed a decrease in beany flavour compound content (hexanal, (E)-2-hexenal, 1-Pentanol and 1-Nonanol) (Kong et al., 2023). Another group of researchers showed PEF application on pea protein isolate and claimed PEF improved the flavour profile of PPI (Guo et al., 2024). Chemical treatments are generally not preferable to alter flavour profiles as the market demands greener approaches.

The biological approach is directed to zymology. Zymology is the study of fermentation science and its biochemical pathways. It involves microorganisms, like bacteria and fungi, which are used according to desired output. Shi et al. showed a decrease in aldehyde volatile and an increase in alcohol volatile in lactic acid fermentation by *Lactobacillus plantarum* of pea protein isolate, which improves the taste of pea protein supported with sensory analysis (Shi et al., 2021). Schindler et al. reported a similar study on pea protein extract, which was pasteurised, acidified to pH 4.5 by HCl, and then inoculated with *Lactobacillus plantarum* L1047 and *Pediococcus pentosaceus* P113. Results revealed a significant decrease in undesirable aroma compounds in the end product (Schindler et al., 2012). There are many studies that have already been published. This approach has a wide range of research scope and needs to be explored at its maximum depth.

2.3.3   Breeding approach used in off-flavuor reduction.

Field pea is considered the oldest grain legume available nowadays. Therefore, many germplasms and cultivars are available. These variations can be used to develop new cultivars of field peas with higher nutritional content and cultivars with desired aromas (Parihar et al., 2020). There is very little research work available related to the breeding approach. Among the huge collection of cultivars, very few cultivars have been studied for the volatile profiles. It is difficult to compare the same cultivars from different years and cultivation sites as they significantly vary in specific volatiles (Azarnia, Boye, Warkentin, & Malcolmson, 2011; Azarnia, Boye, Warkentin, Malcolmson, et al., 2011; Malcolmson et al., 2014) (Trindler et al., 2022).

2.4   Fermentation: In the extraction process of plant proteins.

Using fermentation in the extraction at a specific processing step would be wise. This will save money and decrease the system's complexity, which can help better understand the mechanism of bacterial factories. Fermentation can be used at three different stages. For every stage, we will discuss the complexity of the base matrix, the amount of processing products to be handled, and expense estimation.

2.4.1   Before extraction

In this approach, pea flour is fermented with different bacterial or fungi strains to achieve higher nutritional content, enhance functionality, and improve the aroma profile. The base matrix for fermentation is pea flour, which consists of complex combinations of carbohydrates, starch, proteins, fibres, fat, vitamins, minerals, phytochemicals, and some antinutrients (Wu et al., 2023). This makes it difficult to understand the mechanism of microbes during fermentation. Though many publications have reported that off-flavour notes have reduced after pretreatment with fermentation, understanding the breakdown or degradation of off-flavour compounds and the formation of other flavour compounds is still lacking and not fully documented in research publications. Based on the literature and our preliminary experiments, extraction processes have a 15-20 % protein yield, which means that a large amount of raw material needs to be handled in the production unit, which

requires a big processing plant, a large workforce, and money to cover it all. When one thinks about the fermentation of raw material, these expenses will rise by 4-5 times or even more because, for fermentation, we need to add approximately 6 times the weight of flour and other things to maintain the required condition. Thus, this approach is unsuitable for commercial production in the industry, but results are acceptable at a small scale (Batbayar et al., 2023). Pei et al. reported a significant reduction in undesirable flavour in pea flour after fermentation with lactic acid bacteria (LAB) (Pei et al., 2022). Li et al. showed the effect of 5 different strains of lactic acid bacteria on yellow pea flour with respect to time. They observed that the aroma profile of fermented pea flour depends on strains and fermentation time and concluded that the *L. acidophilus* strain showed an improved aroma profile in fermented pea flour (Li et al., 2021). Some other studies also showed an improvement in the functional properties of the finished product (Batbayar et al., 2023; Byanju et al., 2021; Kumitch et al., 2020).

2.4.2   After extraction

In this approach, the extracted pea protein isolates or pea protein concentrate is fermented to reduce off-flavour compounds and enhance the protein's functionality. In the alkaline extraction-isoelectric precipitation method (AE-IP), the pea flour is solubilized at pH 7.5-9. Centrifugation separates the soluble albumin and globulin from starch, fibers, glutelin, and prolamin. In the second stage, globulins are precipitated by adjusting pH to 4.5-4.8 (Shand et al., 2007). Thus, pellets mostly contain globulins, whereas albumin is separated into supernatant (Schmidt et al., 2022; Tanger et al., 2020). In conclusion, after extraction from the AE-IP method, a major globulin fraction is extracted, which is one advantage of this method as the base matrix is simpler than pea flour for fermentation. Handling fermentation in this approach is convenient, and understanding the mechanisms followed by microbes is easy. But there is a disadvantage that base matrix lacks the essential nutrients for bacterial growth (simple sugars and amino acids) and energy production. At the laboratory scale, the researchers found a significant reduction in off-flavour notes and improved sensory results in final fermented pea protein isolates. Xiang et al. studied the fermentation by a combination of bacteria and yeast (*Saccharomyces cerevisiae and Lactobacillus*

plantarum). They reported a significant removal of volatile components responsible for beany off-flavour (Xiang et al., 2023). Artega et al. performed fermentation of pea protein isolate using enzymes and *Lactobacillus plantarum* and reported that fermentation followed by enzymatic hydrolysis showed higher protein degradation, functional changes, and off-flavour reduction (Arteaga et al., 2022). Another research work from the same author, Artega et al., performed fermentation of commercially available pea protein isolate by 6 different LAB strains for 24 and 48 hours. They revealed that pea protein isolated and fermented with *Lactobacillus plantarum* ranked highest in sensory analysis with the most neutral taste. They also concluded that fermentation negatively affected functional properties (García Arteaga et al., 2021). Similar findings were also reported in which a reduction in off-flavour producing component was observed in lactic acid fermentation of pea protein isolate (Shi et al., 2021). But again, this approach adds one extra step after the extraction process, making it expensive and unacceptable in the industry, as it is not profitable for them.

2.4.3   During extraction

This approach is not widely used and only a few articles have been published. In this approach, the base matrix for fermentation is solubilised protein obtained after mixing pea flour and water at an alkaline pH (7.5-9) and centrifugation to remove starch. This base matrix has two protein fractions, albumin and globulin. Performing fermentation at this step of the extraction process has two major advantages. First, fermentation can achieve acidification in the precipitation step, so no extra step is added. Second, off-flavours are also reduced simultaneously during fermentation. Another advantage is that bacteria can use soluble sugars that are present in pea and that are otherwise lost with albumin supernatant during conventional extraction. In terms of complexity, the base matrix (with one additional albumin fraction of pea protein) is simpler and easy to understand; handling material is also manageable and less expensive than the other two approaches as there is no additional step in processing. Emkani et al. reported an experimental study where precipitation was carried out by using fermentation with 3 different LAB strains (*Streptococcus thermophilus, Lactobacillus acidophilus, and Bifidobacterium lactis*) and their combination for 5–8 hours at 37 °C. They also studied protein fractions (albumin and

globulin) and their thermal properties. As a result, they observed that the combined culture samples acidified faster than others. For fermented samples, protein recovery decreased for the globulin-rich fraction and increased for the albumin fraction, and trivial changes were observed in thermal analysis (Emkani et al., 2021). Another study was reported by the same research group recently. They acidify the protein by fermentation with 2 different LAB strains (*Streptococcus thermophilus, Lactiplantibacillus plantarum*) and their combination at 3 different temperatures (37, 40, and 43 °C). They demonstrated protein composition and some nutritional properties and compared them with control, which is acidified by mineral acid (HCl). In the case of the fermented sample, higher protein recovery was observed in albumin fraction, and antioxidant activity was significantly higher than control. Antinutrient activity was also significantly reduced for fermented samples (Emkani et al., 2023). In both studies, there is a lack of information about the effect of fermentation on protein functionality, off-flavour molecule profiles, bacterial abundance during the fermentation period, and sensory analysis, and very few publications are available that confront this topic.

2.4.4 Conclusion

To fulfil our aim, we need to deeply understand fermentation and extraction. In fermentation specifically, the type and characteristics of microorganisms (bacteria, yeast, or moulds) involved, type of fermentation (homo or hetero fermentation), sugar metabolism pathways, and production of its various end products such as lactic acid, ethanol, acetic acid, CO_2, aldehydes, ketones, etc. In the extraction process, the types of extraction process, the compatibility of an extraction process with fermentation in terms of pH or salt concentration, and the temperature of extraction are also important. To combine fermentation with extraction, we must maintain suitable, favourable conditions for the microorganism community.

2.5 Fermentation

Louis Pasteur, a well-known French scientist, chemist, microbiologist, and pharmacist, coined the term "fermentation". Fermentation is a biological process that produces

chemical changes in organic compounds. Biochemically, it extracts energy from carbohydrates anaerobically. Microorganisms drive this process, degrading the organic matter and producing energy as adenosine triphosphate (ATP). In other words, fermentation is a process where microorganisms grow and change food substances for preservation and transformation purposes. Early cultures pioneered fermentation mainly to keep fresh farm goods and prevent them from spoiling. In food fermentation, the development of undesired microorganisms is prevented by the metabolites produced by the fermenting microorganisms (Di Cagno et al., 2013). This helps in keeping perishable food fresh for a longer time. In addition to preservation, fermentation adds distinctive aroma, flavour, taste, texture, and nutritional content to the food. Bread is a typical example of this case; the main aim of dough fermentation is to give characteristic structure, texture, and organoleptic characteristics to bread after baking. However, fermentation also plays an important role in removing antinutritional compounds and toxins from the food matrix and enhancing nutritional quality. For example, fermented soybean products like tempeh (meaty flavour and texture), natto (a dish from Japan that has a strong flavour and a slimy texture), and soy sauce (a dark brown liquid ingredient with an umami flavour) and in all of them, fermentation reduces antinutrients like phytic acid and trypsin inhibitors and gives a unique aroma and taste to the end product (Chen et al., 2013).

Fermentation is a biological process; unsurprisingly, it involves a diverse array of microorganisms. However, lactic acid-producing bacteria and ethanol-producing yeast are the most widely used microorganisms in food fermentation. Generally, a combination of organisms is used during fermentation, which supports the main bacteria by synergism. *Lactobacillus bulgaricus* and *Streptococcus thermophilus* are used for yoghurt preparation (Kandasamy et al., 2018), *Lactobacillus plantarum* and *Leuconostoc mesenteroides* are used in sauerkraut which is a fermented cabbage (Kandasamy et al., 2018), Korean kimchi is made from fermented cabbage and radish by using LAB strain *Lactilactobacillus sakei* and *Leuconostoc mesenteroides* (Kim et al., 2021). In some products, a combination of LAB and yeast strains is used. Kefir is a fermented dairy beverage in which *Lactobacillus kefiranofaciens*, *Leuconostoc mesenteroides*, and a yeast species *Saccharomyces*

cerevisiae are involved (Blasche et al., 2021). A fizzy drink kombucha is made by fermenting green or black tea by yeast strain *Saccharomyces cerevisiae* and different LAB strains (Harrison & Curtin, 2021).

2.5.1 Bacteria used in manufacture of fermented foods

Diverse bacterial communities are used to manufacture fermented foods, and they are classified in one of the three phyla: proteobacteria, firmicutes, or actinobacteria. *Firmicutes* consist of lactic acid bacteria, a cluster of gram-positive bacteria widely used as key organisms in the manufacture of fermented food. It also includes *Bacillus* and *Brevibacterium* genera, of which some species are used in specific fermented foods (Hutkins, 2007).

Proteobacteria contains a group of gram-negative bacteria. These bacteria are involved in vinegar fermentation, wine spoilage by producing acetic acid, and some alcoholic beverage manufacturing. *Actinobacteria* phyla contains only a few genera indirectly associated with fermented foods. These include *Bifidobacterium, Kocuria, Staphylococcus*, and *Micrococcus*. *Bifidobacterium* does not play any functional role. Rather, it is added only for nutritional purposes. *Kocuria, Staphylococcus, and Micrococcus* are specifically used for meat fermentation with only one purpose: to give the desired flavour and colour. It is worth highlighting that fermented food contains many other organisms whose presence occurs due to accidental contamination (Hutkins, 2007).

2.5.2 The lactic acid bacteria

Interestingly, the term "lactic acid bacteria" is not officially recognised in taxonomy. It is merely a convenient term to describe a collection of bacteria that are functionally and genetically related. Despite its unofficial status, this term holds considerable significance for microbiologists and those studying food fermentations. Hence, it will be frequently employed in this text. Lactic acid bacteria are a group of bacteria that produce lactic acid. They are gram-positive non-motile rods and cocci that do not form spores and have low GC content. These bacteria share similar biochemistry, function, and genetic makeup characteristics. Based on various phenotypic and genotypic differences, they can be

distinguished from other bacteria that produce lactic acid, like *Bacillus*, *Listeria*, and *Bifidobacterium* (Hutkins, 2007).

Lactic acid bacteria have other significant characteristics besides the qualities mentioned earlier. However, it is essential to note that these characteristics apply generally, although exceptions may arise occasionally. Most lactic acid bacteria are catalase-negative, acid-tolerant, aerotolerant (tolerate oxygen), and facultative anaerobes (can live with or without oxygen). They are categorised as heterotrophic chemoorganotrophs based on their requirements for carbon and energy, indicating that they depend on pre-existing organic carbon for both carbon and energy sources (Hutkins, 2007).

Lactic acid bacteria are most favoured for how they metabolise nutrients. They are commonly used in fermented foods because they metabolise sugars and produce lactic acid and other acidic end-products. Based on sugar metabolism, two fermentative pathways exist. In the homofermentative pathway, more than 90% of the sugar is converted entirely to lactic acid. However, the heterofermentative pathway results in about 50% lactic acid, with an equal proportion of acetic acid, ethanol, and carbon dioxide. Lactic acid bacteria typically follow one of these pathways exclusively (obligate homofermentative or obligate heterofermentative), but some species can switch between them depending on the conditions (facultative homofermentative) (Hutkins, 2007).

2.5.3   Fermentation and metabolism basics

Fermentation is defined in a biochemistry textbook as "energy-yielding reactions in which an organic molecule is the electron acceptor". According to the definition, in the case of lactic acid fermentation, pyruvic acid is generated by a glycolytic pathway that serves as an electron acceptor that forms lactic acid. Similarly, in ethanolic fermentation, acetaldehyde is formed by decarboxylation of pyruvate, an electron receptor forming alcohol. While this explanation holds validity for numerous food fermentations, it is somewhat limited in scope. In many fermented foods, important final products are formed through non-fermentative pathways, as traditionally described. For example, the malolactic fermentation process, crucial in winemaking, essentially involves a decarboxylation reaction. However, it doesn't

strictly adhere to the definition provided earlier. This definition doesn't fit well also with the making of tempeh and similar foods fermented by fungi. In these processes, fungi break down soy proteins and complex carbohydrates but don't produce certain glycolytic end products. So, in this discussion, "fermentation" will be used more broadly to include all the different chemical changes that happen when food is fermented (Hutkins, 2007).

From the perspective of microorganisms, fermentation is how they get energy, without much regard for the technical definitions. After all, they need energy to perform work (e.g., transport nutrients and biosynthesis), maintain physical and chemical homeostasis (osmotic and ionic), and grow and replicate. Majorly, energy generated by the fermentation process is in the form of ATP, and usually, it is by the metabolism of sugar. Whether this metabolism leads to the conversion of milk to yoghurt or juice to wine is not the concern of microorganisms. At the same time, these same pathways are responsible for the conversion of milk to spoiled yoghurt or juice to spoiled wine. However, it doesn't matter to microorganisms; it does matter to yoghurt and wine manufacturers (Hutkins, 2007).

Understanding how to manage microorganisms and their metabolic processes is crucial for determining the outcome of fermented foods. It can determine whether one will enjoy a delicious yogurt or end up with spoiled milk that has to be thrown away. This knowledge is the line between success and failure and can even affect a manufacturer's profits. That's why it's important to understand the biochemical reasons behind how microorganisms break down sugars and other substances. This understanding ensures that fermented foods consistently have the intended biological, physical, chemical, nutritional, and sensory qualities (Hutkins, 2007).

2.5.4 Sugar metabolism

Pasteurs, Buchner, Schwann and other biochemists and microbiologists studied the biochemistry behind how microorganisms metabolised sugars. It was logical for these scientists to focus on sugar fermentations in food. This is because products like fermented milk, beer, and wine are vital in industries, and their production relies on sugar fermentation. Therefore, it was crucial to identify the microorganisms involved and the processes they

follow. This understanding would help to enhance and control the production of these goods and prevent them from spoiling (Hutkins, 2007).

2.5.4.1 Homofermentation

Homofermentative lactic acid bacteria use enzymes from the glycolytic Embden-Meyerhoff pathway to metabolise hexoses (sugars with six carbon atoms). Aldolase is the key enzyme of this process, which plays a significant role in the breakdown of fructose-1,6-diphosphate into two smaller molecules of triose phosphates. These triose phosphates participate in reactions that produce ATP, a form of cellular energy (Figure 1). The Embden-Meyerhoff pathway generates two molecules of pyruvate and two molecules of ATP for every molecule of hexose sugar it processes. Afterwards, the lactate dehydrogenase enzyme can transform pyruvate into either L-lactate or D-lactate. In homofermentative metabolism, more than 90% of the initial sugar is converted into lactic acid (Hutkins, 2007).

Crucially, the NADH formed during the glyceraldehyde-3-phosphate dehydrogenase reaction must be re-oxidized by lactate dehydrogenase to maintain the [NADH]/[NAD] balance. Some homofermentative lactic acid bacteria are *Lactococcus lactis, Streptococcus thermophilus, Lactobacillus helveticus,* and *L. delbrueckii* subsp. *bulgaricus* (used as dairy starter organisms); *Pediococcus* sp. (used in sausage cultures); and *Tetragenococcus* (used in soy sauces) (Hutkins, 2007).
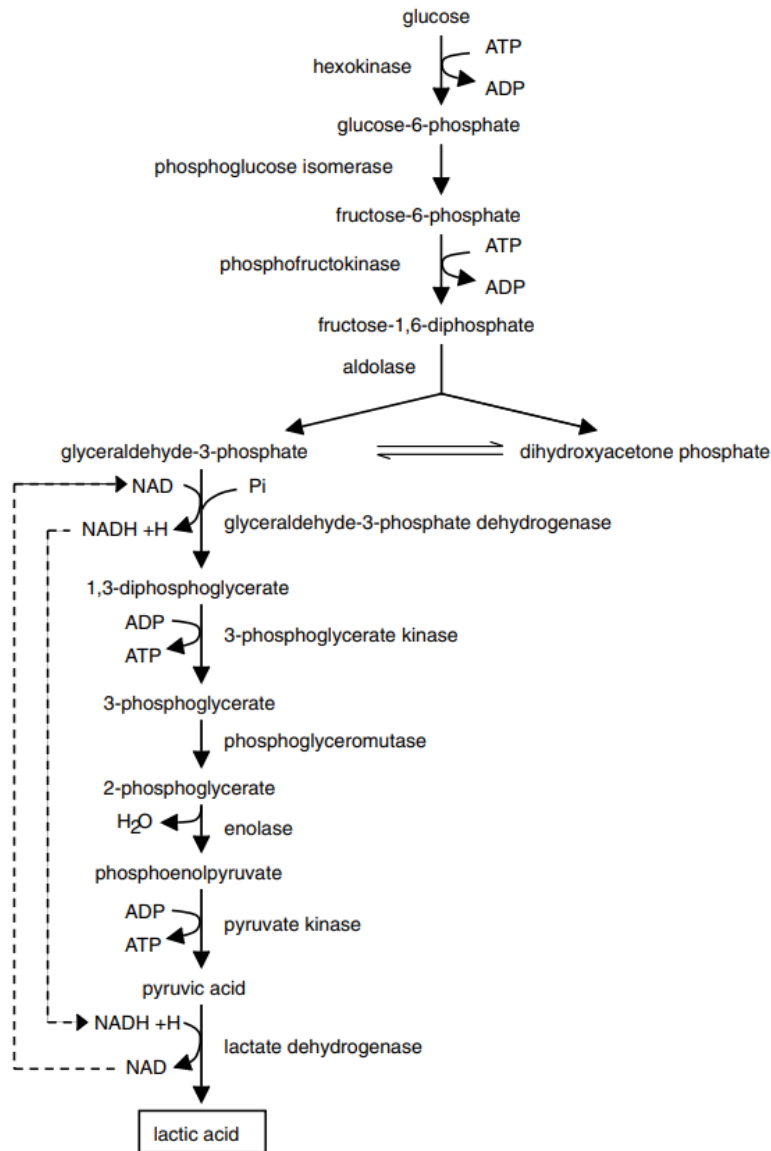
Figure 1. The Embden-Meyerhoff pathway used by homofermentative lactic acid bacteria. The dashed line indicatesd the NAD/NADH oxidation-reduction part of the pathway.(Hutkins, 2007).

2.5.4.2 Heterofermentation

Heterofermentative lactic acid bacteria follow a specific phosphoketolase pathway to metabolise hexoses (Figure 2). Unlike some other bacteria, these lack a particular enzyme called aldolase, but they have another enzyme called phosphoketolase instead. When they breakdown hexoses, they produce roughly equal amounts of lactate, acetate, ethanol, and CO_2, and they only make one unit of ATP for each hexose molecule. Oxidation of NADH and

maintenance of the [NADH]/[NAD] balance occurs via the two reductive reactions catalysed by acetaldehyde dehydrogenase and alcohol dehydrogenase. Many of the lactic acid bacteria used in food fermentations are heterofermentative. Included are *L. mesenteroides* subsp. *cremoris* and *Leuconostoc lactis* (used in dairy fermentations*), L. mesenteroides* subsp. *mesenteroides* and *Leuconostoc kimchii* (used in fermented vegetables), *O. oeni* (used in wine fermentations), and *Lactobacillus sanfranciscensis* (used in making sourdough bread) (Hutkins, 2007).
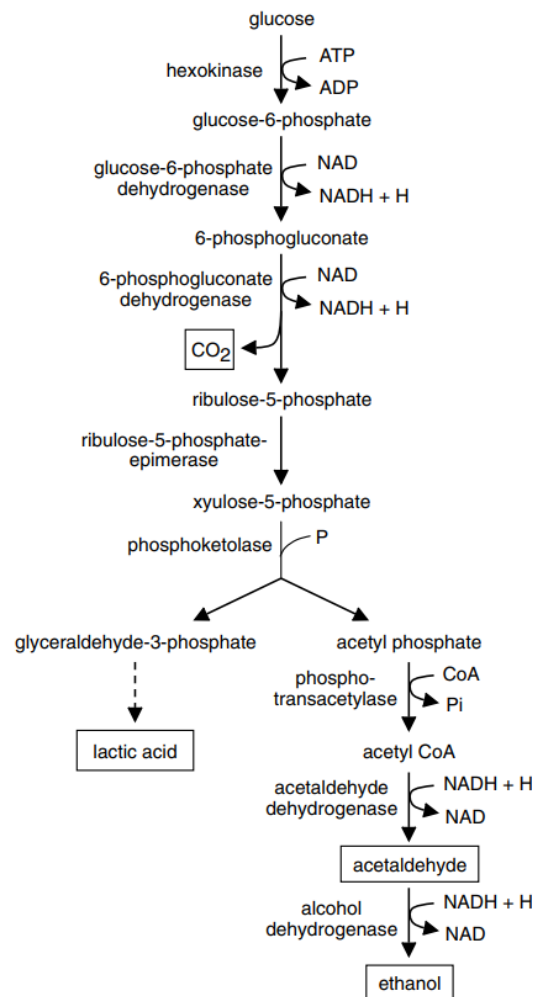
Figure 2. *The phosphoketolase pathway used by heterofermentative lactic acid bacteria (Hutkins, 2007).*

2.5.4.3 Real fermentation scenario

In actual fermentation processes, the amount of product obtained can vary. It depends on different factors like the type and amount of substance being used, the growth temperature, the atmospheric conditions, and the growth phase of the cells. Some of the carbon from carbohydrates is used to make cell mass. However, in certain situations, like when there's not enough sugar or aerobiosis (when there is plenty of oxygen), lactococci bacteria divert from the pyruvate path. Instead of turning it all into lactate, they might use some of it to make other things like acetate and CO_2. This kind of fermentation, called hetero-lactic fermentation, could give the cells more energy or help them eliminate extra pyruvate (Hutkins, 2007).

2.5.4.4 The hetero-lactic fermentation: dealing with pyruvate

Lactic acid bacteria can be obligate homofermentative, obligate heterofermentative, and facultative homofermentative (where both pathways exist) bacteria. Interestingly, obligate homofermentative strains still can produce acetic acid, ethanol, acetoin, CO_2, and other end products besides lactic acid. However, these alternative end products are only produced when pyruvate concentrations are elevated (Hutkins, 2007).

Pyruvate is elevated when the rate of intracellular pyruvate production exceeds the rate at which pyruvate is reduced to lactate (by the lactate dehydrogenase enzyme). Pyruvate can be increased by sugar or amino acids. In either case, pyruvate should be removed because it is toxic to the cells. When extra pyruvate is formed from sugar metabolism, the cell must reoxidise extra NADH produced from glycolysis (upstream in the pathway). Thus, the cell diverts pyruvate to several different pathways and forms multiple end products, respectively, as shown in Figure 3. Additionally, one of these alternate pathways includes a substrate-level phosphorylation reaction, and the cell receives an additional ATP (Hutkins, 2007).

It is important to understand what conditions cause fluctuations in pyruvate formation. First, glycolysis: when fermentation substrates are limiting, glycolytic flux decreases. Specifically, when fructose-1,6-diphosphate concentration is low, lactate dehydrogenase

activity is reduced. Thus, pyruvate is accumulated. Glycolytic flux may also be decreased during growth on undesired carbon sources, like galactose as sugar, resulting in an increase in pyruvate. Simultaneously, lactate dehydrogenase activity is decreased, and pyruvate-formate lyase is activated. This enzyme breaks down the pyruvate into formate and acetyl CoA, further reducing them in ethanol or acetyl phosphate. Acetyl phosphate provides one additional ATP in further reaction (Hutkins, 2007).

However, pyruvate-formate lyase is inactive under aerobic conditions, while other pathways are active. For example, in pyruvate dehydrogenase pathways, pyruvate is decarboxylated by pyruvate dehydrogenase, and CO_2 and acetate are formed (Hutkins, 2007).
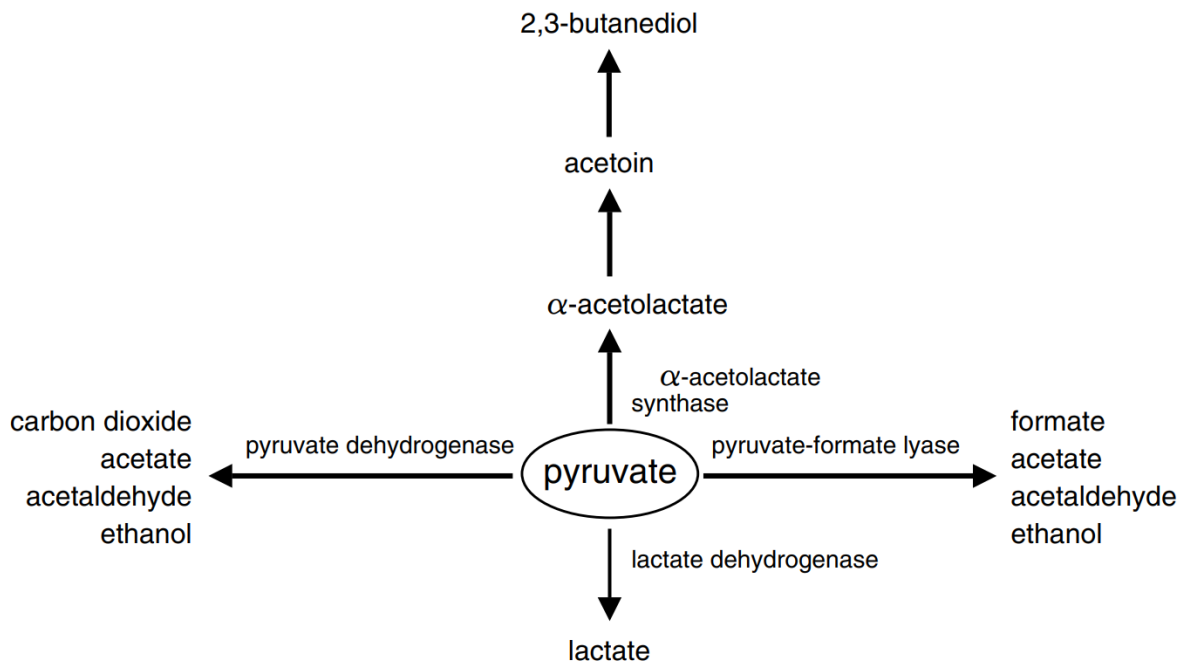
Figure 3. *Heterolactic end products from pyruvate metabolism (Hutkins, 2007).*

2.5.5 Protein metabolism

Like many types of bacteria, lactic acid bacteria can't use inorganic nitrogen and instead need pre-formed amino acids. Most foods only have a small amount of these amino acids. This means that lactic acid bacteria must first be able to degrade proteins and large peptides and then also be able to transport the small peptides and free amino acids that are released during proteolysis. Scientists have well studied how lactic acid bacteria break down

proteins, especially those used in the fermentation of dairy products. The main protein they work on for these bacteria is casein, found in milk. Casein metabolism is not only important nutritionally for the bacteria, but its degradation during cheese manufacturing affects the flavour and texture development. However, protein metabolism is not as crucial in other types of fermentations, like those for vegetables and bread (Hutkins, 2007).

Plant-based protein metabolism by lactic acid bacteria is not well studied, but similar metabolism for casein protein has been explored well. Figure 4 show proteolytic system in lactococci. Milk casein is hydrolysed by a cell envelope-associated proteinase (PrtP) to form oligopeptides. These oligopeptides are then transported across the membrane by the oligopeptide transport system (Opp). The intracellular oligopeptides are then hydrolysed by cytoplasmic peptidases (e.g., PepA, PepC, PepN, and PepX) to form amino acids. Extracellular di- and tripeptides and free amino acids in milk are transported by di- and tripeptide transporters (DtpT, DtpP) and amino acid (AA) transporters. The intracellular di- and tripeptides are then hydrolysed to amino acids. Adapted from Hutkins (2007).

Lactic acid bacteria utilise casein mainly in three steps. First, proteinases break down casein into peptides. Then, the peptides are transported into cells through peptide transport systems. Lastly, intracellular peptidases break down the peptides into individual amino acids within the cells. Each of these steps is explained in detail below (Hutkins, 2007).
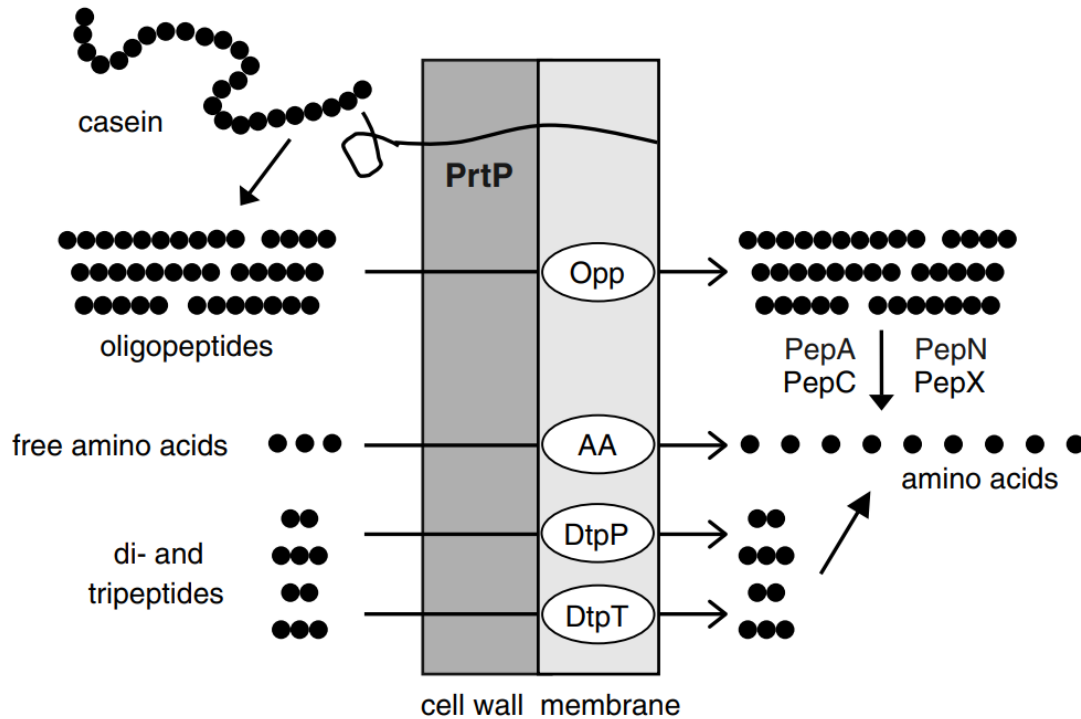
Figure 4. *Proteolytic system in lactococci (Hutkins, 2007).*

2.5.5.1 The proteinase system

Casein hydrolysis by lactic acid bacteria occurs through a cell envelope-associated serine proteinase called PrtP. This enzyme is synthesised as a large inactive pre-pro-proteinase (>200 kDa). The "pre" part includes a leader sequence, which guides the protein across the cytoplasmic cell membrane. The "pro" sequence likely helps the protein stay stable during synthesis. Once both of these parts are removed, mature proteinase remains anchored to the cell envelope. When using casein as a substrate, PrtP produces over 100 products. Most of these are big oligopeptides (up to 30 amino acids), but many are between four and ten residues long (Hutkins, 2007).

2.5.5.2 Peptide transport system

Despite having bulky and large-size peptides, lactococci and other lactic acid bacteria transport peptides directly into the cell without further extracellular hydrolysis. Lactococci and other lactic acid bacteria majorly rely on an oligopeptide transport system (Opp) to transport amino acids. The Opp system transports about ten to fourteen different peptides

of varying sizes (between 4 and 11 amino acid residues). Other transporters found in various strains possess systems for transporting dipeptides and tripeptides (Hutkins, 2007).

2.5.5.3 Peptidases

In the last phase of protein metabolism, intracellular peptidase enzymes break down the peptides gathered in the cell's cytoplasm through the Opp system. More than twenty different peptidases are produced by lactococci and lactobacilli, ultimately generating the pool of amino acids necessary for biosynthesis and cell growth. They are classified into endopeptidases (that cleave internal peptide bonds), exopeptidases (that cleave at terminal peptide bonds) and the latter group consist entirely of aminopeptidases. Lactic acid bacteria need enzymes like endopeptidases, aminopeptidases, dipeptidases, and tripeptidases to efficiently break down peptides stored by the Opp system (Hutkins, 2007).

2.5.6 The genera of lactic acid bacteria

According to taxonomical classification, lactic acid bacteria are classified into 12 genera (Table 2). They are in phylum *Firmicutes* and order *Lactobacillales*. They were grouped into broad phylogenetic clusters based on 16S rRNA sequencing and other molecular techniques (Hutkins, 2007).

Seven out of 12 genera of lactic acid bacteria, *Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus,* and *Tetragenococcus*, are directly employed in food fermentations. *Enterococcus* sp. are commonly present in fermented foods like cheese, sausage, and fermented vegetables, but they are typically not intentionally added. Their presence is usually undesirable because they are indicators of faecal contamination. Similarly, *Carnobacterium* are unwanted as they are considered as organisms that spoil fermented meat products. Additionally, *Aerococcus*, *Vagococcus*, and *Weisella* species are not commonly present in foods, and their importance remains uncertain. The following section will describe the general properties, habitats, and practical considerations of genera relevant to food fermentations (Hutkins, 2007).

Table 2. *Genera of lactic acid bacteria and their properties (Hutkins, 2007).*

Genus	Cell Morphology	Fermentation route	Growth at: 10°C	45°C	Growth in NaClat: 6.5%	18%	Growth at pH: 4.4	9.6	Lactic acid isomer
Lactobacillus	rods	homo/hetero[3]	±[4]	±	±	−	±	−	D, L, DL[5]
Lactococcus	cocci	homo	+	−	−	−	±	−	L
Leuconostoc	cocci	hetero	+	−	±	−	±	−	D
Oenococcus	cocci	hetero	+	+	±	−	±	−	D
Pediococcus	cocci (tetrads)	homo	±	±	±	−	+	−	D, L, DL
Streptococcus	cocci	homo	−	+	−	−	−	−	L
Tetragenococcus	cocci (tetrads)	homo	+	−	+	+	−	+	L
Aerococcus	cocci (tetrads)	homo	+	−	+	−	−	+	L
Carnobacterium	rods	hetero	+	−	−	−	−	−	L
Enterococcus	cocci	homo	+	+	+	−	+	+	L
Vagococcus	cocci	homo	+	−	−	−	±	−	L
Weissella	coccoid	hetero	+	−	±	−	±	−	D, L, DL

[1]Adapted from Axelsson, 2004
[2]Refers to the general properties of the genus; some exceptions may exist
[3]Species of *Lactobacillus* may be homofermentative, heterofermentative, or both
[4]This phenotype is variable, depending on the species
[5]Some species produce D-, L-, or a mixture of D- and L-lactic acid.

2.5.6.1 Streptococcus

The *Streptococcus* group consists of various types found in different places. This group includes bacteria that can cause diseases in humans and animals and those that live in the mouth and intestines without causing harm. One particular species in this group, called *Streptococcus thermophilus*, is used in making fermented foods. *Streptococci* generally are non-motile, facultative anaerobes with an obligate homofermentative metabolism. Their optimum growth temperature is 40 °C to 42 °C. Their nutritional requirements are demanding. *S. thermophilus* is weakly proteolytic and, therefore, relies on pre-formed amino acids. They are salt-tolerant and bile-sensitive, and their range of metabolic functions is somewhat restricted (Hutkins, 2007).

2.5.6.2 Pediococcus

The *pediococci* are cocci shaped homofermentative lactic acid bacteria. They are found in pairs (always spherical), but they don't form chains. *Pediococci* are facultative anaerobes with specific nutritional requirements. They have optimum growth temperatures between 25 °C and 40 °C, but some species can survive at temperatures up to 50 °C. Several *pediococci* are also distinguished from other lactic acid bacteria by their ability to tolerate high acidic conditions (growth at pH 4.2) and high salt (growth at 6.5% NaCl) environments. They are found in diverse habitats, including plant material, milk, brines, animal urine, and beer. They are classified into 6 species; several are relevant to food fermentation. Two

species, *Pediococcus acidilactici* and *Pediococcus pentosaceus*, are naturally found in raw vegetables, where, in desirable conditions, they play a crucial role in the manufacture of sauerkraut and other fermented vegetables. *Pediococci* are also important spoilage organisms in fermented foods, particularly beer, wine, and cider. One species, *Pediococcus damnosus*, poses a significant issue in the beer-making process. This microbe is notorious for generating diacetyl, a substance that is considered a severe flaw in beer production (Hutkins, 2007).

2.5.6.3 Lactobacillus

The *Lactobacillus* genus consists of more than 80 species. This group is the most ecologically, physiologically, biochemically, and genetically diverse in the lactic acid bacteria group. From an ecological perspective, lactobacilli can be found in various environments. Certain species live naturally in plants and vegetables and are commonly present in dairy and meat processing units and in juices, fermented drinks, grains, and cereals. They are also commonly found in the gastrointestinal tract of animals and humans, including the stomach, mouth, and vagina. Strong scientific evidence suggests that these bacteria have wide-ranging "probiotic activity," meaning they support both intestinal and overall health (Hutkins, 2007).

Lactobacilli have the remarkable ability to grow and persist in various environments and conditions due to their diverse physiological characteristics. Although most species are mesophilic, some are psychrotrophic, thermoduric, or thermophilic. The ideal temperature range varies from 30 °C to 45 °C. Some species show high tolerance to salt, osmotic pressure, and low water activity. Acid tolerance is a common feature of *lactobacilli* (many strains prefer an acidic environment); some are also ethanol-tolerant or bile-tolerant. Most species are aerotolerant, although some require strictly anaerobic conditions (Hutkins, 2007).

Despite their diverse metabolic actions, *lactobacilli* are generally fastidious, and many species require nutrient-rich environments. They are not proteolytic or lipolytic; thus, amino acids, peptides, and fatty acids are usually required for rapid growth. Some strains are even

more demanding, requiring various vitamins, nucleotides, and other nutrients. Lactobacilli also need carbohydrates, which they can ferment, and can ferment different sugars depending on their source or habitat. They can ferment common sugars like glucose, fructose, and lactose, as well as sugars found in plants like cellobiose, amygdalin, and trehalose (Hutkins, 2007).

Lactobacillus are fermentative and sub-classified into three groups based on the pathway they use to ferment sugars (Table 3). Group I includes obligate homofermentative species, group II consists of facultative heterofermentative species, and group III involves obligate heterofermentative species (Hutkins, 2007).

Table 3. *Fermentation characteristics of Lactobacillus (Hutkins, 2007).*

Representative strains	% G+C	Growth at 15°C
Group I		
Obligate homofermentative		
Lactobacillus acidophilus	34–37	−
Lactobacillus delbrueckii	49–51	−
Lactobacillus helveticus	38–40	−
Lactobacillus amylophilus	44–46	+
Lactobacillus amylovorus	40–41	−
Lactobacillus crispatus	35–38	−
Lactobacillus gasseri	33–35	−
Lactobacillus jensenii	35–37	−
Group II		
Facultative heterofementative		
Lactobacillus paracasei	46	nd
Lactobacillus curvatus	43	nd
Lactobacillus plantarum	45	+
Lactobacillus sakei	43	nd
Lactobacillus bavaricus	43	nd
Lactobacillus homohiochii	36	+
Lactobacillus coryniformis	36	+
Lactobacillus alimentarius	36	nd
Group III		
Obligate heterofermentative		
Lactobacillus fermentum	53	+
Lactobacillus sanfranciscensis	37	nd
Lactobacillus reuteri	41	nd
Lactobacillus buchneri	45	nd
Lactobacillus brevis	45	nd
Lactobacillus kimchii	35	+
Lactobacillus kefiri	41	nd
Lactobacillus divergens	34	+

2.5.6.4 Bifidobacterium

There is debate about whether the species of *Bifidobacterium* are involved in food fermentation. They are fermentative, but they are not used in the fermentation of any kind of food or found in any raw food material. *Bifidobacteria* mainly live in the intestinal tract, and raised counts in the human gut are linked to less chance of gut infections and better intestinal well-being. Due to their probiotic effects, *Bifidobacterium* species are added to specific food products, like milk, yoghurt, and other fermented dairy products. This makes them an important ingredient in dairy products (Hutkins, 2007).

Taxonomically, there are more than twenty-five different species of *Bifidobacterium*; among them, only about ten are conventionally used as probiotics in the market, including *Bifidobacterium bifidum, Bifidobacterium adolescentis, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium lactis,* and *Bifidobacterium longum*. Until the 1970s, they were classified in the genus *Lactobacillus*. After that, they were found phylogenetically distinct from lactic acid bacteria and classified in entirely different phylum *(Actinobacteria)* (Hutkins, 2007).

Bifidobacterium are gram-positive, non-motile, non-spore-forming rods with a high GC content (55 % to 67 %) bacterial species. Cells are arranged in pairs with a Y or V shape. They are strictly anaerobes, with an optimal growth temperature between 37 °C to 41 °C and an optimum pH between 6.5 and 7.0. They also have demanding nutritional requirements for growth. They metabolise different sugars, including non-digestible oligosaccharides that reach the colon and may provide selective advantages in the colonic environment. They metabolise sugar via the well-known "bifidum" fermentation pathway and yield acetic and lactic acid (Hutkins, 2007).

2.6 Protein extraction methods

The extraction or isolation techniques to get concentrated protein are important, as they give the specific fraction of protein in the end-product based on a technique used. There are two ways to extract pea protein from whole pea flour: dry and wet methods. Dry fractionation is a more efficient method than wet fractionation, as it requires less water, almost no

chemicals, and low energy input (Pulivarthi et al., 2023; Schutyser et al., 2015; Stone et al., 2015). On the other hand, dry fractionation cannot reach a protein purity level similar to that of the wet method (Schutyser & van der Goot, 2011). Dry fractionation uses air classification based on differences in particle density, whereas the wet method uses protein solubility at different pHs.

There are multiple wet fractionation methods. Most commonly, alkaline extraction – isoelectric precipitation and salt extraction – dialysis (Stone et al., 2015) are used and will be described in the next section. Other less commonly used methods are water extraction, acid solubilisation and micellar precipitation (Boye et al., 2010; Lam et al., 2018; Reinkensmeier et al., 2015; Sun & Arntfield, 2011). They all are based on the solubility of different protein fractions in alkali or salt, and based on the method applied, specific protein fractions can be extracted. Furthermore, membrane filtration of solubilized proteins has also gained popularity.

2.6.1 Dry air classification

Air classification is a method to separate pea protein without using water. Pea protein is separated from starch based on its variation in particle sizes, bulk densities, and powder properties of protein and starch granules after grinding pea seeds into pea flour (Arntfield & Maskus, 2011; Tulbek et al., 2017). When the seeds are ground into a powder, the smaller protein fractions (<10 µm) are separated from the bigger starch particles and fibre (between 20 to 70 µm) (Schutyser et al., 2015; Tulbek et al., 2017). To achieve this, the powder is blown with a stream of air in a special chamber called an air classifier. Inside this chamber, there's a spinning wheel with a small opening. This opening lets the small protein part float up and be collected while the heavier and bigger starch and fibre parts are rejected by the wheel (Boye et al., 2010; Schutyser et al., 2015). This way, the concentrated protein and the starch/fibre parts are separated.

To optimise the separation efficiency, the powder needs to be ground fine enough to separate protein and starch but not so much that it damages the starch, making it break into smaller fragments that could mix with the small protein particles (Boye et al., 2010). Peas

are suitable legumes for air classification because their starch is big and uniform in size (Schutyser et al., 2015). Also, peas have low fat, making them better for this process than soybeans.

The dry fractionation method can achieve 50–60% protein concentration and 77% protein recovery in the final pea protein (Schutyser et al., 2015). However, making the protein purer than 60% is hard because the protein and starch are naturally stuck together (Boye et al., 2010). For higher purity proteins, wet methods are considered.

2.6.2 Alkaline extraction – isoelectric precipitation

Dry fractionation separates components based on their particle sizes in pea flour, whereas wet fractionation relies on their solubility differences. The most common method for isolating proteins involves alkaline solubilisation followed by isoelectric precipitation (Arntfield & Maskus, 2011; Taherian et al., 2011). It is well known that pea proteins dissolve best in alkaline conditions. Initially, pea flour is dissolved in a pH between 7 and 11 solution to extract proteins into the liquid phase (Arntfield & Maskus, 2011; Boye et al., 2010; Fredrikson et al., 2001). Subsequently, the solution undergoes centrifugation to separate insoluble components like starch and fibre, leaving the proteins in the supernatant. The supernatant's pH is adjusted to the isoelectric point of pea proteins, typically around pH 4 to 5 (Lam et al., 2018; Tulbek et al., 2017). Globular proteins have a zero net charge at this pH, causing them to aggregate and precipitate due to hydrophobic interactions (Lam et al., 2018; Murray et al., 1981). Another centrifugation step separates the precipitated proteins, forming a pellet, from the remaining soluble carbohydrates (fibre and small sugar) and possibly some albumin proteins in the supernatant, which stay soluble at pH 4.5 due to their higher isoelectric point than globulins (Damodaran & Parkin, 2017; Makri et al., 2005; Thrane et al., 2017). Sometimes, the pellet undergoes washing to remove salts, followed by neutralisation and drying (Boye et al., 2010).

Several aspects of this extraction method can be altered. The pH range for solubilising typically falls between 8 and 10 (Boye et al., 2010; Makri et al., 2005; Shand et al., 2007; Sumner et al., 1981). Usually, increasing the solubilisation pH increases the amount of

protein obtained, but it might lower the purity because starch could also dissolve at highly alkaline pH levels (Reinkensmeier et al., 2015). Moreover, high alkalinity can degrade the protein, causing denaturation and polymerisation, resulting in reduced digestibility and nutritional value (formation of lysinoalanine, alteration of amino acids), decreased taste and texture, and loss of protein functionality (Arntfield & Maskus, 2011). Under alkaline conditions, certain amino acids like cysteine and serine can transform into nephrotoxic lysinoalanine compounds, leading to a reduction in essential amino acids (Lam et al., 2018). Additionally, alkalinity can induce cross-linking of proteins, which decreases protein digestibility and biological availability of amino acids involved in the cross-links (Damodaran & Parkin, 2017). It can also cause the racemisation of amino acids from their natural form (L-) to a form (D-) that the body doesn't absorb well (Damodaran & Parkin, 2017). At very high pH levels, there's increased electrostatic repulsion between protein chains, especially with the ionisation of buried carboxyl, phenolic, and sulfhydryl groups, which causes the protein to unfold. This unfolding exposes hydrophobic ends and sulfhydryl groups of the protein, which encourages protein cross-linking and aggregation (Alizadeh-Pasdar & Li-Chan, 2000). The pH used for extraction should be chosen carefully to get a higher concentration and recovery of protein while minimising the destruction of the protein's structure.

In addition to adjusting pH, other factors in the solubilisation process can be changed to make extraction more effective. The time duration of the solubilisation step usually ranges from 30 minutes to 2 hours, and longer times usually lead to better results (Hoang, 2012; Makri et al., 2005; Taherian et al., 2011). Additionally, doing the solubilisation process multiple times can improve efficiency. After the first solubilisation and centrifugation step, the pellet can be remixed with water to ensure all the soluble proteins are extracted (Makri et al., 2005; Nichols & Cheryan, 1981). The ratio of solid material to water can also affect efficiency, but it is not as important (Hoang, 2012). The flour-to-water ratio is typically between 1:5 and 1:20 (Makri et al., 2005). Using more solid material and less water is often preferred to save water.

The isoelectric precipitation step can be adjusted along with modifying the solubilisation step. The pH precipitating the proteins will affect the final protein fractions, as vicilin,

convicilin, legumin, and albumin proteins differ in their isoelctric point (Damodaran & Parkin, 2017; Derbyshire et al., 1976; Rickert et al., 2004; Rubio et al., 2014). Additionally, the time proteins spend at their isoelectric point will impact overall functionality. It's ideal for proteins to spend as little time as possible at their isoelectric point to avoid irreversible protein aggregation and polymerisation, which cause permanent loss of solubility and overall functionality (Kinsella & Melachouris, 1976).

After the proteins are separated through isoelectric precipitation, they are remixed and neutralised. Then, they can either be filtered or purified further to remove any remaining salts and decrease the ash content. Alternatively, they can skip this step and proceed directly to drying (Cutler, 2004; Nichols & Cheryan, 1981; Tian et al., 1999). The type of drying technique will influence the overall structure and functions of the proteins. Lyophilisation is often used for small-scale operations like those in laboratories. This method preserves the structure of the protein well because it does not involve applying heat (Kinsella & Melachouris, 1976; Stone et al., 2015; Zhao et al., 2013). However, it is costly and time-consuming, so it is not practical for large-scale industrial applications (Sumner et al., 1981). Instead, industrial protein isolates are typically dried using spray drying (Zhao et al., 2013). The specific conditions of spray drying, such as the temperature applied and the size of the particles, will affect how much heat the proteins are exposed to and the final moisture content and size of the protein particles.

Utilising alkaline solubilisation coupled with isoelectric precipitation and carefully selecting extraction parameters facilitates the preservation of the native protein conformation, yielding high protein solubility and overall functional efficacy (Damodaran & Parkin, 2017). Moreover, this process helps achieve protein purity (80-90%) and yield (60-70%) (Boye et al., 2010; Owusu-Ansah & Mc curdy, 1991; Stone et al., 2015; Swanson, 1990; Tian et al., 1999). Alkaline solubilisation, followed by isoelectric precipitation, remains the predominant method on a commercial scale. However, most parameters and conditions utilised in industry often result in protein denaturation and polymerisation, degrading the nutritional and functional attributes of the protein. While there is room for enhancement and

optimisation of extraction/isolation conditions, alternative extraction methodologies are concurrently under investigation.

2.6.3   Salt extraction

Apart from extracting proteins based on differences in solubility at different pH levels, proteins can also be separated based on their solubility at varying salt concentration levels, using a process known as "salting in" and "salting out" (Boye et al., 2010). Initially, proteins are dissolved in a diluted salt solution. The ions in the solution partly cover protein molecules, preventing them from electrostatic interaction with each other and increasing interactions between proteins and water molecules (Duong-Ly & Gabelli, 2014). As the salt concentration increases, more salt ions are present, which replace water molecules from the proteins by fully covering all charges on the protein's surface and competing for water interactions (Novák & Havlíček, 2016; Zhou, 2005). The surface charge elevates hydrophobic interactions among protein molecules, which forms aggregates and precipitates (Novák & Havlíček, 2016).

The salt extraction technique carries a lower risk of protein denaturation, resulting in the preservation of the protein's native structure (Novák & Havlíček, 2016). Salt-extracted protein isolates are typically more resistant to denaturation than those extracted with alkaline methods, mainly because salt helps stabilise the protein's structure (Hermansson, 1986; Sun & Arntfield, 2011). Additionally, salt extraction yields protein isolates with a composition different from alkaline extraction. Unlike alkaline methods, salt extraction retrieves globulins and albumins (Arntfield & Maskus, 2011; Stone et al., 2015). This preservation of structure and combination of globulins and albumins in salt-extracted protein isolates leads to noticeable differences in their functional properties compared to conventionally extracted ones using alkaline methods. But salt extraction obtains protein isolate that is excessively salty, hence it requires a desalting procedure (Murray et al., 1981). Typically, on a laboratory scale, dialysis is the preferred method to eliminate salts while keeping the protein intact, while on an industrial scale, diafiltration is used (Arntfield & Maskus, 2011; Crévieu et al., 1996; Cutler, 2004). However, both dialysis and diafiltration

consume considerable time and generate significant volumes of salty rinse water, which needs appropriate disposal methods (Lam et al., 2018). Alternatively, an approach to removing salt content involves using membrane filtration instead of the "salting out" method, as elaborated in the subsequent section.

2.6.4 Salt solubilisation coupled with membrane filtration

Membrane filtration is becoming more popular for concentrating and purifying pea protein extracts. Once the protein is dissolved, membrane filtration can remove small, undesired substances (salts and sugars) while making the protein more concentrated (Taherian et al., 2011). Instead of using isoelectric precipitation after alkaline solubilisation, membrane filtration has been used more often (Boye et al., 2010; Damodaran & Parkin, 2017; Makri et al., 2005). It can also replace "salting out" the protein after dissolving it in salt. Only one study has used salt solubilisation along with membrane filtration, which is worth more attention because it's a gentle method for separating plant proteins.

The dissolved protein mixture goes through ultrafiltration (UF) to increase the protein concentration and eliminate small molecules. The proteins, which are bigger than the molecular weight cut-off (MWCO) of the membrane, stay on one side of the membrane called retentate, while salts and small molecules like sugars (mono-, di-, and oligosaccharides) can move through the membrane to the other side called permeate (Fredrikson et al., 2001; Taherian et al., 2011). Also, some antinutritional factors, like phytic acid and compounds causing bad taste, are small enough to pass through the membrane. This helps to make the protein more pure and improves its sensory properties (Arntfield & Maskus, 2011; Lam et al., 2018; Taherian et al., 2011).

Ultrafiltration (UF) offers several advantages over alternative methods of concentrating protein, notably it doesn't damage native structure and is able to achieve high protein recovery rates. Pea protein isolates obtained through UF have shown remarkable purity levels of up to 92%, coupled with a protein yield of around 60%, which are comparable to or better than outcomes from other extraction methods (Boye et al., 2010; Fredrikson et al., 2001; Gueguen, 1983; Makri et al., 2005; Taherian et al., 2011). Unlike isoelectric

precipitation, which can degrade the structure of proteins, ultrafiltration is gentle and does not adversely affect protein integrity (Kinsella & Melachouris, 1976; Lam et al., 2018). Protein isolates produced through UF generally exhibit improved solubility and functional properties compared to those prepared using isoelectric precipitation (Boye et al., 2010; Gueguen, 1983; Lam et al., 2018; Vose, 1980). Moreover, UF presents practical advantages over salting out, as it requires lower quantities of salt and water (Taherian et al., 2011). However, UF can entail significant costs due to expenses related to equipment, membranes, and maintenance procedures aimed at preventing membrane fouling.

2.7 The objective of the study

From the above discussion, it has been observed that conventional plant-based protein has an off-flavour issue from a consumer's point of view. It has also been seen that various approaches can be applied to reduce the off-flavour notes (with improved functionality if achievable). As discussed above, fermentation can be one of the finest ways to effectively remove undesired flavour molecules with minimum destruction of native protein matrix with a slight change in functionality. It is a commercially profitable option to use fermentation during the extraction process. It will reduce the processing steps compared to other approaches (after and before the extraction process) and achieve the precipitation of proteins at the same step with the additional benefit of off-flavour reduction.

Our main objective is to reduce the off-flavour in pea protein isolate with nominal damage to the native protein structure. We are interested in following the conventional alkali-solubilised isoelectric-precipitation extraction (AS-IPE) method with an innovative modification in the precipitation step by using LAB fermentation to achieve an isoelectric pH value (i.e. 4.8) in the least time possible and make this process commercially and economically viable. Thus, this work will introduce an innovative protein extraction method with bacteria-based protein acidification until the isoelectric point and with the additional advantage of off-flavour reduction. This method is named alkali solubilised – bacterial-acidified protein extraction (AS-BAPE). We hypothesise that there will be a significant change in the concentration of volatile molecules and targeted molecules for beany flavour,

i.e. aldehydes (hexanal and nonanal). Minimum or no change in fermented protein functionality will be expected compared to conventionally extracted (HCl precipitated) protein. It is based on the fact that lactic acid bacteria have negligible proteolytic activity.

3 Materials and methods

3.1 Materials

3.1.1 Pea flour & proximate composition

We have selected the 'Kirke' variety of field pea, developed in Estonia. The crop year was 2019, the material was stored at -20 °C. The pea flour we used is composed of 87.93 % dry matter, 23.66 ± 0.34 g/100g protein (dry weight basis), and an insignificant amount of fat, 0.94 ± 0.03 g/100 g (dry weight basis). The protein content of flour was determined by the Kjeldahl method with a conversion coefficient of 6.25.

3.1.2 Starter cultures

We have selected five unique commercial starter cultures (SC_1, SC_2, SC_3, SC_4, SC_5). These starter cultures are specifically formulated for a variety of plant-based fermented products. They are all certified safe, kosher, and halal. They are allergen-free, non-GMO, dairy-free, and lactose-free. Their bacterial composition is not shown here due to commercial interests.

3.1.3 Chemicals and instruments

All chemicals used in analytical measurements such as hydrochloric acid, sodium hydroxide, potassium phosphate dibasic and potassium phosphate monobasic, trypsin from bovine pancreas (≥6,000 BAEE units/mg protein, Sigma Aldrich) and Coomassie brilliant blue G-250 were of analytical grade and purchased from Sigma Aldrich.

We used a range of instruments for our analytical measurements. These include a digital overhead stirrer (IKA™ EUROSTAR 60 by Fisher Scientific, Goteborg - Sweden), centrifuge (ROTANTA 460 R by Hettich, Tuttlingen, Germany), freeze dryer (Heto Power Dry PL3000 by Thermo Fisher Scientific, Waltham, MA USA), 32-channel pH monitor iCinac (KPM Analytics, Massachusetts, United States), 48-channel microcalorimeter (TAM IV-48, TA Instruments, New Castle, Delaware, United States), high-performance liquid chromatography (HPLC) system (Alliance 2695 system, Waters Corp., Milford, MA, USA), ultra-performance liquid

chromatography (UPLC) system (Acquity UPLC; Waters Corp., MA, USA), Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA), iSeq100 Sequencing System (Illumina, San Diego, CA, USA), gas chromatograph system (2030; Shimadzu, Kyoto, Japan), mass spectrometer (8050NX Triple Quadrupole; Shimadzu, Kyoto, Japan), homogenizer (Polytron PT 2100, Kinematica AG, Malters, Switzerland), multi-shaker (Thermo Mixer C, Eppendorf, Connecticut, United States), UV-VIS spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), lyophilizer (SCANVAC COOLSAFE, Labogene, Blovstrød, Denmark), and a spectrophotometer (NS810, 3nh Shenzhen Threenh Technology Co., Ltd., Shenzhen, China).

3.2 Methods

3.2.1 Small-scale preliminary experiments

3.2.1.1 *Optimizing the protein extraction process*

The alkali-solubilized isoelectric protein precipitation method is widely used in research and commercially. Optimizing this method leads to high protein recovery, higher protein isolate yield, and minimal loss of functionality, i.e., higher protein nativity. To manufacture this high-quality protein, one can play with the parameters involved in the process. These are the ratio between the raw material and water for the solubilization step, pH at solubilization step, mixing speed, mixing time, mixing temperature during solubilization, first centrifugation speed, protein precipitation pH, mixing time for precipitation step, second centrifugation speed, amount of water to be mixed with precipitated protein at neutralization step.

We aimed to isolate protein from field pea flour. We targeted solubilizing pea proteins at the lowest alkaline pH that still achieved high protein recovery and yield. We needed a lower pH value to be able to ferment the supernatant obtained after the solubilization step, as strong alkalinity inhibits bacterial growth.

The extraction conditions were chosen according to literature. Because reported conditions varied between articles, we decided to test four conditions that were most interesting to us.

We selected pH values of 7.5, 8.0, and 8.5 with two ratios of flour to water: 1:6 and 1:8. The experimental plan is shown in Table 4 and is described below.

The pea flour was weighed into 4 centrifuge bottles (85 or 66 g) and then added 510 g or 528 g of distilled water. We measured the starting pH and added 2M NaOH until the pH was 7.5–8.5. We noted the starting time as soon as NaOH was added, and the amount of NaOH that was added at the beginning until it reached the correct pH value. Bottles were put into the IKA Shaker at 220 rpm and 23 °C for 70 minutes. They were taken out every 10–15 minutes, and pH was corrected by adding 2M NaOH. The added amount of NaOH was recorded for each addition to make a titration curve. After 70 minutes, the samples were centrifuged at 3000 x g for 8 minutes at 21 °C. The supernatants were weighed and separated into different bottles, and the precipitate was eliminated.

The next step was to precipitate the collected supernatant with 4M HCL. We added 4M HCl until the pH was between 4.5 and 4.6 and recorded the added HCl amount and the end pH. Then, all the samples were centrifuged at 3000 x g for 10 minutes at 21 °C. The supernatant and precipitate were collected in separate boxes and weighed.

The last step was to neutralize the precipitate collected from the previous centrifugation. Initially, 70 g of water was added to the precipitate and mixed well. Then, 2M NaOH was added until the pH reached 7.0, and it was mixed well. The colour changed to a brownish shade. Neutralized suspensions were poured into plastic containers, frozen at -80 °C, and lyophilized. The weight of the dried sample was measured at the end of the experiment.

Table 4. *Plan for the preliminary protein extraction optimization experiment.*

Sample Name	N2_1	N2_2	N2_3	N2_4
Extraction pH	7.5	8	8.5	7.5
Flour : water ratio	1:6	1:6	1:6	1:8
Flour weight (g)	85	85	85	66
Added distilled water weight (g)	510	510	510	528

3.2.1.2 Preparation of solubilized pea protein for starter culture screening

The optimum condition was selected from the preliminary experiment, i.e., N2_1 (pH 7.5 and 1:6 pea flour-water ratio). This extraction experiment was upscaled by an upscaling factor of ~13 (8 liters). Pea flour was solubilized in a ratio of 1:6 with water at pH 7.5. After 70 minutes, the samples were centrifuged at 3000 x g for 8 minutes at 21 °C. The supernatant was weighed and collected in different bottles, and the precipitate was eliminated. The supernatant was stored at -20 °C in sterile bags. This supernatant was used as a base fermentation matrix for starter culture screening. It was thawed at +4 °C for 3 days before the starter culture screening experiment.

3.2.1.3 Starter culture stock preparation

Starter cultures were resuspended in 0.85% saline solution and made fresh on every experimental day. 20 DCU dose of starter culture was recommended by the producer for best results. We took the weight and DCU per pack from the package labels and then calculated the amount needed for the stock solution, which was 100x concentrated. The inoculation rate was ~1% (0.96%) of the stock solution (0.5 mL for 51.5 g). Stock solutions were prepared in sterile 100 mL glass bottles. Starter cultures were weighed in a sterile 50 mL tube. To prepare one stock, 50 mL of 0.85% sterile saline solution was poured into a tube with starter culture powder, well mixed with a Vortex, then poured into the 100 mL bottle. This was repeated one more time to makeup 100 mL volume. The bottle was then vortexed until the powder was well dispersed. The bottles were stored in +4 °C until sample inoculation.

3.2.1.4 Starter culture screening

We planned to use 3 major analyses for screening. We analysed growth dynamics (heat flow vs. time curve) by isothermal microcalorimeter, pH graph w.r.t. time obtained from iCinac instrument, and informal sensory reviews given by a trained panellist.

In the experiment, we had five starter cultures and a control sample (without starter culture) subdivided into two groups of temperatures (40 °C and 30 °C). Then, each temperature group had two divisions, one with 0.5% added sucrose and one without sucrose. Table sugar

was added as a source of sucrose. The microcalorimeter and iCinac experiment were performed simultaneously. We have performed this experiment in biological duplicates.

Initially, all glass bottles and microcalorimeter vials were autoclaved. Before the experiment, pH probes in the iCinac instrument were calibrated with a standard pH (4 & 7) buffer. All probes were sterilized with an antibacterial solution. The microcalorimeter was also calibrated at the same temperature as the experiment, and the baseline was corrected.

The pea protein solution was defrosted at +4 °C. For the sample with sucrose, sucrose was mixed properly with pea protein matrix in a big bottle and then 51.5 g was poured into 100 mL bottles for the iCinac experiment.

After arranging everything, the experiment was started by pre-warming the samples in a water bath for iCinac. After attaining 40 °C temperature, samples were inoculated with 0.5 mL of respective starter cultures according to plan and well mixed. At the same time, after inoculation, 2 mL of inoculated sample was taken out and pipetted into a vial and capped hermetically for microcalorimeter analysis. pH probes were sterilized with an antibacterial solution, rinsed with sterile water, and inserted into the bottles. Then, the vials were hung in microcalorimeter wells per the protocol. The calorimeter experiment was run for 40 hours, and pH monitoring by iCinac was done for 18 hours.

After finishing the experiments, the pH vs. time graph was plotted from iCinac data, the heat flow vs. time graph was plotted from microcalorimeter data, and a trained sensory panellist performed sensory analysis on the iCinac samples and recorded observations.

We aimed to select 2 starter cultures out of 5 by considering the acidification rate (it must reach pH 4.8-4.6), growth dynamics (analysed heat flow curves), and a brief informal sensory evaluation of the fermented sample (pea-like smell and taste, any other off-note, colour, viscosity, etc.).

3.2.2 Large-scale protein extraction and fermentation

From the preliminary experiments we selected optimal protein isolation and fermentation conditions: pH 7.5 at 1:6 flour:water, SC_1 and SC_2 fermented at 40 °C without added

sugar. An experimental scheme was prepared (Figure 5) where protein was precipitated by using HCl or fermentation by two starter cultures from the same initial protein supernatant.
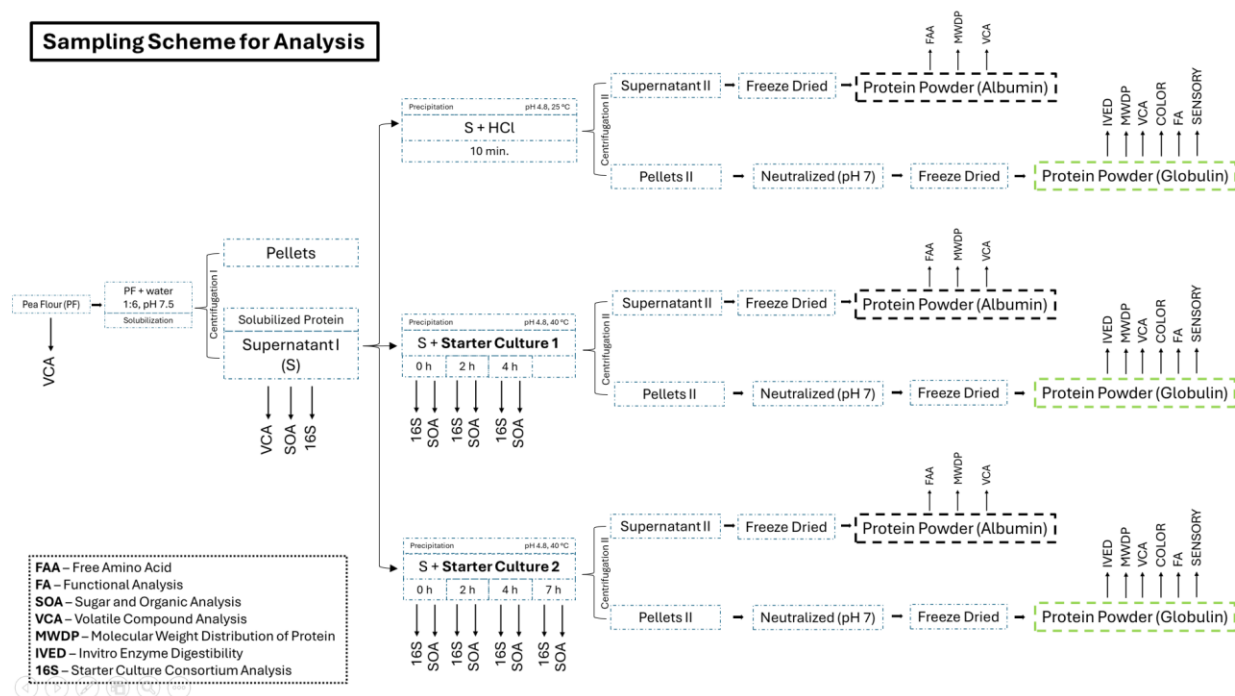
Figure 5. *A scheme for the large-scale protein extraction experiment and sampling points for various analyses listed in the bottom left corner.*

As also shown in Figure 5, we preplanned the sampling for various analyses. We took a sample from pea flour, supernatant I (S) and albumin and globulin protein powder of each treatment for volatile compound analysis. 2 samples from supernatant I and 2 samples in every 2 hours (0 h, 2 h, 4 h and/or 7 h) from precipitation step of fermentation experiments (S+SC_1 & S+SC_2) were taken for sugar & organic acid and starter culture consortium analyses which revealed sugar consumption and organic acid formation or degradation during fermentation and abundance of bacterial species. A sample from albumin protein powder of each treatment was taken for free amino acid analysis which shows degradation of protein during fermentation. As free amino acid is soluble in water and separated in supernatant part after second centrifugation, the globulin precipitate was not analysed. One sample from albumin and globulin protein powder of each treatment were taken to analyse

molecular weight distribution of protein. Functional analysis (includes water and oil holding capacity, foaming capacity and stability, emulsion activity and stability, surface hydrophobicity, water solubility index, protein solubility), in-vitro enzymatic digestibility, colorimetry, and sensory analysis were performed for globulin protein powders.

3.2.2.1 Preparation of solubilized pea protein

13800 g of distilled water at room temperature was put into a 25 L plastic bucket, and then 2300 g of pea flour was poured into the water while stirring. We measured the starting pH and added 2M NaOH until pH 7.5. The bucket was stirred with a vertically mounted stirrer (IKA EUROSTAR 60) at 150 rpm at 23 °C for 60 minutes. Every 5 minutes, the pH was corrected. After extraction, the suspension was split into centrifuge bottles and centrifuged at 3000 x g for 8 min at 21 °C. Supernatants and sediments were collected into buckets, and their weights were recorded. The supernatant was poured through a 250 µm sieve to remove some floating flakes. The precipitate was eliminated. The remaining supernatant was then split into three parts of ca 4 kg. The first part was precipitated with HCl, and the other two were inoculated with SC_1 and SC_2 and fermented.

3.2.2.2 Starter culture stock preparation

The starter cultures were prepared fresh during the protein extraction. The preparation protocols were followed exactly as described in the preliminary experiments section.

3.2.2.3 Protein precipitation - Fermentation

Took two 5 L bottles (autoclaved) and poured 3960 g of supernatant into each, then put these bottles into preheated water baths to warm them up until 40 °C (took 25 min to warm up). Tracked the temperature with long glass thermometers sterilized with EtOH and washed with sterile water. Bottles were covered with aluminium folium. Then put sterilized long magnetic stirrer rods into the bottles and put them onto magnetic stirrer plates and started mixing. Then we inoculated each bottle with 40 mL of stock solution of starter culture, mixed well. The starting time was recorded. Then inserted presterilized pH sensors into the bottles and measured and recorded the starting pH. Put the bottles into the oven onto magnetic stirrers with medium rotation speed. The bottle openings were covered with aluminium foil,

which also held the pH sensors. Fermentation pH was recorded every 5–10 minutes. Fermentation lasted until the pH dropped below 4.8 and then stopped by simultaneous cooling and centrifugation. The suspension was then centrifuged at 3000 x g for 10 min 21 °C. Supernatant and precipitate were collected into a plastic pitcher and weighed.

3.2.2.4 Protein precipitation – Chemical

Chemical precipitation started with ca 4 kg of supernatant. 4M HCl was added until pH was 4.8, recorded added HCl amount and end pH. Protein colour changed from light brown to cream white as soon as pH dropped below ca 6.5. Suspension was centrifuged at 3000 x g for 10 min 21 °C. Supernatant and precipitate were collected into a plastic pitcher and weighed.

3.2.2.5 Precipitate neutralization – Chemical.

Distilled water was added to the precipitate (1:1, water: precipitate) and mixed with spoons. Added 2M NaOH until pH reached 7.0, mixed well. Poured the neutralized suspension into pre-weighed boxes, bottles washed with a bit of distilled water to get the remaining protein, put into -40 °C freezer and lyophilized.

3.2.2.6 Freeze drying

Samples were frozen and then dried in a -96 °C lyophilizer (SCANVAC COOLSAFE, Labogene, Blovstrød, Denmark). After drying measured the weight of all samples. Powders were collected into zip-lock bags, mixed. All functional analyses (WHC, OHC, WSI, FA, FS, EA, ES, PS, SH), colorimetry, in-vitro enzymatic digestibility, free amino acid, molecular weight distribution analysis, gas chromatography, and sensory analysis were analysed using the freeze-dried powders.

3.2.3 Starter culture consortium

3.2.3.1 Microbial cell separation and genomic DNA extraction

Microbial cells from samples were isolated aseptically. For that, 10 mL of each sample was diluted in 40 mL of sterile 0.85% NaCl, 0.05% Tween 20 solution (Sigma), vortexed thoroughly, and centrifuged at 300 × g for 10 min at 6 °C (Hettich ROTANTA 460R, fixed angle

rotator). To pellet the microbial cells, the supernatant was transferred to a new 50 mL tube and centrifuged at 5000 × g for 15 min at +6 °C (Hettich ROTANTA 460 R, fixed angle rotator). The pellet was washed in 2 mL of sterile 0.85 % NaCl solution, transferred to a 2 mL tube, and centrifuged at 10000 × g for 10 min at room temperature (Sigma1-14 microcentrifuge). The supernatant was aspirated, and the pellet containing the microbial cells was stored at -20 °C until gDNA extraction.

For gDNA extraction, cells pellets were re-suspended in 250 µL 1 x PBS and subjected to gDNA extraction by ZymoBIOMICS™ DNA Miniprep Kit (Zymo Research) according to the manufacturer's instructions. The concentrations of the extracted DNAs were quantified by a Qubit™ 3 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using Qubit dsDNA HS and BR Assay Kits (Thermo Fisher Scientific).

3.2.3.2 16 S library preparation, next-generation sequencing, and data processing

Amplicon libraries targeting the V4 region of the 16S rRNA gene by the primer pair 515F/806R were prepared according to Illumina's dual indexing protocol as published in (Kazantseva et al., 2021). Multiplexed and normalized libraries were sequenced with iSeq100 Sequencing System (Illumina, San Diego, CA, USA) using iSeq 100 i1 Reagent v2 and 2 × 150 cycles paired-end sequencing protocol. DNA sequence data were analysed as published before (Espinosa-Gongora et al., 2016; Kazantseva et al., 2021; McDonald et al., 2016) by open-source BION-meta program (https://github.com/nielsl/mcdonald-et-al, Danish Genome Institute, Denmark) according to the author's instructions.

3.2.4 Sugars and organic acids

Liquid samples collected during fermentation were vortexed for 30 s, then centrifuged at 14,000 × g for 10 min at room temperature. The supernatant was filtered through a prewashed 3 kDa molecular weight cut-off filter (Amicon® Ultra-0.5, Merck KGaA, Germany). Concentrations of sugars and organic acids were measured with a high-performance liquid chromatography (HPLC) system (Alliance 2695 system, Waters Corp., Milford, MA, USA) equipped either with an Aminex HPX-87P column for sugars or Aminex HPX-87H for organic acids (7.8 × 300 mm, 9 µm particle size, Bio-Rad Laboratories, Inc., CA, USA). A BioRad

Micro-Guard Cation P guard column (4.6 × 30 mm, 9 μm particle size) with isocratic elution of ultrapure water at a flow rate of 0.6 mL/min at +85 °C was used for sugar analysis. H guard column (4.6 × 30 mm, 9 μm particle size) with isocratic elution of 5 mM H_2SO_4 at a flow rate of 0.6 mL/min at +35 °C was used for organic acid analysis. Waters 2414 refractive index detector was used for the detection and quantification of substances, which was paired with a Waters 2487 Dual Absorbance Detector for organic acid analysis. Standards that were used in the analysis are listed in Table 5.

Table 5. *Standards used in the HPLC analysis.*

Compound	Supplier	Purity	CAS number
Acetic acid	Honeywell Fluka	≥98%	64-19-7
Citric acid	Sigma-Aldrich	≥98%	77-92-9
Malic acid	Sigma-Aldrich	≥98%	6915-15-7
Succinic acid	Sigma-Aldrich	≥98%	150-90-3
Lactic acid	Acros Organics	≥98%	867-56-1
Formic acid	Honeywell Fluka	≥98%	141-53-7
Propionic acid	Sigma-Aldrich	≥98%	79-09-4
Isobutyric acid	Acros Organics	≥98%	79-31-2
Butyric acid	Sigma-Aldrich	≥98%	107-92-6
Isovaleric acid	Acros Organics	≥98%	503-74-2
Valeric acid	Alfa Aesar	≥98%	109-52-4
D-Gluconic acid solution	Sigma-Aldrich	45-50% in Water	526-95-4
D-(+)-Galacturonic acid monohydrate	TCI	>=97.0%	91510-62-2
D-Glucuronic acid	Megazyme kit	-	12/3/6556
Fumaric acid	Acros Organics	99+%	110-17-8
D-(+)-Mannose	Sigma-Aldrich	≥99%	3458-28-4
D-Fucose	Carbosynth	≥97%	3615-37-0
Stachyose hydrate	Acros Organics	≥97%	54261-98-2
D-(+)-Galactose	Sigma-Aldrich	≥98%	59-23-4
D-Fructose	Supelco	≥98%	57-48-7
N-acetyl-D-glucosamine	Sigma-Aldrich	≥98%	7512-17-6
Mannitol	Supelco	≥98%	69-65-8
Sucrose	Supelco	≥98%	57-50-1
D-Glucose	Supelco	≥98%	50-99-7
D-(+)-Xylose	Sigma-Aldrich	>=99%	58-86-6
N-acetyl-D-galactosamine	Carbosynth	>=98.0%	1811-31-0
Ethanol	Honeywell Riedel-De-Haen	≥98%	64-17-5

3.2.5 Molecular weight distribution of proteins

Samples (0.1 g) were weighted into 25 mL flasks and dissolved with MilliQ water in ultrasonic bath (VWR ultrasonic cleaner) during 15 min. Then samples were centrifuged at 14,000 × g for 10 min at room temperature (MicroCL 21R Centrifuge, Thermo Fisher Scientific). The supernatant was filtered through a 0.22 µm PTFE filters (Millipore S.A.S., Malsheim, France). Molecular weight markers were ferritin from equine spleen Type I in saline solution - F4503-25MG Sigma-Aldrich (Steinheim, Germany) with molecular weight 480 000 Da, α-Lactalbumin (from bovine milk Type I, ≥85% (PAGE), lyophilized powder - L5385-25MG) Sigma-Aldrich (Steinheim, Germany) - 14 000 Da, aprotinin (from bovine lung lyophilized, ~80% (HPCE), crystalline (fine), white, ≥3500 U/mg - 10820-25MG) Sigma-Aldrich (Steinheim, Germany) – 6500 Da, vitamin B12 – 1350 Da, and glutathione 307 Da.

Analysis was performed on an ultra-performance liquid chromatography (UPLC) system (Acquity UPLC; Waters Corp., MA, USA), including a binary solvent manager, a sample manager, and a photodiode array detector (PDA), controlled by Waters Empower™ 3.0 software (Build 3471, Waters Corp., MA, USA). Separations were performed on Waters ACQUITY UPLC BEH SEC, 125 A, 1.7 µm (Waters, Milford, MA, USA) operated at +30 °C. The injection volume was 2.0 µL, flow rate 0.3 mL/min, and absorbance was recorded at 220 nm. The running time was 15 min. Mobile phase was 100 mM sodium phosphate buffer solution (pH 6.8). Empower software (Waters Corp., MA, USA) was used for data processing. The standard curve was constructed using linear regression on log(molecular weight) as Y and retention time as X.

3.2.6 Free amino acids

Samples (0.1 g) were weighted into 25 mL flasks and dissolved with MilliQ water in ultrasonic bath (VWR ultrasonic cleaner) during 15 min. Then samples were centrifuged at 14,000 × g for 10 min at room temperature (MicroCL 21R Centrifuge, Thermo Scientific). The supernatant was filtered through a 0.22 µm PTFE filters (Millipore S.A.S., Malsheim, France). Samples were diluted 10 times with 0.1 N HCl. Prior to injection, free amino acids were derivatized with 6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (Waters Corp., MA,

USA) according to the manufacturer's procedure. 0.1 mM selenomethionine was used as an internal standard. Analysis of free amino acids was performed on an ultra-performance liquid chromatography (UPLC) system (Acquity UPLC; Waters Corp., MA, USA), including a binary solvent manager, a sample manager, and a photodiode array detector (PDA), controlled by Waters Empower™ 3.0 software (Build 3471, Waters Corp., MA, USA). Separations were performed on Waters Acquity UPLC AccQ•Tag Ultra Column (2.1 × 100 mm, 1.7 µm particle size) operated at +55 °C. The injection volume was 1.0 µL, the amino acids were eluted at a flow rate of 0.7 mL/min, and absorbance was recorded at 260 nm. The running time was 11 min. Empower software (Waters Corp., MA, USA) was used for data processing.

3.2.7 Volatile compounds

Extraction of volatiles was carried out using solid phase microextraction (SPME). 60 mg of sample was weighed into a 10 mL SPME vial, equipped with a magnetic stirrer. 940 µL of milliQ water was added to the vial to make 6% solution in water. SPME fiber (30/50µm DVB/Car/PDMS Stableflex, length 1 cm) was used to adsorb/absorb volatile compounds from the headspace (HS) for 40 minutes at 50 °C. The absorbed/absorbed volatile compounds were subsequently desorbed into a GC injection port for 5 minutes.

Identification and quantification of volatile compounds was performed using gas chromatograph system (2030; Shimadzu, Kyoto, Japan) equipped with mass spectrometer (8050NX Triple Quadrupole; Shimadzu, Kyoto, Japan). A ZB5-MS column (30 m length × 0.25 mm i.d. × 1.0 µm film thickness; Phenomenex, Torrance, CA, USA) was used with helium as a carrier gas at linear velocity of 35 cm s^{-1}. The oven was programmed to ramp up from 40 °C at a rate of 5 °C/min to a temperature of 190 °C and at a rate of 25 °C/min from 190 °C to 280 °C with an additional holding time of 3 minutes (total run time 36 min). Mass spectra were obtained at an ionization energy of 70 eV with a mass-to-charge ratio scan range of 35 to 300. For each sample, 3 analytical replicates were made.

Non-targeted identification of volatile compounds was carried out using GCMS solution software (Shimadzu, Japan) and retention indices. Experimental retention indices were

calculated using the retention times of the eluting compounds normalized to the retention times of adjacent n-alkanes. The identification of the compounds was verified by comparing experimental retention indices to NIST17 and FFNSC libraries. Semi-quantitative approach against an internal standard (4-methyl-2-pentanol; 333 ppb) was used to quantify identified volatile compounds (in IS ppb-eq.).

3.2.8 In-vitro enzymatic digestibility

The protein content of lyophilized samples was determined according to the Kjeldahl method. The in-vitro protein digestibility (IVPD) was assayed by the methods of (HSU et al., 1977) and (Espinosa-Ramírez et al., 2018) with some modifications. Briefly, 10 mL of aqueous powder suspension containing 6.25 mg protein/mL was prepared. Samples were placed in a 37 °C water bath, and the pH was adjusted to 8.00 using 0.1 N NaOH and/or 0.1 N HCl while stirring. Trypsin at a concentration of 1.6 mg/mL was maintained in an ice bath, and the pH was adjusted to 8.00 with 0.1 N NaOH and/or 0.1 N HCl. Then, 1 mL of the enzyme solution was added to the protein suspension and continuously stirred at 37 °C. The trypsin had an activity of >6000 BAEE units/mg proteins. The pH drop was recorded at 15 seconds after the addition of the enzymatic solution and at 1-minute intervals for 10 min. The IVPD percentage was calculated using Eq. (1) (HSU et al., 1977), where x is the end pH after 10 min.

$$y = 210.464 - 18.1x \quad \text{(Equation 1)}$$

3.2.9 Functionality analysis

3.2.9.1 *Water and oil holding capacity and water solubility index*

The methods for measuring oil-holding capacity (OHC), water-holding capacity (WHC), and water solubility index (WSI) at room temperature were adopted from (Jakobson et al., 2023). Briefly, 1 g of powder was suspended in 10 mL of distilled water or rapeseed oil, gently mixed for 30 min at room temperature, and centrifuged at 10,000 × g for 15 min; the supernatant was carefully removed by using pipets without disturbing the pellet layer and the remaining precipitate was weighed. WHC and OHC were expressed as the weight of water or oil held by 1 g of powder dwb (dry weight basis). The water supernatant was collected and oven-

dried to calculate the WSI, expressed as the percentage of dissolved solids to the initial powder weight (dwb).

$$Water\ Holding\ Capacity\ (WHC)\ [g/g] = \frac{weight\ of\ water\ absorbed\ by\ sample}{weight\ of\ sample\ taken\ (dwb)} \times 100$$

$$Oil\ Holding\ Capacity\ (WHC)\ [g/g] = \frac{weight\ of\ oil\ absorbed\ by\ sample}{weight\ of\ sample\ taken\ (dwb)} \times 100$$

$$Water\ Solubility\ Index\ (WSI)[g/g] = \frac{weight\ of\ disolved\ sample\ in\ supernatant}{weight\ of\ sample\ taken\ (dwb)} \times 100$$

3.2.9.2 Foaming capacity and stability

Foams were prepared by dispersing 0.20 g of powder in 20 mL of distilled water in a 50 mL centrifuge tube, following the protocols (Jakobson et al., 2023; Sharma et al., 2023) with some modifications. The samples were frothed at room temperature inside a 50 mL graduated centrifuge tube using a Polytron PT 2100 homogenizer (Kinematica AG, Malters, Switzerland) equipped with a ⌀ 12 mm probe at a speed of 22,000 rpm for 1 min. Foaming capacity (FC) was calculated by measuring the height of the sample volume before (V_1) and the height of the foam after frothing (V_2). The change in foam volume (V_t) after 1 h of standing was recorded. Foam stability was calculated by dividing the height of foam (V_t) after 1 hour by the initial height of foam (V_2).

$$Foaming\ Capacity\ [\%] = \frac{v_2}{v_1} \times 100$$

$$Foaming\ Stability\ [\%] = \frac{v_t}{v_2} \times 100$$

3.2.9.3 Surface hydrophobicity

The surface hydrophobicity was evaluated according to the method of (Cao et al., 2016; Sharma et al., 2023) with some modifications, which is based on measuring complex formation at 585 nm, due to hydrophobic interactions between the basic and aromatic amino acids residues of the proteins and the anionic form of Coomassie Brilliant Blue G - 250 (CBBG) stain. This method was standardized for partially soluble proteins. Weigh the powder sample into a 1.5 mL tube by maintaining a concentration of 5 mg/mL of actual protein content to make a 1.2 mL protein solution in 20 mM phosphate buffer at pH 6.0. Vortex the tube till the whole sample gets mixed thoroughly. Add 300 µL of a CBBG solution (0.1 mg/mL) prepared in ultra-pure water. A control sample (without protein) was prepared by combining 1.2 mL of the phosphate buffer with 300 µL of the CCBG solution. A sample blank (without dye) was also prepared by adding the exact amount and concentration of the sample added with 1.2 mL of the phosphate buffer. Samples were agitated in a multi-shaker (Thermo Mixer C, Eppendorf, Connecticut, United States) at 2000 rpm for 4 minutes at room temperature and then centrifuged at 2000 g for 10 min at 4 °C. The supernatant was transferred to another 1.5 mL tube without disturbing the pellet using a pipette and centrifuged again under the same conditions. Finally, the supernatant absorbance at 585 nm was measured using a UV-VIS spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) by base zeroing with blank (Phosphate buffer).

CBBG bound amount was calculated using the following:

$$\text{CBBG Bound [µg]} = \frac{Ab_{\theta\,\text{Control}} - Ab_{\theta\,\text{Sample}} + Ab_{\theta\,\text{Sample Blank}}}{Ab_{\theta\,\text{Control}}} \times 30 \text{ µg}$$

3.2.9.4 Emulsion activity and stability

Emulsions were prepared by dispersing 0.24 g of powder in 12 mL of distilled water and 12 mL of sunflower oil, following the protocols of (Sharma et al., 2023)(Jakobson et al., 2023).

First, samples were mixed at room temperature inside a 50 mL graduated centrifuge tube using a Polytron PT 2100 homogenizer (Kinematica AG, Malters, Switzerland) with a ⌀ 12 mm probe at 11,000 rpm for 1 min. Subsequently, to determine emulsion activity (EA), the samples were centrifuged for 5 min at 1100× g at 20 °C. EA was calculated by measuring the height of the emulsified layer (H_1) and the total height of the liquid (H_T). For the determination of emulsion stability after heat treatment (ES), samples were first heated in a water bath at 80 °C for 30 min, then cooled in an ice-water bath for 15 min, and finally centrifuged for 5 min at 1100× g at 20 °C. For the ES calculation, the height of the emulsified layer after 30 min (H_2) was recorded and divided by the height of the emulsified layer (H_1).

$$Emulsion\ Activity\ [\%] = \frac{H_1}{H_T} \times 100$$

$$Emulsion\ Stability\ [\%] = \frac{H_2}{H_1} \times 100$$

3.2.9.5 Protein solubility

Protein solubility was determined by the protocol described in (J. Yang et al., 2021) with some modifications. 0.3 g of lyophilized powder was mixed with 10 mL 0.02 M phosphate buffer PB (pH 7). The mixture was vortexed vigorously and mixed in a tube rotator for 30 minutes at room temperature. Then centrifuged at 4636 x g for 10 minutes at 23 °C. The supernatant was collected in another tube, and its protein content was determined by the Lowry method using Modified Lowry Protein Assay Kit (ThermoFisher Scientific, Waltham, MA, USA) following the instructions by the manufacturer. Protein solubility was calculated by taking the ratio of protein concentration in the supernatant to the actual protein concentration in the initial suspension calculated from the Kjeldahl analysis.

$$Protein\ Solubility\ [\%] = \frac{Protein\ conc.\ in\ supernatant}{Protein\ conc.\ in\ intial\ suspension} \times 100$$

3.2.10 Colour

Samples were placed in 8 cm × 8 cm transparent plastic mini-grip bags, forming a 5-mm-thick layer, and the colour was measured at three different spots using a portable spectrophotometer (NS810, 3nh Shenzhen Threenh Technology Co., Ltd., Shenzhen, China) set to measure in the CIELAB colour space at D65 and 2°.

3.2.11 Sensory analysis

The sensory evaluation of the products was carried out by 8 assessors (average age 33 ± 8) with previous training and experience in evaluating similar types of samples. Prior to the assessment sessions, additional training was conducted with selected samples to allow the assessors to familiarize themselves with the samples and to refine the assessment method if necessary. The final assessment method was based on relevant scientific sources, developed in-house protocols, and discussions with the sensory panel. The samples were prepared as 6% water dispersions in filtered drinking water and served at room temperature (21 °C) in transparent sniffing glasses with cover slips. The evaluation was performed independently by the assessors. All samples were coded with random three-digit codes and the order of assessment was randomized for each assessor according to the Williams' Latin Square design. The evaluation was carried out in a dedicated sensory room where any external factors that could interfere with the evaluation were eliminated, in accordance with ISO 8589:2007.

The descriptive sensory analysis (Quantitative Descriptive Analysis) used a 10-point scale, where 0 - "none", 1 -"very weak", 5 - "moderate" and 9 - "very strong". The samples were assessed in two replicates in one session. The evaluation started with odour modality, followed by taste and texture. The attributes and their definitions are shown in Table 6. During the evaluation, additional comments on all modalities could be added if desired. The assessors had a reference sample (another commercial pea protein isolate) in all the assessment sessions, to help them navigate the scale better. Data collection was carried

out using RedJade sensory software version 6.1.1 (RedJade Sensory Solutions LLC, Martinez CA, USA).

Table 6. *The attributes and their definitions used in the sensory analysis*.

Attribute	Definition	Scale 0-9
ODOUR		
Overall intensity	Overall odour intensity	
Characteristic raw material	Odour associated with a specific raw material (e.g. cereals, legumes).	
Sour	Perceivable sourness associated with vinegary, yoghurt-like, fermented, citric, etc	
Sweet	Perceivable sweetness associated with sugar, caramel, toffee, etc.	
Off-odour intensity	Non-characteristic odours (aged, chemical, dusty, rancid, metallic, animal, earthy, papery, etc.).	
TASTE		
Overall intensity	Overall taste intensity	
Characteristic raw material	Taste associated with a specific raw material (e.g. cereals, legumes).	0 - none; 1 – very weak; 5 – moderate; 9 – very strong
Sour	Perceivable sourness associated with vinegary, yoghurt-like, fermented, citric, etc.	
Sweet	Perceivable sweetness associated with sugar, caramel, toffee, etc.	
Salty	Perceivable saltiness associated with table salt, minerals, etc.	
Bitter	Perceivable bitterness associated with caffeine, quinine, etc.	
Astringent	Perceivable astringency (dry, sharp, mouth puckering)	
Off-taste intensity	Non-characteristic taste (aged, chemical, dusty, rancid, metallic, animal, earthy, papery, etc.).	
Aftertaste intensity	Perceived aftertaste intensity of the sample 5 s after swallowing	
TEXTURE		
Graininess (size)	Size of perceivable grains in the sample	0 - none; 1 – very small; 5 - moderate; 9 – very big

Graininess (amount)	Amount of perceivable grains in the sample	0 – no particles; 1 – few particles; 3 – some particles; 5 – several particles; 7 – many particles; 9 – mostly particles

3.2.12 Statistical analysis

Preliminary fermentation in iCinac and microcalorimeter were performed in duplicates. The protein content of all samples by the Kjeldahl method, in-vitro enzymatic digestibility, water and oil holding capacity, water solubility index, foaming capacity and stability, emulsion activity and stability, surface hydrophobicity, protein solubility and colourimetry were measured in triplicates. The starter culture consortium analysis was analysed by single samples, and sugar, organic acids, molecular weights distribution of protein, free amino acid and sensory assessment were performed in duplicates. Statistical analysis was done in IBM SPSS Statistics Version 29.0.0.

4 Results and discussions

4.1 Small-scale preliminary experiment results

4.1.1 Optimized protein extraction process

Four experiments were conducted to screen out the best protein extraction conditions, varying the extraction pH and water-to-flour ratio (Table 7). Three pH values (7.5, 8.0, and 8.5) and two ratios (1:6 and 1:8) were planned. The best conditions were analysed based on the final protein content, protein isolate yield (PIY), and protein recovery (PR). The initial pH of the pea flour and water solution was between 6.40 and 6.60, which was close to a neutral pH of 7. For protein solubilization the pH was increased with NaOH to the planned value and then readjusted every 20 minutes until the end of extraction after 1 hour and 15 minutes. It was observed that with the 3rd addition of NaOH, the pH of the solution became stable, and the reason was that when protein solubilizes completely, the pH becomes stable. It is important to note that the acidification method, solubilization pH, and dilution ratio affect the final product's yield, protein content, and recovery. Among all protein isolates obtained by various conditions, the highest protein content was 82.82% in the N2_4 sample (pH 7.5, 1:8). Whereas the highest protein isolate yield and protein recovery was seen in N2_3 (pH 8.5, 1:6), i.e. 17.78% and 61.22%, respectively, and lowest was observed in N2_1 sample. This can be due to the fact that at higher alkaline pH, protein solubility increases, but at very high pH it can also cause protein denaturation and dissolve starch (Arntfield & Maskus, 2011; Reinkensmeier et al., 2015). Higher alkaline pH caused more browning in the final protein isolate, which is undesirable. High pH also does not favour bacteria-growing conditions to start fermentation. The dilution ratio was also considered an important factor in commercial scalability; a higher dilution ratio means higher operational costs, which is also not a good option for the industry. It was observed that for all trials, protein content, recovery, and isolate yield were statistically different, but practically, the difference is negligible or does not impact commercial production. According to the aim of this research, the lowest pH values with similar or higher protein content, protein isolate yield and recovery were required. Thus, the sample at a lower pH and water-to-flour ratio, i.e. N2_1 (pH 7.5,

1:6), was selected for further extraction experiments. Though N2_1 has low PIY (16.91%) and PR (58.61%), according to the authors, this difference compared to other samples will not affect the further experiment significantly and was negligible for this work.

Table 7. *Preliminary protein extraction experiments and optimized experiment results (chemical precipitation).*

Sample Name	Solubilization				Precipitation			Results							
	Extraction pH	Flour : Water ratio	Start pH	End pH	Acidification by	Start pH	End pH	Dry matter content (%)	Protein content (%) dwb	Protein Isolate yield (%) dwb	Protein recovery (%) dwb	L	a	b	Colour
N2_1	7.5	1:6	6.41	7.5	HCl	7.46	4.58	93.2	82.00	16.91	58.61	78.76	1.34	19.69	
N2_2	8	1:6	6.42	7.99	HCl	7.89	4.57	93.5	82.70	17.23	60.23	76.38	2.53	19.19	
N2_3	8.5	1:6	6.44	8.49	HCl	8.44	4.58	94.1	81.47	17.78	61.22	74.79	2.93	17.31	
N2_4	7.5	1:8	6.54	7.49	HCl	7.45	4.58	93.8	82.82	17.36	60.76	80.21	1.41	17.83	
Additional Protein Extraction (at optimised parameters) for Starter Culture Screening															
N3_1	7.5	1:6	6.44	7.49	HCl	7.48	4.57	95.8	80.08	17.71	59.93	79.14	1.48	19.52	

The optimized extraction condition at pH 7.5 and 1:6 water-to-flour ratio (N3_1), was applied to prepare additional protein solution for starter culture screening by small-scale fermentation. This time, extraction was performed on an 8-liter scale (scaled up by 13 times). The supernatant part was separated by centrifugation and then used as a protein solution in fermentation experiments for the screening process. The supernatant had 2.9% protein content and 5.4% dry matter content. A part of this solution was processed further for precipitation and neutralization steps to analyse the protein content, yield, and recovery.

Protein content, protein isolate yield, and protein recovery were recorded as 80.1%, 17.7%, and 59.9%, respectively. Protein recovery and yield were slightly enhanced, but fraction purity was lower; nevertheless, the parameters were approximately similar to those of the small-scale experiment. Small variations can be due to the scaling-up process. No

significant change in colour was observed in the sample compared to the small-scale experiment.

4.1.2 Starter culture screening and fermentation kinetics

Two experiments (microcalorimetry and pH monitoring by iCinac) were conducted to screen the starter cultures. Five different stater cultures (SC_1, SC_2, SC_3, SC_4 & SC_5) and control samples (do not contain any starter culture) at two temperatures (30 and 40 °C) and two sucrose % (0% and 0.5%) were analysed in duplicates. The microcalorimeter experiment was run for 40 h, and pH monitoring by iCinac was carried out for 18 h to observe fermentation dynamics. Heat flow curves and pH-time graphs were obtained from the analysis. At 40 °C temperature, as seen in Figure 6, SC_1, SC_4, and SC_5 were the fastest starter cultures and didn't show any lag phase. Whereas SC_2 and SC_3 were slightly slower than the other three starter cultures. Ideally, the control sample should not show any heat flow values (as no starter culture was added), but interestingly, it was showing. It was due to the presence of native bacteria present in flour. Native bacteria were alive in the protein solution as the method did not involve a pasteurization step. Regardless, inoculated samples finished fermentation before the control started to ferment. SC_1, SC_5, SC_2, and SC_3 displayed single peaks during fermentation. However, in the control sample, three small peaks were observed, which can be due to metabolic switch to a different nutrient or a shift in bacterial consortium. Sugar-added samples showed higher heat flow values Figure 7, which means a greater amount of metabolic products were produced, but there was no significant improvement in the acidification rate. pH monitoring data revealed that SC_1 was the fastest acidifier among all samples and reached pH 4.8 in 220 min (3.67 h). Whereas SC_4, SC_5, SC_2, and SC_3 took 274, 274, 348, and 424 minutes. The time taken by samples with sugar was not significantly different compared to those with no sugar, as shown in Figure 8. This means that sugar addition did not enhance the acidification rate to reach pH 4.8. Whereas SC_3 was the most acidic sample after 18 hours, it could be due to the presence of acid-tolerant LAB strains. It is important to note that after 18 hours, every sample with additional sugar was more acidic than the sample without sugar (Figure 9), and

thus, it can be concluded that sugar played an important role in acid production in a longer fermentation process.

Figure 6. *Heat flow curve for control and SC_1, SC_2, SC_3, SC_4, and SC_5 samples at 40 °C without sugar.*

Figure 7. *Heat flow curve for control and SC_1, SC_2, SC_3, SC_4, and SC_5 samples with and without sugar at 40°C.*
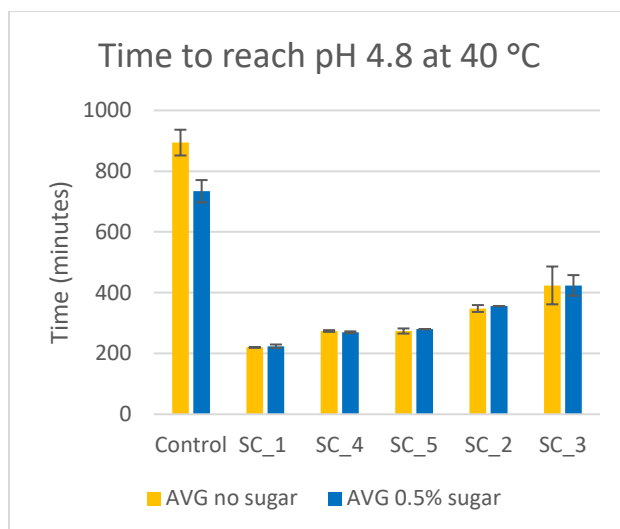
Figure 8. *The time taken by samples (with and without sugar) to reach pH 4.8 at 40 °C.*

Figure 9. *The pH values of samples (with and without sugar) after 18 hours of fermentation at 40 °C.*

In sensory assessment, the control sample had a clear pea-like smell and taste. SC_1 had a sour vegetable or cabbage and a weak pea-like smell, whereas it had a strong acidic and weak pea-like taste. SC_4 and SC_5 had similar smell profiles but had a strong pea-like taste. SC_2 had the most neutral smell and weak intensity of sourness & pea-like smell. It had strong acidic like tartaric acid and a roasted taste. SC_3 had a neutral but spoiled milk-like smell, and it was the most acidic sample with no pea-like taste. Interestingly, a pinkish colour was observed in the sample after fermentation.

Some variations were also observed at 30 °C temperature; SC_1, SC_4, SC_5, and SC_2 were the fastest starter cultures and showed a small lag phase in the growth curve, as shown in Figure 10. Whereas SC_3 was slower than the other four starter cultures. Similar to the 40 °C temperature the control showed a delayed heat flow. Higher heat flow was observed in the control sample compared to the starter culture samples. It was due to mesophilic native bacteria present in flour, which best grows in the temperature range between 30 to 38 °C. These mesophilic bacteria may possibly interfere with added starter culture, which deviates from our fermentation aim. SC_1 and SC_5 displayed a single peak during the fermentation period. However, in SC_2, SC_3, and the control sample, multiple small peaks and some fluctuations were observed, and it can be due to metabolism switch, consortia shift, or native bacteria interference. Sugar-added samples showed slightly higher heat flow values Figure 11, which means a greater amount of metabolic products were produced. Similar to 40 °C, pH monitoring data revealed that SC_1 was the fastest acidifier among all samples and reached pH 4.8 in 440 min (7.3 h), which was 2 times more than the time taken at 40 °C. Whereas SC_4, SC_5, SC_2, and SC_3 took 536, 508, 524, and 642 minutes. The time taken by samples with sugar was not significantly different compared to those with no sugar, as shown in Figure 12. This means that sugar addition did not enhance the acidification rate to reach pH 4.8 in this case, too. Whereas SC_2 was the most acidic sample after 18 hours, it could be due to the presence of acid-tolerant LAB strains. But after longer fermentation at 18 h point, all sugar-containing samples were found to be more acidic than the non-sugar samples Figure 13.
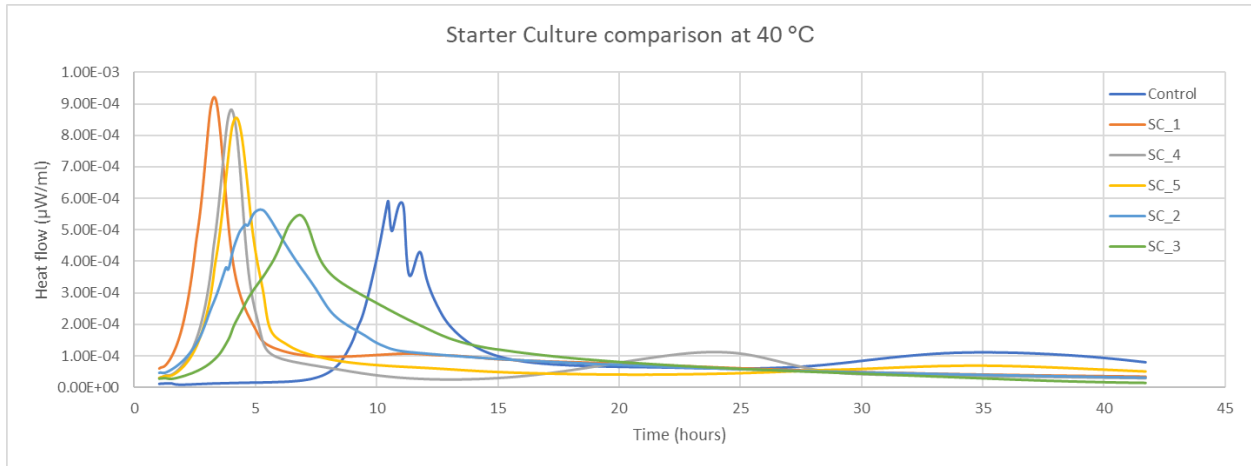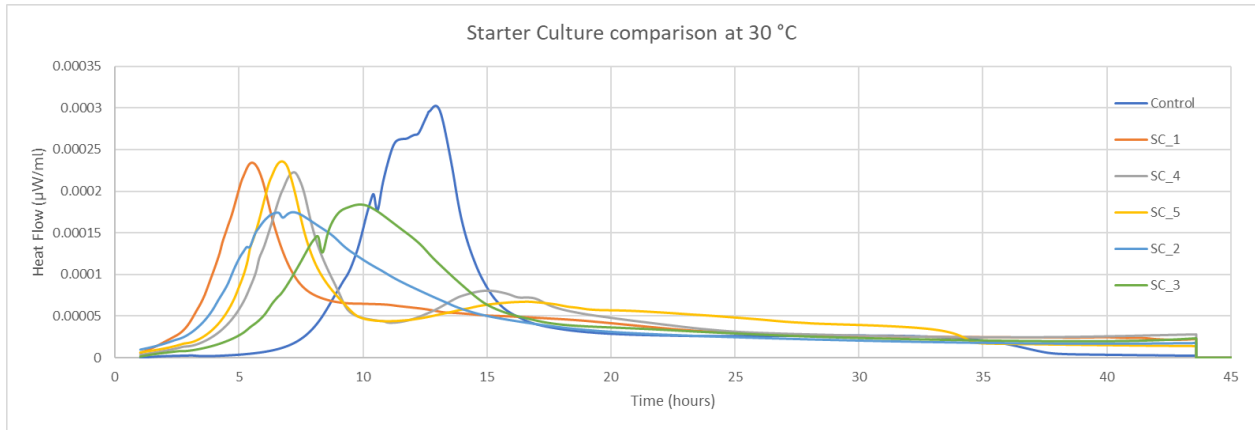
Figure 10. *Heat flow curve for control and SC_1, SC_2, SC_3, SC_4, and SC_5 samples at 30 °C without sugar.*
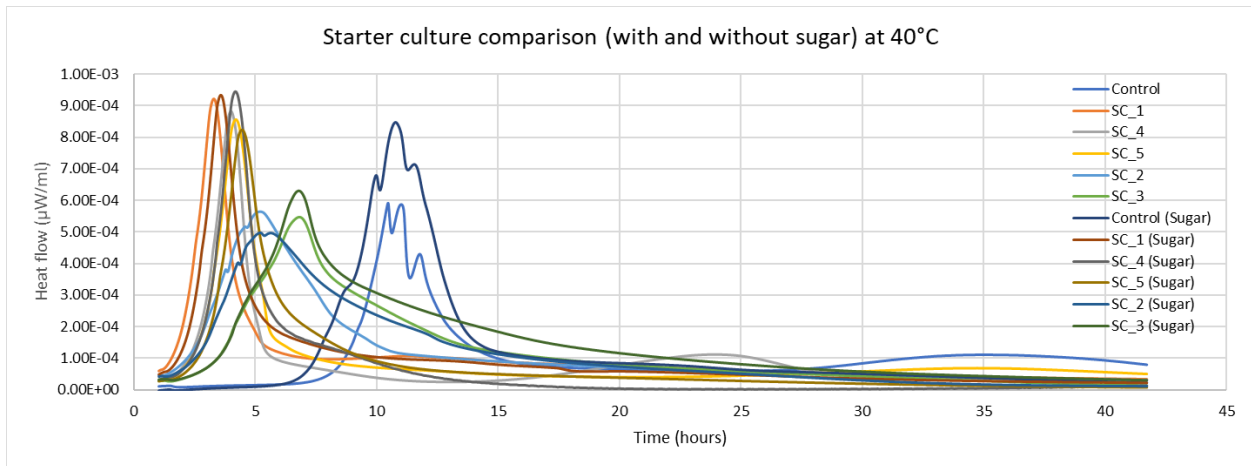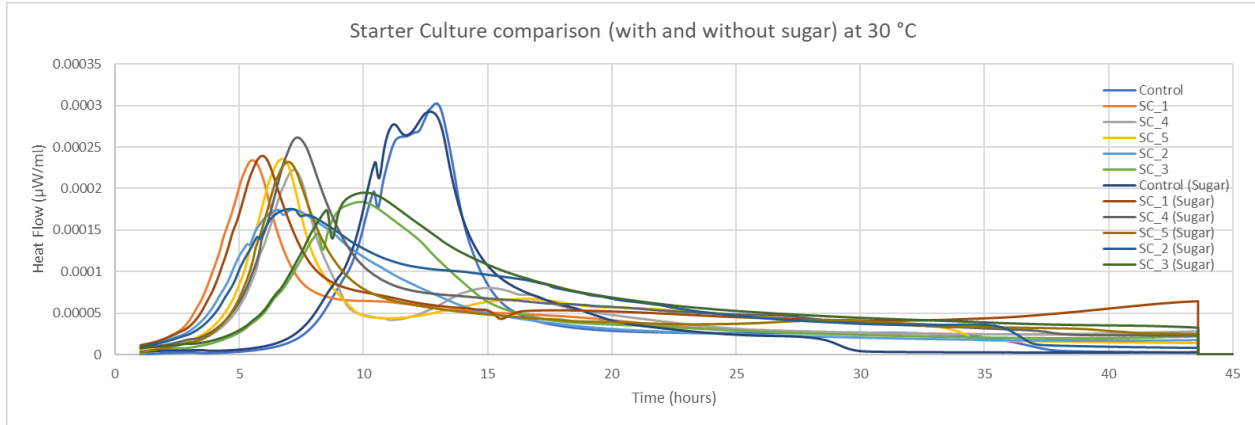

**Figure 11.** *Heat flow curve for control and SC_1, SC_2, SC_3, SC_4, and SC_5 samples with and without sugar at 30 °C.*

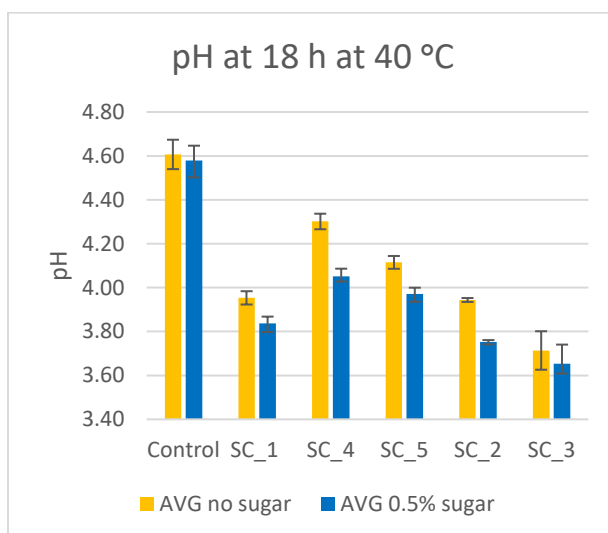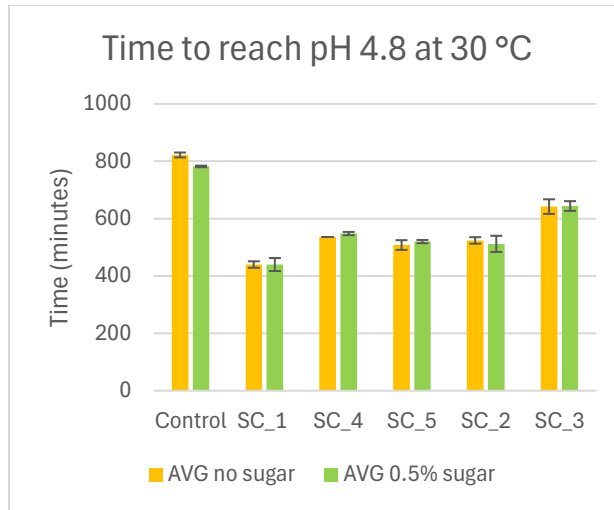**Figure 12.** *The time taken by samples (with and without sugar) to reach pH 4.8 at 30 °C.*
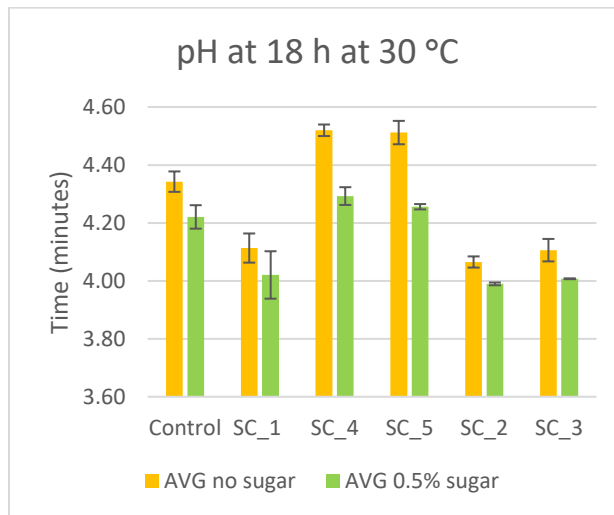
Figure 13. *The pH values of samples (with and without sugar) after 18 hours of fermentation at 30 °C.*

In sensory assessment, the control sample had a clear pea-like smell and taste. SC_1 had an acidic and weak pea-like smell, whereas it had a strong acidic but sweet note and a strong pea-like taste. SC_4 and SC_5 had similar weak pea and weak acid smell profiles but had mild pea-like taste. In aftertaste, bitterness and astringency were also noted. SC_2 had a weak pea and fermented cabbage or vegetable-like smell. It was the most acidic sample, like tartaric acid, which has bitterness & astringent aftertaste, and a pinkish colour was also observed in the fermented solution. SC_3 had a strong acidic but rotten egg-like smell, and it had strong acidic with a meat or bouillon-like taste.

After analysing several parameters for every sample, SC_1 was found to be the fastest starter culture at both temperatures, while SC_2 was the best sample in the sensorial aspect, meaning the least pea-like smell and taste. The temperature of 40 °C was preferred for fermentation because most of the bacteria in the starter culture are thermophilic, and at this temperature, fermentation was least affected by native bacteria. It was observed that the acidification rate was not improved by additional sugar. Thus, it was not opted for further experiments. In conclusion, SC_1 and SC_2 were selected for large-scale fermentation and will be studied deeply.

4.2 Large scale extraction

Large-scale experiments were carried out for three selected samples at a 4-liter capacity. SC_1 and SC_2 starter cultures were selected in the preliminary trial. Three globulin samples include Control_g (precipitated by HCl), SC_1_g (which was precipitated by SC_1 stater culture) and SC_2_g (which was precipitated by SC_2 stater culture). Corresponding albumin fractions that were not precipitated and remained in the supernatant are named Control_a, SC_1_a, and SC_2_a. In the solubilization step, 14 liters of pea flour-water slurry were initially prepared by maintaining 7.5 pH and approximately 12 liters of supernatant were recovered after centrifugation. The supernatant was then divided into three parts to prepare the control acidified by HCl and to ferment with SC_1 and SC_2. SC_1 sample took 235 minutes (3 hours & 55 mins) to reach pH 4.7 whereas SC_2 sample took almost double time 420 minutes (7 hours) to attain pH 4.8. These results are obvious because we selected two starter cultures, of which one was the fastest acidifier (SC_1) and the other one was the best flavour modifier but slow in acidification (SC_2). Before drying, the control sample had a smooth, buttery texture and was light yellow in colour. The fermented sample was slightly hard and compact in texture and darker in shade. The compact texture can be due to a change in the water absorption of protein powder (the precipitate had less water), and the dark appearance could be due to long hours of heating at 40 °C during fermentation, which may cause browning or Maillard reactions (Nursten, 2002). The protein content of fermented samples was slightly higher than the control sample reported in Table 8. Protein recovery of Control_g, SC_1_g and SC_2_g samples were similar and reported as 63.11%, 63.16%, and

63.72%. At the end of the experiment, pea protein isolates (protein content > 80%) were recovered. All three samples achieved similar protein isolate yield. In conclusion, fermentation-assisted acidification resulted in a similar yield and quality of protein as the conventional alkali method.

Table 8. Large-scale experiment conditions, fermentation time, protein concentration, protein yield, recovery, and colour values of precipitated globulin samples.

Sample Name	Solubilization				Precipitation				Results							
	Extraction pH	Water : flour ratio	Start pH	End pH	Acidification by	Start pH	End pH	Time taken to reach pH 4.7, minutes	Dry matter content (%)	Protein content (%) dwb	Protein Isolate yield (%) dwb	Protein recovery (%) dwb	L	a	b	Colour
Control_g	7.5	1:6	6.54	7.5	HCl	7.5	4.77	5	98.41	83.63	17.86	63.11	73.86	2.24	22.02	
SC_1_g	7.5	1:6	6.54	7.5	SC_1	7.17	4.77	235	98.42	84.20	17.75	63.16	70.76	2.87	21.74	
SC_2_g	7.5	1:6	6.54	7.5	SC_2	7.24	4.79	420	97.91	84.63	17.81	63.72	67.04	3.65	20.39	

4.3 Molecular distribution of proteins

Both albumin and globulin fractions obtained by conventional and fermentation processes were analysed by size exclusion chromatography (Figure 14). The highest peaks at 411, 410, and 377 kDa can be attributed to coagulated protein particles. Fermented samples showed higher coagulation than the control sample after acidification, and SC_2_g samples had the highest relative abundance of high molecular weight particles, followed by SC_1_g. Higher coagulation could be due to longer precipitation time, as Kinsella & Melachouris concluded in their study that a longer precipitation step results in irreversible aggregation of protein particles (Kinsella & Melachouris, 1976) and in the present study fermented samples took 4 to 7 hours to precipitate. In contrast, the control sample was precipitated in a few minutes which may result in a lower degree of irreversible coagulation. The SC_2 sample took an

extra 3 hours for fermentation than SC_1. Thus, it showed a higher number of coagulated particles. Long hours of heating during the fermentation process also attributed to coagulation. Heating causes the denaturation of proteins, but in the present work, it had a marginal impact on protein degradation and coagulation because the temperature (40 °C) was lower than the denaturation temperature of pea proteins (75–85 °C) (Lu et al., 2020). Globulins are divided into two subfractions 11S (legumins) and 7S (viciline) and conviciline. The peaks observed at 75 kDa (near RT 3.6 min) can be ascribed to convicilin (Schmidt et al., 2022) and peaks at 30–31 kDa (near RT 4 min) can be dissociated viciline trimers (Gatehouse et al., 1982). The peaks below 15 kDa (after RT 4.4 min) probably belong to the albumin fractions. Peaks for low molecular weight peptides have appeared in all albumin fraction samples, which can be explained by the degradation of proteins caused by bacteria. Before fermentation, some peptides were formed during alkali treatment (protein solubilization step) of pea flour. Thus, a similar amount of peptides were available for all three samples. After 3 min RT, a crossover of globulin peaks was observed, and from this point, the concentration of peptide was lower in the fermented sample. This may be due to the consumption of peptides by starter culture species in fermented samples, whereas in the control sample, there was no further consumption of peptides as no starter culture was added. On the other hand, in the albumin fraction of fermented samples (supernatant part), multiple peaks of higher intensity compared to the control sample were observed. It seemed a reversed condition as of globulin fraction. Multiple peaks depict a higher number of peptides, but they are low molecular weights peptides. They were produced during fermentation by bacterial degradation of bigger peptides and resulted in multiple small peptides. Peptide degradation can occur by proteolytic enzymes released by different starter culture species (Kieliszek et al., 2021). However, in control samples, peptides remained intact and not degraded in small fragments as there was no bacterial action. These peptides may become soluble in the supernatant (albumin fraction) part and separate from pellets (globulin fraction). Thus, appeared in the albumin fraction. Overall, fermentation contributes to peptide degradation, which results in the formation of low

molecular weight proteins and peptides (<10 kDa) and higher irreversible coagulation of protein particles.
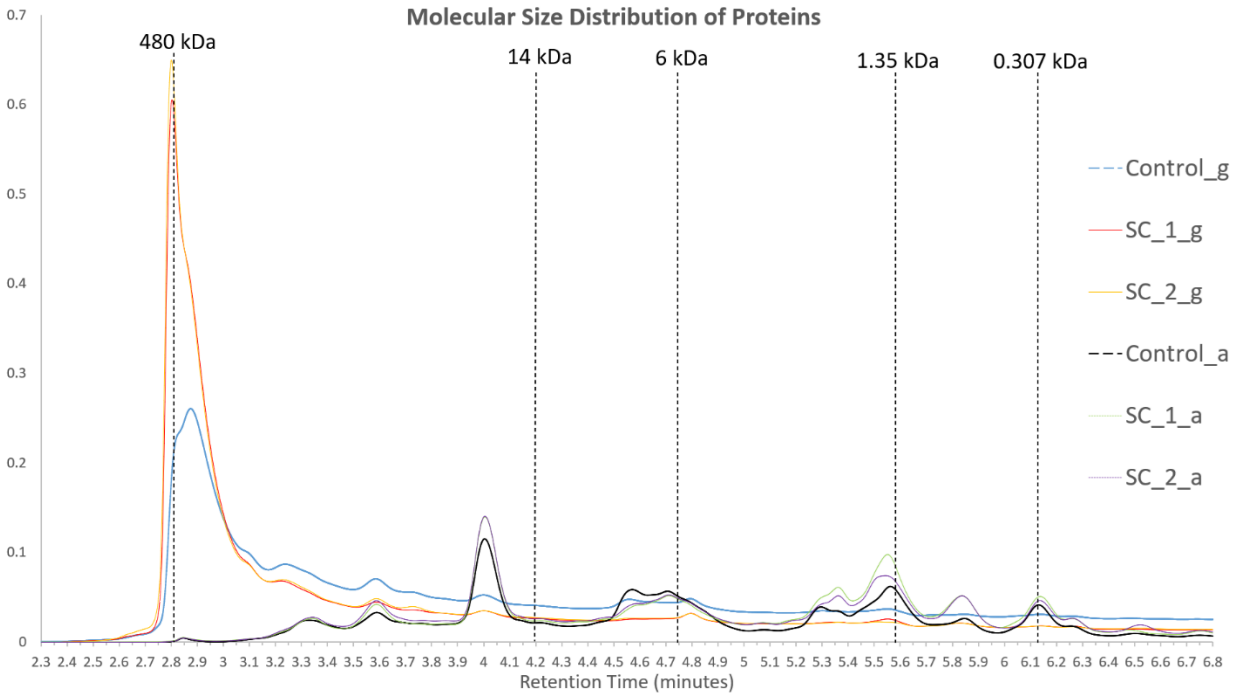
Figure 14. Molecular size distribution of proteins in control and fermented samples SC_1 and SC_2.

4.4 Functional properties

The globulin fractions precipitated by HCl and fermentation were used for assessment of various physicochemical and tech-functional properties. The results are listed in Table 9, but the discussion is presented in the following sections.

Table 9. The different functional properties of pea globulin fractions precipitated by commercial HCl acid (Control_g) and fermentation by lactic acid bacteria with different starter cultures SC_1 and SC_2

Analysis	Control_g	SC_1_g	SC_2_g
Water Holding Capacity [g H2O g^{-1}]	0.78 ± 0.02[a]	1.53 ± 0.02[b]	1.68 ± 0.03[c]
Oil Holding Capacity [g oil g^{-1}]	1.65 ± 0.02[a]	1.66 ± 0.01[a,b]	1.70 ± 0.02[b]
Surface Hydrophobicity [CBBG Bound (µg)]	20.82 ± 0.88[a]	22.72 ± 0.65[b]	21.17 ± 0.34[a,b]
Water Solubility Index [%]	74.0 ± 1.4[b]	55.8 ± 0.2[a]	54.2 ± 0.5[a]
Protein Solubility [%]	37.0 ± 4.4[a]	37.2 ± 1.0[a]	38.2 ± 2.5[a]
Foaming Capacity [%]	45.5 ± 2.2[a]	66.7 ± 1.1[b]	83.3 ± 1.1[c]
Foaming Stability [%]	16.9 ± 2.2[a]	21.1 ± 1.1[b]	15.4 ± 1.1[a]

Emulsion Activity [%]	37.2 ± 1.0[a]	37.8 ± 1.0[a]	38.3 ± 0.0[a]
Emulsion Stability [%]	97.0 ± 2.6[b]	88.2 ± 2.7[a]	92.7 ± 2.5[a,b]
Digestibility (Trypsin) [%]	79.7 ± 0.3[b]	77.1 ± 1.1[a]	76.9 ± 0.4[a]

Values are expressed as means ± standard deviation of triplicate measurements. Values sharing a letter within a row are not statistically different.

4.4.1 Solubility, water and oil holding capacities, and surface hydrophobicity

The solubility of all three samples was determined in two ways: water solubility index (WSI) and protein solubility (PS). Water solubility was calculated by measuring the weight of water-soluble particles of pea protein isolate (PPI) and reported as WSI%. This analysis is more oriented towards particle structure and solvation properties of particles. In pea protein isolate, the particle is termed a complex matrix consisting mostly of protein (~80 %), some carbohydrates and phenolic compounds, but also bacterial cell mass in the case of fermented samples. In the present study, the water solubility index of Control_g, SC_1_g and SC_2_g was reported as 74%, 55.8%, and 54.2% Table 9. A significant decrease in WSI in fermented samples can be due to long hours of heating and precipitation, which results in irreversible coagulation or aggregation of PPI particles. This ultimately causes a loss in the solubility of particles (Kinsella & Melachouris, 1976). Another term for assessing solubility is protein solubility. It is determined by analysing the protein content of the supernatant obtained after pea protein isolate solution centrifugation. Protein content can be determined by any molecular assay for protein quantification, such as the Lowry, Bradford, or Biuret method. Interestingly, the protein solubility of all three samples was similar, and no significant difference was observed. Whereas the water solubility index of fermented samples was significantly lower compared to the control sample. It might be because of the different methodologies applied to analyse and measure a different component (WSI – the weight of PPI powder particles and PS – % of soluble protein at a molecular level) in both methods. Further research will be needed to interpret the results from two analyses.

The water-holding capacity of food material can be described as the ability to hold added water under force like centrifugation, pressing, or heating. (Hermansson, 1985) defined

WHC as the structural property and ability of the food matrix to entrap water and prevent water from being leached out from the three-dimensional structure of the protein powder. This property has direct implications for new product development from plant-based proteins. During cooking, water leaches out from plant-based products. The porosity of a three-dimensional structure is increased or decreased by applying external forces that influence WHC and OHC. There are two significant ways in which porous structure is formed. First, the drying techniques play a significant role. For example, freeze-dried quinoa protein isolate had higher water holding capacity compared to spray-drying and vacuum-drying (Y. Shen et al., 2021), and similar findings were reported by (Zhao et al., 2013) for rice protein where freeze-dried sample had higher water capacity than spray-dried powder. The second way is the nature of the process or condition by which protein unfolding and folding occurs. The process can be physical (heating, ultrasonication, micro fluidization, etc.), chemical (enzyme treatments), or biological (fermentation). In the present study, during fermentation, microbes produce and release the enzymes that cleave the bonds between protein and nonnutritive compounds inside the complex food matrix of protein, carbohydrates, and non-nutritive compounds. It creates small voids inside the food matrix and makes it porous and permeable, and thus, these matrices, after fermentation, have a higher ability to hold water inside their structure (Alrosan et al., 2023). In our study, WHC was higher for both fermented samples than samples precipitated by HCl (1.68 > 1.53 > 0.78 g_{H2O} g^{-1}) shown in Table 9. This can be due to the breakdown of a complex matrix of protein, carbohydrates, and nonnutritive compounds by lactic acid bacteria during fermentation. SC_2-precipitated pea protein had higher WHC than SC_1-precipitated pea protein, which can be due to the longer fermentation time taken by SC_2. A longer fermentation means more consumption of compounds by bacteria and higher breakdown of a complex matrix of protein by bacteria. Water holding capacity can also be understood by relating to WSI. WHC has a negative correlation with the water solubility index when measured by the method used in this work. If the particles of PPI were less soluble, then they would settle down at the bottom and hold more water in their porous structure, which ultimately gives higher water-holding capacity values to PPI powder (Jakobson et al., 2023).

The oil holding capacity of PPI was least affected by fermentation. The OHC of Control_g, SC_1_g, and SC_2_g samples were reported as 1.65%, 1.66%, and 1.70%, respectively. As discussed above, bacteria formed a porous structure while fermenting the pea protein solution. This porous structure holds oil similar to water. Interestingly, oil holding capacity was not improved but water holding capacity was improved by fermentation. This difference may be rather attributed to the method peculiarities, as explained above. Surface hydrophobicity was also analysed for all three samples, and the values are reported in Table 9. Surface hydrophobicity values for control and fermented samples were statistically different, but practically, they were similar. This means that fermented samples have a similar quantity of hydrophobic groups as the control sample. This observation also supports the unchanged oil-holding capacity of fermented samples. No new hydrophobic groups were exposed in the fermented samples.

4.4.2 Foaming capacity & stability and emulsion activity & stability

The ability of proteins to create stable foams with air, known as foaming or whipping, is crucial in many food products such as angel food cakes, sponge cakes, candy, meringue, whipped toppings, icings, fudges, nougats, and similar items. Food foams typically comprise of gas bubbles (air) spread throughout a liquid. This liquid also contains a soluble surfactant, which helps stabilize the bubbles and keep them dispersed evenly. The surfactant reduces the surface tension of the liquid, making it easier for the liquid to change shape and spread out more, even against its own natural tendency to resist spreading. Ideal foaming proteins have the ability to create extended layers around air droplets and undergo a specific, possibly crucial level of natural denaturation. This process stabilizes the foam and establishes its structural framework. However, complete denaturation should be avoided because it leads to the fragility of membranes and the collapse of the foam. A consistent layer of soluble protein is necessary to provide some flexibility to the membrane, allowing it to adjust to changes in volume, such as contraction after leakage (Kinsella & Melachouris, 1976). The foaming capacity (FC) of Control_g, SC_1_g, and SC_2_g samples were reported as 45.5%, 66.7%, and 83.3%, respectively. A significant increase in the values of the foaming capacity of fermented samples was observed, and the SC_2_g sample had

the highest FC. This may be due to the ability of the protein sample to denature partially, which helps extend membranes around the air droplets and make the foam. Fermentation has caused the porosity in PPI particles, making them more easily partially denature by applying external forces. Whereas a control sample had not undergone any treatment, which makes its particles hard to unfold. SC_2_g sample showed the highest values of FC due to having a more susceptible particle matrix for denaturation, which was formed by the longest (7 hours) hour of fermentation. Thus, the fermented sample reported a higher foaming capacity as they were more susceptible to partial denaturation by homogenization. A research study observed that the foaming capacity of fish protein concentrate (FPC) was actually given by the soluble protein in FPC while the remaining denatured peptides acted as a foam stabilizer (Kinsella & Melachouris, 1976). In the present study, foaming stability was not significantly changed for fermented samples. This might be due to a similar quantity of denatured protein in all samples. It can also be supported by the free amino acid analysis, which revealed that the amount of completely denatured protein (free amino acids content) was almost similar among the control and fermented samples. Moreover, only the globulin part was taken for foaming capacity analysis, and a large quantity of degraded protein was separated into the albumin part, which was discarded later. Thus, overall foaming capacity values are low and similar for all samples.

Proteins are often investigated for their ability to create food emulsions. Generally, emulsion capacity, efficiency, or activity decreased when protein concentration increased, above a critical concentration (Kinsella & Melachouris, 1976). It's possible that when the protein concentration decreases, more polypeptides unfold while they are being sheared in the emulsifying process. This unfolding is helped by the peptide chains sticking to the lipid droplets due to their hydrophobic nature. Consequently, a larger amount of protein becomes available, leading to an improved emulsifying efficiency. Yasumatsu et al. developed the emulsion activity method indicating proteins' emulsifying capacity (Yasumatsu et al., 1972). They revealed that the emulsifying activity and stability closely correlated with its soluble protein content and supported their finding by comparing studies on soy products. Emulsion activity and stability for all three samples are reported in Table 9.

The emulsion activity did not differ in all three samples. Emulsion stability was also found to be almost similar for all the samples, including the control. It can be due to the similar soluble protein content of control and fermented samples that have been determined in the protein solubility analysis. In conclusion, emulsion activity and stability also remained unchanged with minor fluctuation due to unchanged soluble protein content.

4.5 Bacterial Consortia

The heat curves from microcalorimetry showed shifts and changes in the metabolism of starter cultures during fermentation. To explicitly investigate dynamic shifts between starter cultures, 16s rRNA sequencing was performed, and samples were analysed every 2–3 hours. Figure 15 and Figure 16 show the relative abundance of bacterial species from the whole consortia. Before the pea solution was fermented, the native bacteria formed 2.8-11.5% of the total population. The native bacteria were exceeded by starter cultures and decreased almost to 0% after 2 hours and completely depleted at the end of fermentation. The native microbiota was dominated by *Pantoea agglomerans* and *Pediococcus pentosaceus,* which can be found in pea seeds (Chartrel et al., 2021). *Pediococcus pentosaceus* is not considered as native bacteria species of pea plant. Its presence in pea solution can be due to some environmental conditions.
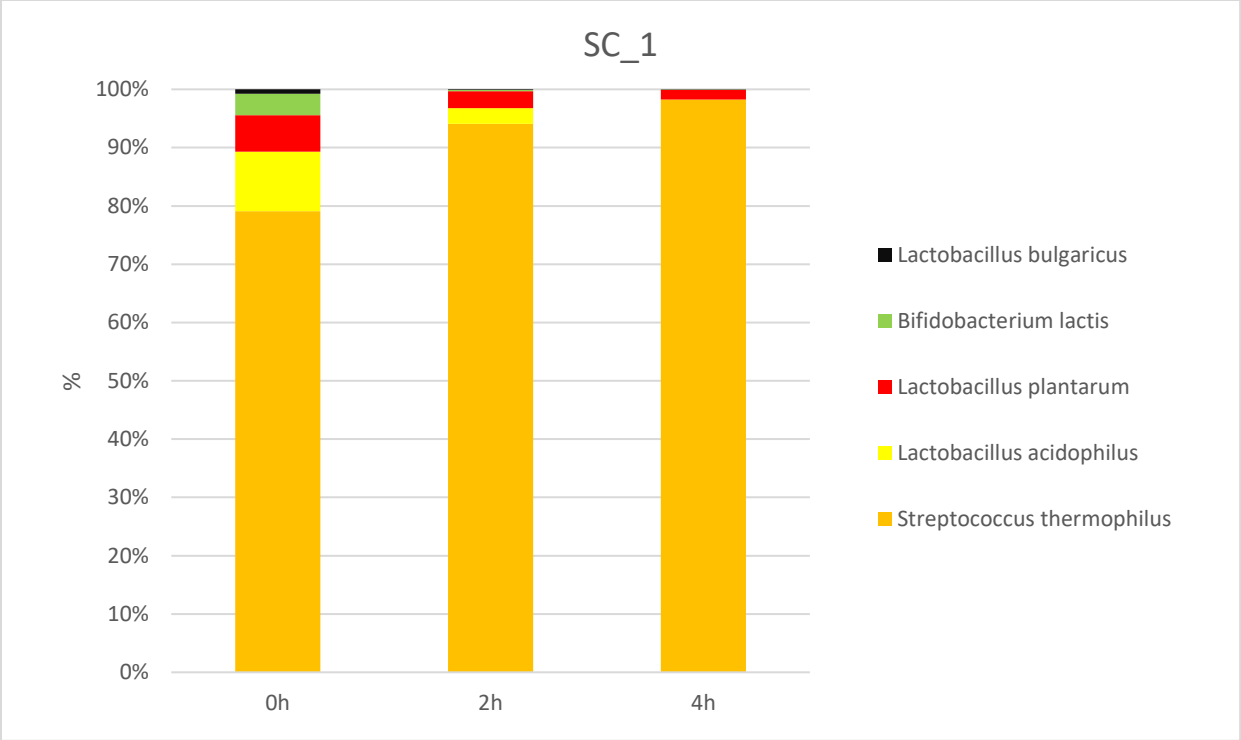
Figure 15. *Bacterial consortia of SC_1 sample fermentation at 0, 2, and 4 hours.*
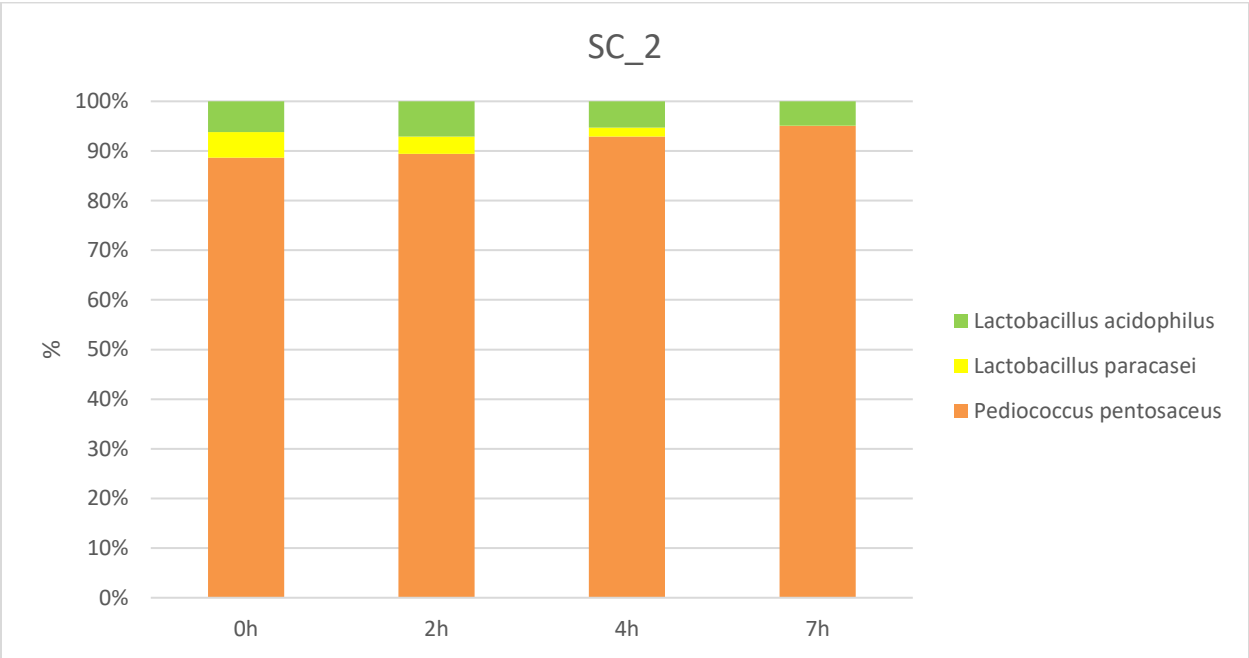
Figure 16. *Bacterial consortia of SC_2 sample fermentation at 0, 2, 4, and 7 hours.*

In SC_1 and SC_2 samples, *Streptococcus thermophilus* and *Pediococcus pentosaceus* were the dominant species throughout the fermentation. Their dominance is mainly due to their prevalence in starter culture right from the start of the experiment. In SC_1, they accounted for 79% and SC_2, for 88% of the total bacterial consortia.

Sampling of SC_1 fermentation media at 0 hours showed *that S. thermophilus (79%) was the dominating species, followed by L. acidophilus (10%), L. plantarum (6%), B. Lactis (4%),* and *L. bulgaricus*, which was nearly 1%. S. thermophilus dominated throughout the fermentation, depicting an almost monoculture environment. Some 2–3% presence of *L. acidophilus and L. plantarum* were observed throughout the fermentation. At 4 hours (end point) only two species were found S. thermophilus (95%) and L. plantarum (2%). Heat flow curves show that SC_1 had a single growth phase (Figure 6), indicating that pH limitation decelerates the growth of S. thermophilus. Still, other bacterial species were not strong enough to overtake the culture. Adamberg et al. showed that *S. thermophilus* St20 was acid-sensitive and unable to grow below pH 5 (Adamberg et al., 2003), similar to the present work.

SC_2 has three different species in the culture: *Pediococcus pentosaceus*, *L. acidophilus* and *L. paracasei* as shown in Figure 16. At the beginning of fermentation, P. pentosaceus was the dominating species followed by *L. acidophilus* (6%) and *L. paracasei* (5%). *P. pentosaceus* was dominating throughout the fermentation, *L. paracasei* decreased with time and *L. acidophilus* increased after 0 hours but decreased after 2 hours and then remained constant till 7 hours (end point). Two peaks were observed in the heat curve, the first one, may indicate the *L. acidophilus* accelerated growth at 2 hours. The second peak may indicate a takeover by *P. pentosaceus* again. At the end of fermentation (at 7 hours), only *Pediococcus pentosaceus* (94%) and *L. acidophilus* (5%) were detected.

Generally, the bacterial species that dominated at the start of fermentation were the most abundant until favourable conditions. In the present study, *S. thermophilus* and *P. pentosaceous* were the dominating species. It seems that starting conditions were more favourable for *S. thermophilus*; thus, it gradually increased from 79% to 95% but declined around pH 5, which was a limiting factor for its growth. Studies in milk revealed that during

the initial hours of fermentation, *S. thermophilus* is the main lactose degrader, and then at later stages, other bacteria increase their presence (Kütt et al., 2023). Compared to other LAB bacteria, in addition to dominant *S. thermophilus*, *L. acidophilus* was constantly present in the SC_2 consortium. The dominance of two bacterial species throughout fermentation indicates that these species may not compete for the same carbon sources. While *S. thermophilus* try to metabolize mono- and disaccharides, *L. acidophilus* may ferment different fractions from sugar, and they also have the capability to ferment sugars found in plants like cellobiose, amygdalin, and trehalose (Hutkins, 2007). This observation indicates that *L. acidophilus* should be considered an important species for fermenting plant-based material in combination with *P. pentasaceus* to achieve fast acidification and better flavour reduction.

4.6 Sugars and organic acids

The concentration of sugars and organic acids was monitored every 2–3 hours until the end of fermentation, as shown in Figure 17 and Figure 18, respectively. Initially, at 0 hours, oligosaccharides (5.9 g/L), disaccharides (3.9 g/L)—predominantly sucrose (3.70 g/L)—were present in good proportion compared to other sugar molecules like glucose (0.33 g/L), fructose (0.18 g/L), and galactose (0.18 g/L). The results show that after 4 or 7 hours, both starter cultures significantly metabolized the bigger sugar moieties (disaccharides and oligosaccharides). In the SC_1 sample, during the first 2 hours, the starter culture degraded the disaccharides and oligosaccharides, which resulted in a decrease in the concentration of oligo- and disaccharide and an increase in the concentration of some simpler sugar moieties like glucose, fructose, and galactose. After 2 hours, the starter culture heavily decreased the oligosaccharides, disaccharides, and some specific simple sugars. Sucrose was a highly metabolized sugar followed by glucose and galactose. Interestingly, fructose concentration was continuously increased throughout the fermentation. It may be due to the dominant *S. thermophilus* species does not prefer to metabolize fructose and is likely to ferment other sugar sources (sucrose, glucose, and oligosaccharides). In the SC_2 sample, throughout the fermentation, bigger sugar molecules were degraded similarly to the SC_1 sample, resulting in a decrease in oligo- and disaccharides and an increase in the

concentration of simple sugars such as glucose, fructose, and galactose. After 2 hours, SC_2 starter culture started metabolizing simple sugars like fructose and glucose along with di- and oligosaccharides. Interestingly, galactose concentration was still elevated until 4 hours and fructose, sucrose, glucose, and disaccharides were slowly consumed until 4 hours, then their concentration dropped drastically. It can be due to the synergistic metabolism of two dominant *P. pentosaceus* and *L. acidophilus* species. These two species were found dominant (*P. pentosaceus*—94 % and *L. acidophilus*—5 %) after 4 hours, till the end of fermentation in the SC_2 sample. Simple sugar molecules like glucose and fructose were completely consumed by bacteria, and some disaccharides (1.7 g/L) and oligosaccharides (5.3 g/L) still remained in the sample at the end of fermentation. It may be due to unfavourable pH conditions, which may be a limiting factor for the growth of bacteria.
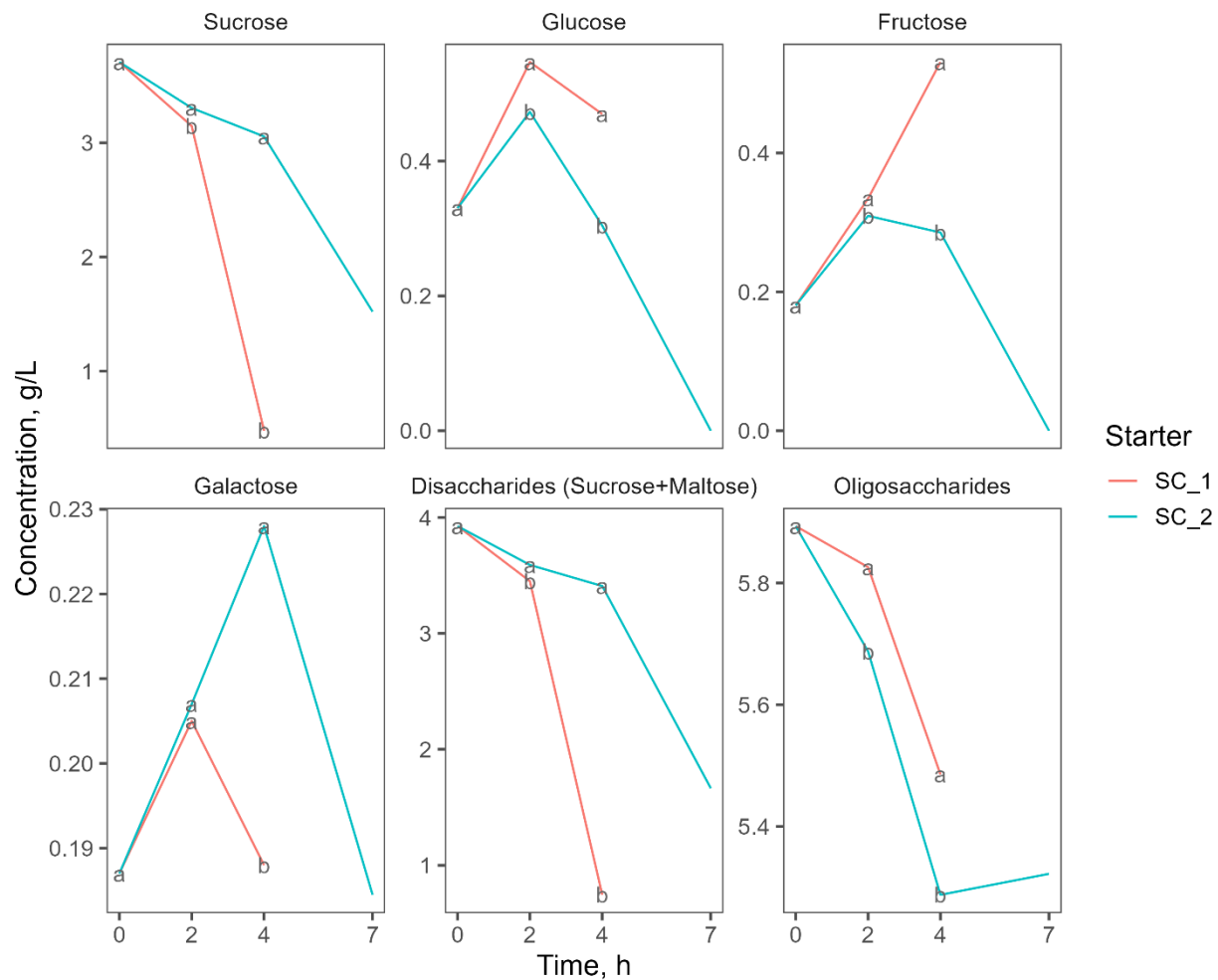
Figure 17. *Sugar composition (mono-, di-, and oligosaccharides) of control and fermented samples during the fermentation process.*
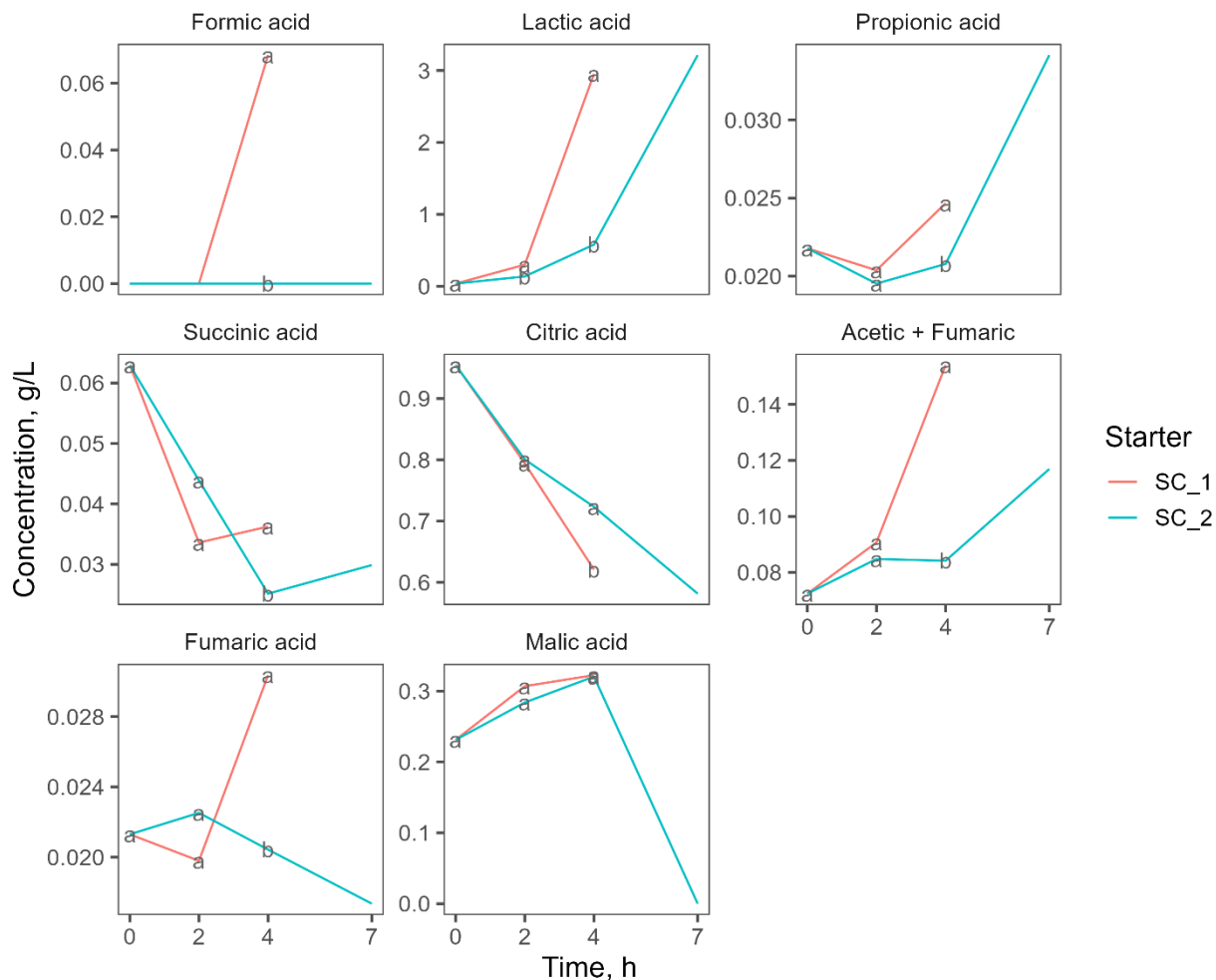
Figure 18. *Organic acids composition of control and fermented samples during the fermentation process.*

The organic acid results from the HPLC analysis show that both starter cultures ferment the pea solution homofermentatively (Figure 18). In both samples, the only acid produced in high concentration was lactic acid (~3 g/L). Some other acids, like formic acid (produced only in SC_1), propionic acid, acetic acid, fumaric acid, and malic acid, were produced in very low concentrations (0.02–0.09 g/L). Homolactic fermentation is a redox-neutral reaction where glucose is metabolized to lactic acid, as ATP can be produced during glycolysis without accumulating excess NADH inside the cells (Kütt et al., 2023). The SC_2 sample produced the highest concentration of lactic acid (3.21 g/L), and the SC_1 sample produced a little less, 2.95 g/L. This can be due to the longer fermentation time and high

limiting factor for lower pH values of the SC_2 starter culture. The SC_2 starter culture took 420 min to reach 4.7 pH (as SC_1 took only 235 min), and *Pediococcus* species also have a higher tolerance for low pH values. Thus, produced more metabolic products, specifically lactic acid, in the SC_2 sample. Some small amounts (0.08 g/L) of acetic acid were produced in the SC_1, followed by the SC_2 sample, maybe by some other species (*L. plantarum*, *L. paracasei*), but it was not consumed during metabolism; thus, its concentration was elevated throughout the fermentation. Some organic acids like citric acid, malic acid, and succinic acid were present in small quantities from the beginning. However, all were consumed by the end of fermentation in both samples. In the SC_1 sample, the dominating *S. thermophilus* species could not utilize malic acid may be due to the absence of an enzyme in the reductive pathway of the TCA cycle (Kütt et al., 2023). Thus, malic acid concentration increased at the end of fermentation. Whereas, in SC_2, after 4 hours, malic acid was depleted sharply, which can be due to malo-lactic fermentation. In malo-lactic fermentation, malic acid (sharp acidic note) is converted into softer lactic acid by bacterial species. *Lactobacillus* and *Pediococcus* species are responsible for this type of fermentation (Gil-Sánchez et al., 2019), and similar species were present in the SC_2 sample after 4 hours in the present work. Citric acid was also decreased throughout the fermentation process. *L. plantarum* in SC_1 and *L. paracasei* in SC_2 samples are probably the responsible species for citric acid degradation. Analysis of NCBI protein database indicates that the genomes of *Lactobacillus plantarum* and *Lactobacillus paracasei* harbor genetic sequences responsible for encoding enzymes essential for the degradation of citric acid (Kütt et al., 2023).

The free amino acid (FAA) concentration was measured in the dried supernatant part (albumin fraction) resulting from the final centrifugation of acidified protein. The results reveal that the free amino acid content was marginally increased for fermented samples compared to the Control_a sample (4874 mg/100 g), which indicates the degree of protein denaturation. Among fermented samples, SC_2_a (5293 mg/100 g) had more FAA content than SC_1_a (5112 mg/100 g). Thus, SC_2 had more peptide-degrading starter culture species, or peptide degradation may result from longer process and heat supplied during

fermentation. In fermented samples, some free amino acids like Asp, Arg, Glu, Phe, and also GABA were increased, and Asn and Glu were decreased mostly. In the SC_1_a sample specifically, Arg, Phe, Ser, Pro, and GABA were increased and Glu, Asp, Asn were decreased compared to the control sample. Whereas, in the SC_2_a sample, Asp greatly increased compared to the control sample, followed by GABA, Glu, Phe, and Pro. On the other hand, Asp has decreased significantly, followed by Ala and Arg. The increased concentration of certain free amino acids is likely due to the consumption of peptides by bacteria, as it is energetically viable to metabolize peptides composed of multiple amino acids and excrete surplus amino acids (Kevvai et al., 2014).

4.7 Digestibility

The digestibility of all samples was analysed using the trypsin enzyme. The digestibility of Control_g sample was reported as 79.7%, and for fermented samples, it was reported as 77.1% for SC_1_g and 76.9% for SC_2_g (Table 9). No significant difference was observed between the samples, but the digestibility of fermented samples was slightly decreased compared to the control. This can be explained by the amount of peptides available in a sample for trypsin degradation. From the molecular weight distribution analysis, a lower amount of peptides (coagulated high molecular weight proteins were not considered) was observed in the globulin fraction of fermented samples than in the control, which reveals that fewer peptides were available for trypsin degradation in the fermented sample. Thus lowered the digestibility values for fermented samples. The difference between control and fermented samples might be also due to the presence of trypsin inhibitors in fermented samples, blocking trypsin activity and slowing down peptide digestion. Kormin et al. showed that a bacteriocin (Plantaricin BS2) produced by LAB species (*Lactobacillus plantarum* BS2) had inhibitory activity towards trypsin enzymes (Kormin et al., 2001). On the other hand, Cabuk et al. reported that with an increase in time, fermentation has increased the non-nutritive compounds (phenols and tannins), but decreased the enzyme inhibitors present in pea protein and enhanced its in-vitro digestibility (Çabuk et al., 2018). Only some LAB species produce bacteriocins during long fermentation hours. That's why it is important to

select the specific non-bacteriocin-producing LAB species for fermentation and optimize fermentation time to enhance the digestibility of fermented plant protein.

4.8 Volatile Compounds

In total, 99 volatile compounds were detected among all samples. All compounds were enlisted (not shown, available on request). A principal component analysis (PCA) and a partial least squares regression (PLSR) biplots were made to understand the high-dimensional data. In Figure 19, the PCA plot shows 67.9% of the original data after dimensionality reduction and was plotted to observe the volatile compound association with different samples like pea flour, protein extract (the supernatant used for fermentation), albumin and globulin parts of the control and fermented samples. It classified volatile compounds and samples into 3 big groups. One group consisted of pea flour and pea extract, which was unsurprising as pea extract was only solubilized in water with pH adjustment, and no major modification was performed. The second group contained an albumin fraction of the control and fermented samples. It has been observed that the albumin fraction of control was slightly shifted from other fermented samples. It may be because of the high quantity of water-soluble volatile compounds that were formed during fermentation, separated with the albumin section. Thus, different volatile compounds were associated with fermented and control samples. The third group showed a cluster of 3 samples, globulin fraction of control and fermented samples. It was hard to understand the difference between the globulin fraction of control and fermented samples based on volatile compounds by this PCA plot, as the albumin fraction of these samples showed observable differences. Thus, PLSR was plotted to classify the globulin fraction of the control and fermented samples and correlate them with the sensory odour profile. The PLSR plot in Figure 20 classified 3 globulin samples into three groups, which depicted that all three samples differed from each other in volatile profile. Aldehyde group molecules are well known for generating off-flavour smells like beany, grassy or pea-like in plant proteins (Engels et al., 2022). The control sample (globulin part) correlated highly with a pea-like odour, which was analysed by the sensory panel. Hexanal and nonanal may be the potential volatile compounds responsible for the pea-like or beany or grassy smell as they have a high

correlation with the Control_g sample. Whereas SC_1_g sample also has a significant aldehyde concentration comprising benzaldehyde (almond), 2-methylbutanal (phenolic or coffee). Interestingly, these molecules impart sweet, almond-like or phenolic notes instead of a pea-like smell, which may mask the off-flavours. SC_2_g sample grouped with sweet and sour odours, which are sensory parameters. It was observed that many sweet-smelling compounds from aldehyde, alcohol, and ketone were in high concentrations like acetaldehyde (ethereal or fruity), 1-hexanol (ethereal or fruity), acetoin (sweet, buttery, creamy, dairy), and 2,3-butanedione (buttery, sweet, creamy, pungent, caramellic). All these volatile molecules correlated highly with the SC_2_g sample and sweet odour sensorial parameter. This might be possible that these sweet-smelling aromatic compounds mask the leftover pea-like odour, as fermentation degraded major pea-like off-flavour producing compounds and overall reduced the off-flavour note efficiently.

Figure 19. *Principal component analysis of gas chromatography data*.

Figure 20. *Partial least squares regression for GCMS and sensory data.*

Total volatile acid content (Figure 21) was increased in fermented samples compared to the control sample, but after centrifugation, a major part was separated into supernatant; thus, the precipitated globulin fraction has the least acid content and smell. SC_1_a has high acetic acid content followed by SC_2_a sample. Acetic acid (vinegar odour) and butanoic acid (sour or cheese odour) were the most dominating volatile acids present in the sample. Ester content was increased in the fermented samples, but a major proportion was transferred to the supernatant part, and again, a very small quantity was available in the globulin fraction of protein. SC_1_a has higher total ester content than all the other samples. Major contributing esters were methyl ester of nonanoic acid (sweet or fruity aroma) and methyl ester of hexadecanoic acid (oily, waxy, fatty). In plant proteins, the aldehyde group is majorly responsible for beany, grassy, or green flavour in plant proteins. Major dominating

aldehyde molecules were reported to be hexanal, nonanal, acetaldehyde, benzaldehyde, and butanal-2-methyl (Engels et al., 2022). In raw pea flour, hexanal was found to be the dominating aldehyde molecule which is responsible for beany or grassy aroma. The globulin fraction of the control sample had the highest hexanal content, and after fermentation, it decreased in the SC_2_g sample, followed by SC_1_g. Sweet-smelling aldehydes acetaldehyde (ethereal or fruity), benzaldehyde (almond), and 2-methylbutanal (phenolic or coffee) were also increased in globulin fraction of both fermented samples compared to the Control_g sample. Fermented samples had higher alcohol content than the control sample, but a major part of the alcohol was dissolved in the supernatant and separated from the globulin fraction (precipitated part). The globulin fraction of the SC_2 sample has the highest amount of alcohol content (1-hexanol) among all globulin samples. 1-hexanol (ethereal or fruity), 2-ethyl-1-hexanol (citrus, fresh, floral, oily), and 2,3-butanediol (fruity, creamy, buttery) were the most dominating volatile alcohols detected in SC_2_g sample. Ketones were the most affected volatile group by fermentation. Lactic acid bacteria drastically increased the concentration of volatile compounds in the ketone group. Interestingly, a major proportion was soluble in the supernatant and separated from the globulin fraction, but still, the globulin fraction of the fermented sample had a higher ketone content than the control. 4-methyl-2-pentanone was dominating and responsible for sharp, green, and herbal notes in the pea flour and protein solution used for fermentation. However, it was diminished during extraction, and two other molecules (acetone and 2-octanone) were increased, which usually impart ethereal or apple (acetone) and green or herbal (2-octanone) notes that may be responsible for sweet and pea odour in the control sample. Whereas in fermented samples, the dominating volatiles were acetoin (sweet, buttery, creamy, dairy), 2-heptanone (cheese, fruity, coconut), 2,3-pentandedione (pungent, sweet, butter, creamy), 2,3-butanedione (buttery, sweet, creamy, pungent, caramellic) and 2-nonanone (fresh, sweet, green, weedy). There was a sharp decrease in the concentration of 4-methyl-2-pentanone and 2-octanone in fermented samples which was probably responsible for green or pea-like aroma in pea flour and protein solution used for fermentation. A decrease in the concentration of these two ketone molecules may result in

a reduction in pea-like odour. In Figure 20, the PLSR plot also showed that pea odour correlated with these two molecules, thus supporting the volatile analysis result.
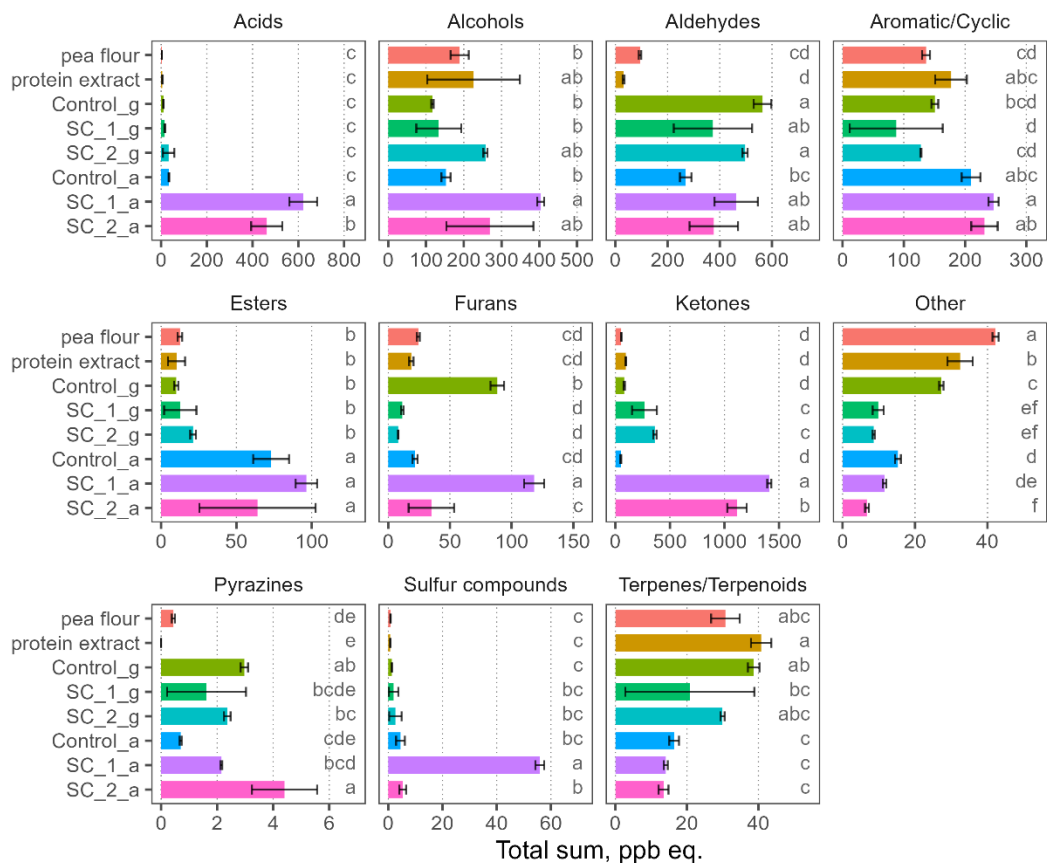
Figure 21. *Gas chromatography data of all raw, control and fermented samples grouped by functional group.*

4.9 Sensory

Pea odour was significantly decreased in fermented samples than in control, as revealed by the sensory analysis data as shown in Figure 22. Some aldehyde and ketone molecules may be responsible for pea-like, beany, green, grassy, or earthy aromas. In aldehydes, hexanal molecules with green or grassy aroma were relatively low in fermented samples (in globulin fraction) compared to the control. Whereas two ketone molecules (4-methyl-2-pentanone and 2-octanone), which have a green or pea-like aroma, were present in the control sample, but in fermented samples, they were relatively low in quantity. Fermented samples found sweeter than the control samples. Esters, aldehydes, ketones and alcohol may be

responsible for the sweet odour in the pea protein isolate (globulin fraction). Generally, ester majorly contributes to sweet odour and due to very low threshold value, it imparts smell at a low concentration. In volatile analysis, it was observed that though the fermented sample produced more ester molecules, the major fraction was soluble in the supernatant and mixed with the albumin part. However, some ester molecules remained in the globulin part, which may impart a sweet odour to the final product. Sweet aroma aldehyde molecules were also increased in the fermented sample as concluded from the volatile analysis. Acetaldehyde and benzaldehyde may be responsible for sweet aroma or taste. As per volatile compound analysis, ketone was separated with the supernatant part (albumin fraction), but the remaining ketones cumulatively may slightly contribute to sweet or fruity aroma and support the sweet odour in the sensory of fermented samples. Sour odour was perceived more in the fermented samples. It can be due to acid production (majorly lactic acid) during fermentation. Major portion of acid was neutralised and then also separated with supernatant in fermented samples, thus globulin fraction had least sourness which was only sensed (smell) but not effectively distinguishable in taste. Interestingly, bitter taste and astringency in aftertaste were significantly reduced in fermented samples (Control_g > SC_1_g > SC_2_g). Saponins and tannins compounds are responsible for bitterness and astringency (Soares et al., 2020). Fermentation may have degraded these phenolic compounds which effectively reduced bitterness, astringency, and overall intensity of aftertaste. Shi et al. (2021) and García Arteaga et al. (2021) revealed that the fermentation with *L. plantarum* significantly decreased the bitter taste and as well as beany and grassy flavour in pea protein isolates. Similar findings were reported by Markkinen et al. (2019) where fermentation with *L. plantarum* significantly reduced the phenolic compounds of sea buckthorn juice. Whereas, Al-Qaisi et al. (2024) reported contrary results where fermentation enhanced the phenolic molecules in pea protein isolates. Therefore, impact of fermentation on phenolic compounds seems highly depend on the used strain or starting material. The benefit of impacting phenolic compounds is debatable. On one hand, the astringency or bitterness of pea proteins could be affected by changing the phenolic content and composition, whereas the health-promoting activities, often associated with phenolic

compounds in pea proteins, can be affected in a negative direction. Overall, there was no off-odour observed in all three samples and fermented samples were observed most neutral sample in overall taste intensity.
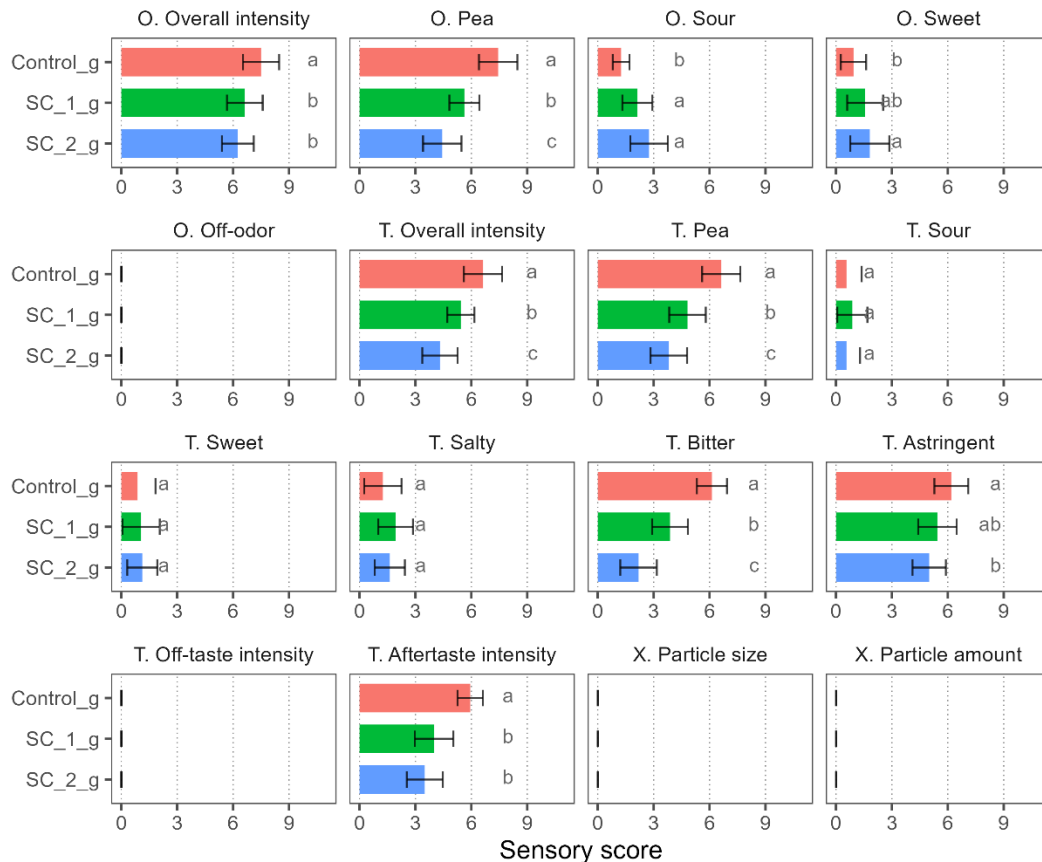
Figure 22. Sensory ratings for different odour and taste attributes of control and fermented SC_1 and SC_2 samples.

4.10 Colour

Figure 23 shows the appearance of globulin samples that have been fermented by SC_1 and SC_2 starter cultures and the control sample. SC_2_g was observed to be darker than the SC_1_g sample, and the Control_g had the lightest colour shade. Longer hours of fermentation at elevated temperature turned the colour darker and browner. The interpretation generated from visual observation was consistent with the determined L, a, and b values reported in Table 10. The change in L, a, and b from the Control_g sample was

calculated as ΔL, Δa and Δb, shown in Table 10. ΔL represents the lightness change. It was negative for all fermented samples, which means fermentation has decreased the lightness of the globulin samples and made them darker with increased fermentation time. Δa values signify the redder colour, which means reddishness increases with an increase in Δa value. Fermented samples have positive Δa values, and the SC_2 sample reported a greater Δa value than SC_1, which depicts SC_2 as a reddish shade. Δb values signify yellow to blue colour, and more positive Δb values show yellow appearance and negative values represent blue shade. The change in b values was slightly negative, which means that the yellowness of the fermented sample decreased with fermentation time. Overall, fermentation has increased the intensity of darker shades with an increase in fermentation time. This might be related to browning or Maillard reactions due to prolonged heating at 40 °C, but it is also possible that fermentation has degraded the natural pigment molecules which were lighter in shade or formed new darker pigments. Similar findings were reported by other researchers (Kaleda et al., 2020; Schaffner & Beuchat, 1986), in which colour degradation of plant extract was reported by lactic acid bacteria fermentation.

Table 10. *Colour values (L, a, and b) for control and fermented samples.*

Sample Name	Color Analysis						
	L	a	b	ΔL	Δa	Δb	Colour
Control	73.86	2.24	22.02	0	0	0	
SC_1_g	70.76	2.87	21.74	-3.10	0.63	-0.29	
SC_2_g	67.04	3.65	20.39	-6.82	1.41	-1.64	

Figure 23. *Freeze-dried protein powder of control and fermented samples. Globulin fraction (top) and albumin fraction (bottom).*

5 Conclusion

In the fermented samples, *Streptococcus thermophilus* or *Pediococcus pentosaceus* were the dominating LAB strains. The *Pediococcus* fermented sample had a stronger pea taste and odour reduction, and, interestingly, bitterness was also reduced significantly from 6.1 to 2.2 (sensory scale 0–9) compared to the conventionally produced sample. A greater amount of higher molecular mass protein fraction and slightly higher peptide content were observed in the fermented samples. Fermentation enhanced water holding and foaming capacities and decreased the water solubility index. Unsubstantial changes were observed in other functional attributes. The study showed that LAB fermentation can be used for pea protein precipitation and can simultaneously greatly reduce typical off-flavours. The changes in off-flavour profile and techno-functional properties depend on the used starter culture.

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