Effect of Fermentation on Nutritional and Sensory Properties of Textured Pea Protein for Plant-Based Meat Analogues

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ABSTRACT

The demand for plant-based meat analogues (PBMAs) continues to rise, fuelled by environmental and health motivations. However, their nutritional quality remains a concern due to the presence of anti-nutritional factors (ANFs) such as phytic acid (PA), which can reduce the bioavailability of essential minerals and hinder protein digestibility. Additionally, sensory limitations and the frequent use of additives further hinder broader consumer acceptance. To address these issues, this study explored lactic acid fermentation as a natural pre-treatment for textured pea protein (TPP), a common ingredient in PBMAs, aiming to improve both nutritional and sensory properties. Three commercial starter cultures containing *Latilactobacillus sakei, Leuconostoc carnosum,* or *Lactococcus lactis* were used to ferment TPP. Key parameters evaluated included protein content, PA levels, and *in vitro* protein digestibility (IVPD). The fermented TPP was then incorporated into plant-based burger prototypes for sensory analysis. Fermentation led to a significant reduction in PA levels by up to 14.6% with *L. sakei* and *L. carnosum*, while IVPD remained unchanged, ranging from 34.0% to 34.7% in all samples. Protein content remained stable throughout the fermentation process. Sensory evaluation revealed a significant increase in yeast-like aroma and flavour, especially in samples fermented with *L. lactis* and *L. carnosum*. Non-significant trends toward enhanced sweetness, umami, and firmness were observed across fermented samples, while pea-like and hay-like notes remained largely unchanged. Overall, lactic acid fermentation demonstrated promise as a natural pre-treatment for TPP, particularly in reducing PA levels and enhancing sensory attributes in PBMA, underscoring its potential for clean-label innovation. However, the findings also highlighted the need for further research and methodological refinement to fully optimize both analytical methods and fermentation applications in the plant-based sector.

Keywords: Pea protein, meat analogue, textured vegetable protein, lactic acid fermentation, phytic acid, protein digestibility, sensory properties

POPULAR SCIENCE SUMMARY

As more people seek healthier and more sustainable food options, plant-based meat analogues (PBMAs) are gaining popularity. These products aim to mimic meat, but they often fall short when it comes to taste, texture, and nutritional quality. A common ingredient in many PBMAs is textured pea protein (TPP), which consists of fibrous, meat-like pieces made from concentrated yellow pea protein that has been milled and processed using heat, water, pressure, and mechanical force. However, yellow peas naturally contain compounds called anti-nutritional factors (ANFs), such as phytic acid. These compounds can interfere with the body's ability to absorb essential minerals and may also reduce how efficiently the protein is digested and utilized.

This study explored whether lactic acid fermentation, a natural process also used to make yogurt and sauerkraut, could serve as a simple, clean-label method to improve the nutritional and sensory properties of TPP. Three types of beneficial bacteria commonly used in food fermentation were used to explore if they could lower phytic acid levels, influence protein content, improve protein digestibility, and enhance the taste and texture of TPP.

The results were promising. Fermentation successfully reduced phytic acid levels, which could help the body absorb and make better use of essential minerals. Protein content remained stable, however, laboratory tests mimicking human digestion showed no improvement in how well the protein was digested. To evaluate the effects on flavour and texture, the fermented TPP was used in plant-based burger prototypes. Fermentation resulted in a stronger yeast-like aroma and flavour, similar to the savory boost provided by yeast extract, which is often added to create meaty notes. This natural enhancement could reduce the need for added flavorings in PBMAs. Fermentation also slightly improved sweetness, umami, and firmness, qualities that can make plant-based meats more appealing without relying on additional additives. However, some common plant-based flavours, such as pea and hay-like notes, remained unchanged.

Overall, this study suggests that fermentation could be a valuable tool for improving the nutritional quality, flavour, and texture of PBMAs. It supports the development of cleaner-label products with fewer additives and more natural sensory enhancements. However, more research is needed, including more targeted experiments to better understand and optimise the fermentation process, as well as consumer testing, to assess how well fermented plant-based products meet the expectations of today's health- and quality-conscious consumers.

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LIST OF ABBREVIATIONS

Abbreviation	Definition
ANF	Anti-nutritional factor
ANOVA	Analysis of variance
DW	Dry weight
FDW	Freeze-dried weight
FLc	Fermented, *L. carnosum*
FLl	Fermented, *L. lactis*
FLs	Fermented, *L. sakei*
IVPD	*In vitro* protein digestibility
L. carnosum	*Leuconostoc carnosum*
L. lactis	*Lactococcus lactis*
L. sakei	*Latilactobacillus sakei*
PA	Phytic acid
PBB	Plant-based burger
PBMA	Plant-based meat analogue
SGF	Simulated gastric fluid
SIF	Simulated intestinal fluid
SSF	Simulated salivary fluid
TI	Trypsin inhibitor
TPP	Textured pea protein
TVP	Textured vegetable protein
UF	Unfermented

1. INTRODUCTION

Plant-based protein sources are becoming increasingly popular as a sustainable, ethical, and healthy alternative to animal-based proteins. One of the strongest arguments for shifting toward plant-based alternatives is their potential to significantly reduce the environmental impact of our diets, by lowering greenhouse gas emissions and decreasing land and freshwater usage (Bunge et al., 2024). Given that our food system is one of the largest contributors to climate change, transformation in how we produce and consume food has never been more urgent. Plant-based proteins present a promising solution to mitigate these challenges while promoting a more sustainable food system (UN, n.d.).

However, while many advantages support a shift from animal- to plant-based proteins, concerns remain regarding their nutritional quality and the presence of anti-nutritional factors (ANFs) such as phytic acid (PA), tannins, and trypsin inhibitors (TI), that hamper protein digestibility, and bioavailability of essential nutrients in the human body (Emkani et al., 2022). A major concern is the limited absorption and utilization of non-heme iron and zinc due to naturally occurring PA in plants, which binds these minerals and significantly inhibits their uptake (Gupta et al., 2015). Additionally, the undesirable beany flavours and soft textures of many plant proteins present sensory challenges, and the frequent use of additives, often conflicting with clean-label demands, further limits their broader market appeal (Giacalone et al., 2022; Elhalis et al., 2023a). With iron and zinc deficiencies being major global health concerns, improving their absorption is crucial. Likewise, protein is essential for human life; hence, enhancing its digestibility is equally important. Addressing these challenges requires exploring alternative methods to improve protein digestibility, reduce PA, and enhance sensory qualities, supporting the growing market for plant-based proteins.

To increase consumer acceptance of plant-based protein sources, particularly in Western markets, there has been growing focus on developing substitutes that closely replicate the appearance and texture of traditional meat products. These substitutes, known as plant-based meat analogues (PBMAs), are commonly made using textured vegetable protein (TVP). TVP is a versatile ingredient mainly produced through extrusion, a process that uses heat, pressure, and mechanical force to transform plant proteins into fibrous structures that mimic the texture of meat. While extrusion has demonstrated some effectiveness in reducing ANFs, improving protein digestibility, and enhancing sensory characteristics, there is still potential for further enhancement in both nutritional and sensory qualities (Duque-Estrada et al., 2023; Elhalis et al., 2023a). As such, exploring pre-treatments, such as fermentation, of TVP before its incorporation into PBMA formulations presents an interesting area of focus for this degree project.

1.1 Collaboration, project background and previous research

This degree project was carried out in collaboration with Solina Sweden, a company at the forefront of alternative protein innovation, with a strong focus on enhancing taste, texture, nutritional quality, and sustainability. Given the well-established impact of lactic acid fermentation on the sensory properties of food, Solina has conducted internal evaluations to explore the potential of fermenting TVP derived from yellow peas prior to its use in PBMA. While preliminary findings suggest promising sensory effects, the impact of fermentation on the nutritional properties remains largely unexplored.

Previous research has examined the physical, chemical, and nutritional effects of fermenting soy-based TVP using *Bacillus* species (Maung et al., 2020; Ojokoh & Wei, 2011; Kim et al., 2010; Kim & Lee, 2010). However, similar studies on textured pea protein (TPP) produced from yellow peas remain limited. While some work has explored the fermentation of pea protein prior to extrusion or the combination of fermented, untextured pea protein with unfermented extruded protein (Valtonen et al., 2023; Kaleda et al., 2020), there is still a lack of understanding regarding how fermentation specifically affects TPP. Given that lactic acid fermentation has been identified as a promising strategy for enhancing both the nutritional and sensory qualities of pea proteins (Valtonen et al., 2023; Kim et al., 2023; Elhalis et al., 2023b; Shi et al., 2021; Xiang et al., 2023), exploring its application to TPP constitutes a valuable focus for this degree project.

1.2 Aim and objectives

The aim of this degree project is to investigate the effect of lactic acid fermentation as a pre-treatment for TPP to enhance the nutritional and sensory properties of PBMAs. The focus on nutritional properties is specifically on reducing PA levels and improving protein digestibility, as these are two key concerns associated with plant-based protein sources, and factors influencing consumer acceptance. To achieve this, the following objectives have been established:

- Develop the fermentation process of dry TPP using the lactic acid bacteria *Latilactobacillus sakei*, *Leuconostoc carnosum* and *Lactococcus lactis*.
- Assess the effect of fermentation on protein content and *in vitro* protein digestibility.
- Assess the effect of fermentation on PA content.
- Incorporate the fermented TPP in the formulation of a plant-based burger.
- Evaluate the sensory properties of the developed product.

2. LITERATURE REVIEW

2.1 Plant-based meat analogues

The primary goal of PBMAs is to replicate conventional meat in terms of nutritional value, texture, flavour, and physicochemical properties (Younis et al., 2023). However, replicating the distinct flavour, texture, and protein quality of meat remains challenging, often requiring the addition of various ingredients to achieve comparable results. Typically, PBMAs consist of a combination of water, textured and non-textured plant proteins, plant-based fats, as well as binding, flavouring, and colouring agents, each adjusted depending on the desired product.

The plant protein plays a key role in achieving the desired texture, as it can be processed through various techniques into fibrous structures similar to that of meat (Younis et al., 2023). Additionally, the plant protein is one of the key contributors to the nutritional profile of the final product. Soy protein is the most used protein source for PBMAs due to its availability, low cost, texturizing properties, and high-quality amino acid composition, which makes it relatively comparable to the protein quality of animal-based products (Sun et al., 2021). However, alternatives such as cereals, and other legume proteins like pea, lupin, faba bean, and chickpea are becoming increasingly popular as alternatives to soy, particularly due to soy's intense demand, which contributes to deforestation, monocultures, and subsequent losses in biodiversity (Song et al., 2021).

Pea (*Pisum sativum*) proteins, including pea flour (20-25% protein), pea protein concentrate (50-75% protein), and pea protein isolate (>80% protein), are considered one of the most promising alternatives to soy proteins (Younis et al., 2023; Boukid et al., 2021). Unlike soy and wheat, pea protein is low allergenic, giving it a compelling advantage as a protein source for PBMAs. Additionally, pea proteins contain a high nutritional value, are cost-effective and widely available. However, despite containing all essential amino acids, pea protein has lower quality and digestibility than animal proteins due to its limited levels of sulfur-containing amino acids, including methionine and cysteine, as well as the presence of ANFs, which also hinder mineral absorption (Shanthakumar et al., 2022). In addition, pea proteins are associated with undesirable flavours and aromas (Xiang et al., 2023). The process from raw pea seed to TPP has been documented with some impact on these challenges, which will be described in the following sections.

2.1.1 Pea protein extraction

Pea proteins are obtained through an extraction process from pea seeds. Before extraction, the seeds undergo various pre-treatments, including cleaning, drying, sorting, dehulling, and splitting to facilitate protein extraction while preserving their techno-functional properties (Shanthakumar et al., 2022). Depending on the desired type of pea protein, different extraction methods are used: wet extraction is commonly applied for pea protein isolates, while dry

fractionation is typical for concentrates. In this degree project, a blend of pea protein concentrate and isolate was used for TPP production; therefore, both extraction methods are described below.

Dry fractionation involves milling and air classification and is typically preceded by dehulling to slightly reduce ANFs and bitter compounds (Shanthakumar et al., 2022). After milling, the flour is separated by a spiral air stream into a fine, protein-rich fraction (pea protein concentrate) with 50–75% protein dry weight (DW) and a coarse, starch-rich fraction (Assatory et al., 2019; Boukid et al., 2021). Wet extraction typically includes alkaline solubilization followed by isoelectric precipitation. After dehulling, milling, and defatting the pea seeds, proteins are extracted in water under alkaline conditions, separated by centrifugation, then precipitated by adjusting the pH to their isoelectric point. The resulting protein is washed, neutralized, and freeze-dried to yield a pea protein isolate typically exceeding 80% protein DW. The extracted protein can then be used in texturization applications such as TPP production.

2.1.2 Textured pea protein

The texturization process of pea protein into fibrous, meat-like textures can be carried out in various ways, including extrusion, shear cell technology, and electrospinning (Baune et al., 2022). The TPP used in this project was produced by extrusion, hence, this technique will be further described.

Extrusion transforms dry, powdery material into a structured product using moisture, heat, pressure, and mechanical force (Baune et al., 2022). It is typically categorized as low-moisture extrusion (LME) or high-moisture extrusion (HME). HME yields moist, fibrous TVP suitable for fresh, whole-muscle PBMA, while LME produces a drier, spongier and more porous product that is shaped into mince, chunks, or flakes and further dried for shelf stability. Upon rehydration, LME TVP develops a chewy, juicy texture ideal for applications like burgers and sausages (Vila-Clarà et al., 2024). The TPP used in this project was produced via LME.

Extrusion can influence protein digestibility, amino acid composition, and anti-nutrient levels. In general, thermal processing enhances protein digestibility by denaturing and unfolding proteins, making them more accessible to digestive enzymes. Moreover, heat treatment is a key factor in reducing or inactivating ANFs, thereby further improving digestibility (Duque-Estrada et al., 2023; Nikmaram et al., 2017). However, extrusion has also been reported to be less effective in degrading PA in legumes (Ojokoh & Wei, 2011). Additionally, protein denaturation during high-temperature processing can lead to aggregation, which may reduce enzyme accessibility and thus lower digestibility (Drulyte & Orlien, 2019). Elevated temperatures can also cause amino acid loss through Maillard reactions and protein aggregation, potentially comprising protein quality (Duque-Estrada et al., 2023; Hejdysz et al., 2022; Drulyte & Orlien, 2019). Determining an exact temperature at which extrusion negatively affects protein digestibility and quality is challenging, as factors such as moisture content, screw speed, and processing time also influence the overall outcome. For instance, one

study reported reduced digestibility of faba beans at extrusion temperatures above 115°C, whereas others observed improved digestibility at temperatures up to 180°C in various legumes and cereals (Hejdysz et al., 2022; Pismag et al., 2024). Thus, while extrusion can enhance certain nutritional properties, it may simultaneously compromise others, depending on the specific food matrix and processing conditions.

Moreover, LME has been shown to reduce off-flavours like “green” and “fatty” odours in pea protein (Ebert et al., 2022). In addition, extrusion can generate new flavour notes via cross-reactions such as the Maillard reaction, though it may simultaneously produce undesirable beany compounds (Yang et al., 2023). Overall, the effects of extrusion on protein quality, digestibility, ANF levels, and sensory properties depend on various factors, including the food matrix, protein type, and key processing parameters such as temperature and time.

2.2 Anti-nutritional factors

Anti-nutrients, or ANFs, are naturally occurring plant compounds that can interfere with the digestion, absorption, and utilization of essential macro- and micronutrients in the human body (Singh et al., 2023). Thus, ANFs, such as PA, tannins and TI, pose a challenge to the nutritional quality of plant-based foods, particularly those rich in legumes, cereals, nuts and seeds. However, ANFs are not solely associated with negative effects. Research have shown that when consumed in lower concentrations, they may offer health benefits, including anti-cancer, antioxidant, anti-inflammatory, and prebiotic effects, as well as potential as natural preservatives (Singh et al., 2023). Yet, given the generally high PA content in legumes, effective reduction strategies remain crucial for minimizing its anti-nutritional effects.

2.2.1 Phytic acid

Phytic acid (myo-inositol hexaphosphoric acid, IP_6) is a naturally occurring compound found in plant seeds, legumes, cereals, and nuts. It is commonly present in its salt form, phytate, and serves as the primary storage form of phosphorus in mature plants, accounting for 50–85% of their total phosphorus content (Gupta et al., 2015). PA is stored in the globoid crystal within the protein bodies of legume seed, thus, its content generally increases in proportion to higher protein concentration (Carnovale et al., 1988; Gupta et al., 2015). Compared to whole pea seeds, pea protein concentrate has been documented to contain more than twice the amount of PA, increasing from 0.85% to 1.90% DW (Carnovale et al., 1988).

Reducing PA levels in plant-based foods is essential for improving their nutritional quality. The negatively charged phosphate groups in PA bind strongly to positively charged minerals, forming insoluble complexes that impair the absorption of essential minerals such as iron, zinc, calcium, and magnesium (Amat et al., 2023). This is particularly concerning given that deficiencies in iron and zinc, linked to anaemia, immune dysfunction, and impaired growth,

remains major global health issues. With plant-based diets on the rise, reducing PA content is a vital step toward combating micronutrient malnutrition (Gupta et al., 2015).

In addition, PA can form complexes with proteins, which may impair their digestibility. The formation of these complexes is influenced by factors such as the protein matrix and pH (Amat et al., 2023; Carnovale et al., 1988). Below a protein's isoelectric point, PA binds to positively charged proteins via electrostatic interactions. Above this pH, where both PA and proteins carry negative charges, ternary complexes may form through cation bridging (Amat et al., 2023; Nassar et al., 2021). These interactions are also affected by the plant protein source and the accessibility of amino terminal groups, making the specific protein conformation a key determinant of PA binding (Amat et al., 2023). As such, reducing PA content does not always translate to improved protein digestibility. While previous studies highlight PA's role in limiting digestibility, other ANFs should also be considered as contributors to digestive interference.

Reduction strategies

Humans are unable to break down the majority of PA consumed, making its breakdown largely dependent on mechanical processing or the activity of exogenous phytase from plant or microbial sources (Arsov et al., 2024; Gupta et al., 2015; Nikmaram et al., 2017). Various techniques have been documented for reducing PA in pea seeds, including dehulling, milling, extrusion, soaking, germination, and fermentation. As previously noted, the TPP used in this degree project likely underwent several of these steps, such as dehulling, milling, and extrusion. However, combining extruded TPP with phytase-activating methods like fermentation could potentially further reduce PA levels (Nikmaram et al., 2017).

Phytase is an enzyme that hydrolyses PA into lower myo-inositol phosphate forms (IP_5, IP_4, IP_3, IP_2, IP_1), which have a reduced capacity to bind minerals (Gupta et al., 2015). When exposed to moisture, such as during soaking, naturally occurring phytase in plants becomes active and initiates PA hydrolysis (Samtiya et al., 2020). Soaking can also cause water-soluble PA to leach into the soaking medium, however, this may also lead to the loss of minerals and soluble proteins (Gupta et al., 2015). Fermentation offers a more targeted approach, as both plant- and microbe-derived phytase can be active, potentially resulting in significant PA degradation. Nevertheless, the hydrolytic efficiency of any phytase depends on factors such as time, temperature, pH, and resistance to proteolysis. Additionally, for microbial phytase, the specific strain's phytase-producing capacity is a key determinant of its effectiveness (Gupta et al., 2015; Kaleda et al., 2020).

2.3 Protein digestion

Protein digestion is a complex process that occurs in multiple stages along the gastrointestinal tract. It involves enzymatic hydrolysis, where proteins are broken down into smaller peptides and eventually into free amino acids, which are then absorbed into the bloodstream. The key initial phases include: 1) the salivary phase, 2) the gastric phase, and 3) the intestinal phase.

2.3.1 Protein digestibility

Protein digestibility refers to the ability to which a protein can be broken down into absorbable amino acids and smaller peptides during digestion (Goodman, 2010). Along with amino acid composition, digestibility is a key factor in determining the nutritional quality of a protein source, as it affects the bioavailability of essential amino acids necessary for human nutrition. Additionally, proteins with higher digestibility have been linked to improved health outcomes (Kaur, et al., 2022).

The extent of protein digestibility is influenced by both exogenous and endogenous factors. Exogenous factors include interactions between proteins and other compounds, while endogenous factors relate to the intrinsic structure of the protein itself. Thus, pea proteins, and plant proteins in general, have lower digestibility compared to animal proteins due to exogenous factors, such as interactions with ANFs, and endogenous factors, such as their compact and rigid protein structure (Park et al., 2010). However, by altering these factors through various processing techniques, such as chemical, physical, or enzymatic methods, the digestibility of plant proteins can be enhanced (Kaur et al., 2022).

2.3.2 *In vitro* protein digestibility

A widespread method for determining protein digestibility of foods is through *in vitro* protein digestibility (IVPD) studies, where the human digestive system is simulated in a laboratory setting. Unlike *in vivo* methods, *in vitro* studies are more cost-effective and less time-consuming while being considered equally reliable for assessing the protein digestibility of foods (Kaur et al., 2022). Additionally, IVPD provides a solid basis for comparing results across different studies, as standardized protocols have been developed for this purpose.

2.4 Lactic acid fermentation

Fermentation is a biochemical process carried out by bacteria and fungi. By utilizing part of the food substrate for growth and reproduction, the microorganisms produce different enzymes and metabolites that can enhance food preservation, texture, flavour, and the bioavailability and digestibility of nutrients (Emkani et al., 2022). Lactic acid bacteria (LAB) are a group of facultative anaerobic gram-positive bacteria that produce lactic acid as their primary metabolite during fermentation, leading to an acidification and pH reduction of the food product. In addition, depending on the specific genus and species, different metabolic profiles and enzyme activities have been observed, resulting in the production of various primary and secondary metabolites in the final product. Consequently, different LAB can contribute different sensory and nutritional profiles of fermented foods (Harlé et al., 2020).

Lactic acid fermentation has been shown to increase the protein content of peas through microbial biomass growth, secretion of microbial proteins, and carbohydrate consumption (Çabuk et al., 2018a; Skalickova et al., 2022). However, the extent of this effect varies

depending on the legume substrate and the specific microorganism involved (Emkani et al., 2022).

LAB can be divided into two main groups based on their fermentation metabolism and the end products formed during carbohydrate breakdown for energy production (Emkani et al., 2022). Homofermentative LAB primarily use the Embden-Meyerhof-Parnas pathway to convert glucose into lactic acid as the main end product. In contrast, heterofermentative LAB use the phosphoketolase pathway, generating a mix of metabolites such as lactic acid, ethanol, and acetic acid (Giacon et al., 2022). Heterofermentative LAB are further categorized as obligate or facultative, with facultative species able to shift between homo- and heterofermentative pathways depending on the sugar source and environmental conditions (Gänzle, 2015).

In addition to carbohydrate catabolism (glycolysis), the catabolic breakdown of proteins (proteolysis) and lipids (lipolysis) is also essential for bacterial growth. During proteolysis, LAB degrade proteins into smaller polypeptides and free amino acids, which can then be further metabolised and used in biosynthetic processes (Emkani et al., 2022). This activity contributes to the potential of fermentation to enhance protein digestibility in legumes, as discussed in the following paragraph.

2.4.1 Effect on protein digestibility

Lactic acid fermentation can improve the protein digestibility of legumes through multiple mechanisms. It can reduce ANFs that inhibit digestive enzymes, as well as stimulate the production of proteolytic enzymes that facilitate the breakdown and release of proteins from the food matrix. For instance, LAB have been shown to reduce TIs, which interfere with trypsin, a key enzyme involved in protein digestion in the human gastrointestinal tract. This effect is partly attributed to the secretion of proteolytic enzymes by LAB, which degrade TIs into smaller, inactive fragments (Çabuk et al., 2018a). Additionally, LAB have demonstrated the ability to mitigate the impact of insoluble protein complexes formed with ANFs such as tannins and PA. Through enzyme production and hydrolytic activity, LAB can lower the concentration of these compounds, thereby enhancing overall protein digestibility (Knez et al., 2023).

2.4.2 Effect on phytic acid

The level of PA present in legumes can be reduced though the metabolic activity of lactic acid fermentation. Through a combination of acidification, activation of endogenous legume phytase, and the production of microbial phytase, PA is hydrolysed, reducing the inhibitory effects on both protein digestion and mineral absorption (Samtiya et al., 2020). Furthermore, the production of organic acids during lactic acid fermentation has been reported to facilitate the formation of soluble mineral complexes, thereby preventing the formation of insoluble mineral-PA complexes. As a result, mineral bioavailability can be enhanced (Hemalatha et al., 2007).

2.4.3 Effect on sensory properties

The metabolic activity of LAB contributes to the production of a wide range of enzymes and metabolites, which holds promise for enhancing the sensory properties of plant-based protein sources. For example, lactic acid fermentation has been reported to initiate protein gelation through its proteolytic activity, thereby improving textural properties of PBMA. Additionally, acidification have been reported to contribute to less soft textures due to acid-induced gelation (Valtonen et al., 2023; Ren & Li, 2022).

Furthermore, LAB can mitigate off-flavours in plant proteins by converting aldehydes like hexanal, linked to "hay" and "beany" notes, into less odorous alcohols and carboxylic acids via alcohol and aldehyde dehydrogenase activity (El Youssef et al., 2020; Fischer et al., 2022). In addition, LAB fermentation can enhance the aroma and taste of PBMA by producing flavour-enhancing metabolites (Figure 1) (Emkani et al., 2022). For example, proteolysis releases peptides and amino acids that can interact with other compounds, potentially enhancing sweetness and saltiness, masking bitterness and sourness, and increasing umami through the presence of glutamic and aspartic acids (Valtonen et al., 2023). However, fermentation may also introduce undesirable notes, such as soapy or medicinal aromas, depending on the starter culture used (Kaleda et al., 2020).

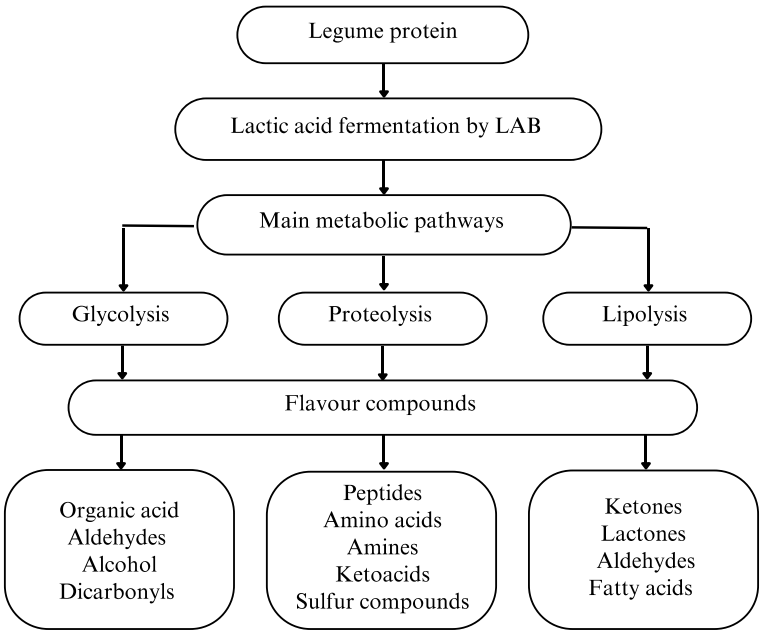

Figure 1. Overview of the main metabolic pathways in lactic acid fermentation contributing to the production of flavour compounds, adapted from Emkani et al. (2022).

2.5 Starter cultures

Given the potential of lactic acid fermentation to reduce PA levels, improve protein digestibility, and enhance sensory characteristics, three different starter cultures, each involving a distinct LAB species, will be evaluated in this degree project. The species, *Latilactobacillus sakei* (*L. sakei*), *Leuconostoc carnosum (L. carnosum),* and *Lactococcus lactis* (*L. lactis)* are generally recognised as safe by the U.S. Food and Drug Administration and are included in the Qualified Presumption of Safety system by the European Food Safety Authority, indicating a long history of safe use in food.

2.5.1 *Latilactobacillus sakei*

L. sakei, formerly known as *Lactobacillus sakei,* is a facultative heterofermentative LAB naturally found in fresh meat, fish, and seafood. Additionally, it has been isolated from various fermented products such as kimchi, sauerkraut, and sake (Champomier-Vergès et al., 2001; Mani-López et al., 2024). As a starter culture, it is most used in fermented dry sausages, where it enhances taste and aroma, as well as safety by producing bacteriocin to suppress pathogenic and spoilage bacteria. However, *L. sakei* is increasingly employed as a starter culture for plant-based fermentations due to its well-documented bioprotective properties. Nevertheless, few studies have investigated its effects, particularly concerning its impact on nutritional and sensory quality, of plant-based protein sources.

L. sakei strains have shown phytase activity in previous studies, demonstrating its ability to degrade PA (Carrizo et al., 2016; Kang et al., 2015). Regarding protein digestibility, Bartkiene et al. (2015) reported a significant increase in IVPD of both lupin- and soybean-derived proteins when fermented with *L. sakei*, compared to untreated samples as well as those undergoing spontaneous fermentation. Moreover, Lee et al. (2024) examined the sensory effects of an *L. sakei* strain in the production of black soybean yogurt. The results demonstrated its ability to generate flavour-enhancing volatile compounds, underscoring its potential to improve the flavour profile of legume-based foods.

2.5.2 *Leuconostoc carnosum*

L. carnosum is a facultative heterofermentative LAB naturally found in fresh cold-stored meat products. A key characteristic is its ability to produce bacteriocins, which inhibits the growth of pathogenic species such as *Listeria monocytogenes* (Candeliere et al., 2021). Although *L. carnosum* has primarily been studied in meat fermentation, its demonstrated ability to enhance the flavour of fermented meats, in addition to improve shelf-life, presents an interesting aspect to explore for PBMA as well (Jacobsen et al., 2003). Additionally, *Leuconostoc* species, in general, have been recognized for their roles in fermenting plant-derived foods, contributing to both improved nutritional value and enhanced sensory properties (Shin et al., 2015). For example, Wätjen et al. (2023) evaluated seven plant-derived *Leuconostoc* strains for use as freeze-dried starter cultures in soy-based cheese fermentation. Although the study primarily focused on flavour profiles related to cheese, the results were noteworthy, as all strains

effectively metabolized and reduced off-flavours commonly linked to soy and legumes, such as hexanal.

Although specific studies on *L. carnosum*'s ability to reduce ANFs and enhance protein digestibility are limited, research on the *Leuconostoc* genus suggests potential. Saizen et al. (2023) reported improved IVPD in fermented peanut slurry using a starter culture containing *L. lactis* subspecies and *Leuconostoc* species, attributed to protease activity and peptide release. Similarly, Rizzello et al. (2019) found that *Leuconostoc kimchii* exhibited both proteolytic and phytase activity in faba bean dough, reducing phytic acid by 38% after 24 hours and 55% after 48 hours of fermentation at 25 °C.

2.5.3 *Lactococcus lactis*

L. lactis is a homofermentative LAB, commonly recognized for its role in the dairy industry, where it plays a crucial role as a starter culture for a variety of fermented dairy products (Chen et al., 2015). Furthermore, it is a well know bacteriocin producing specie, protecting a wide range of food products from pathogenic and spoilage bacteria (Onyeaka & Nwabor, 2022).

The proteolytic activity of *L. lactis* has been extensively studied. Nájera-Domínguez and Gutiérrez-Méndez (2013) analysed 21 strains from various sources, including raw-milk cheeses, legumes, cereals, root vegetables, and commercial dairy starters. While proteolytic activity varied among strains, those from commercial starters generally showed medium to high activity, likely due to adaptations for milk protein degradation. Similarly, García-Cano et al. (2019) identified an *L. lactis* strain with the highest proteolytic activity among 137 LAB strains isolated from dairy, underscoring the species' potential to break down proteins, thereby improving protein digestibility.

Several *L. lactis* strains have demonstrated phytase activity, highlighting the species' ability to degrade PA in food products (De Angelis et al., 2003; Khodaii et al., 2013; Zhou et al., 2021). However, studies on its effectiveness in reducing PA in peas, and legumes in general, remain limited. Moreover, *L. lactis* has demonstrated potential to enhance the sensory qualities of pea-based PBMA. In a study by Valtonen et al. (2023), fermentation of pea protein concentrates with *L. lactis* significantly reduced pea-like odour, enhanced umami flavour and modified the texture of plant-based sausages.

In summary, although the selected species are commonly used in food applications, their potential as starter cultures to enhance the nutritional and sensory qualities of PBMA formulated with TPP remains largely unexplored. This underscores the need for further research into the specific effects of *L. sakei*, *L. carnosum*, and *L. lactis*, making it a relevant and compelling focus for this degree project.

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Starter cultures and TPP

The commercial freeze-dried starter cultures from the Vertera® (VEGA™) SAFEPRO® range (Chr. Hansen, part of Novonesis, Denmark) were used in this degree project. Their species composition and batch numbers are displayed in Table 1. The commercial TPP (PPEAT2003, EU) was obtained from Solina and was based on yellow peas. The proximate nutritional composition per 100 g of DW was 7.0 g of fat, 5.0 g of carbohydrates, 7.0 g of fibre, and 70.0 g of protein.

Table 1. The commercial freeze-dried starter cultures, species composition and batch number used for this degree project.

Starter culture	LAB species	Batch number
Vertera® (VEGA™) SAFEPRO® 01	*Latilactobacillus sakei*	3758975
Vertera® (VEGA™) SAFEPRO® 02	*Leuconostoc carnosum*	3714024
Vertera® (VEGA™) SAFEPRO® 03	*Lactococcus lactis*	3714934

3.1.2 Chemicals and enzymes

The chemicals, enzymes, and their quantities used for IVPD are presented in Table 2 and Table 3. Milli-Q water was used for all IVPD solutions and dilutions, referred to as "water" in the text. The phytic acid assay kit was obtained from Neogen's Megazyme range (Neogen® Megazyme International Ltd, Ireland). Hydrochloric acid, calcium chloride dihydrate and sodium hydroxide (VWR Chemicals, Belgium). Ammonium carbonate, ammonium molybdate, ascorbic acid, pancreatin from porcine pancreas, α-Amylase from porcine pancreatin, and soluble corn starch (Sigma Aldrich, USA). Magnesium chloride hexahydrate, potassium chloride, potassium dihydrogen phosphate, porcine bile extract, porcine pepsin, sodium chloride, sodium hydrogen carbonate, and trichloroacetic acid (Merck Millipore, Germany). Sulphuric acid (Scharlab, Spain).

3.2 Methods

A flow chart of the experimental design is displayed in Figure 2. All fermentations were conducted in duplicate, and each fermented sample was analysed in duplicate, resulting in a total of four replicates (n = 4). Prior to analysis, the samples were freeze-dried to obtain results based on freeze-dried weight (FDW).

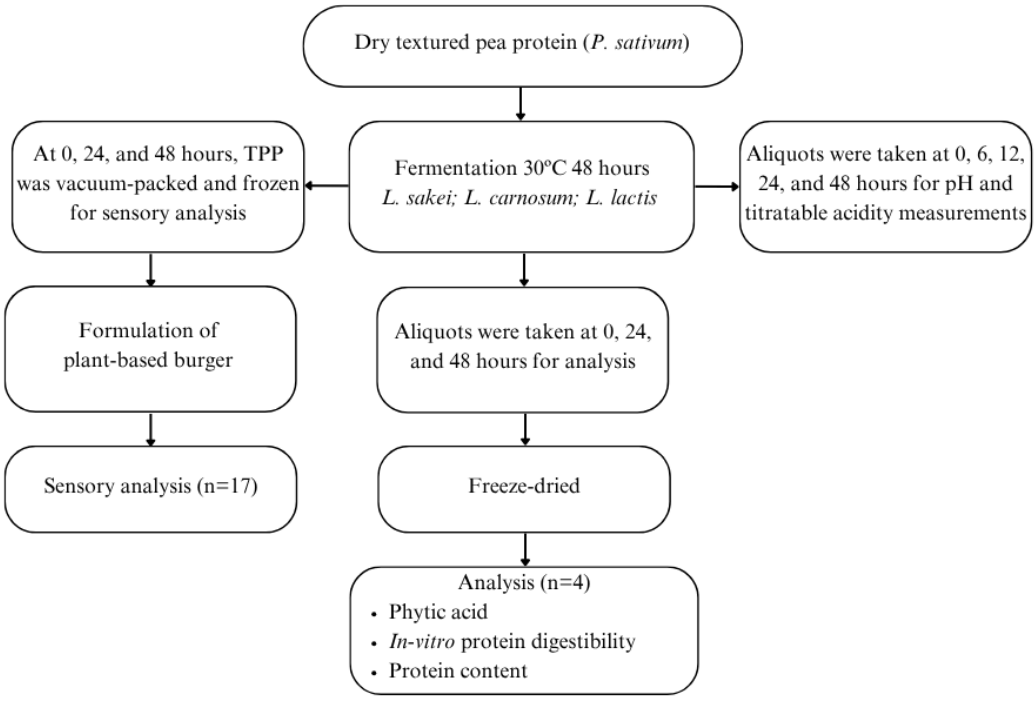

Figure 2. Flow chart of the experimental design.

3.2.1 Fermentation

A whole 25 g bag of each freeze-dried starter culture was dissolved in 475 g of distilled water to create a "culture water" solution. The manufacturer recommends preparing the entire 25 g bag to ensure the correct culture dosage of 0.025% in the final PBMA product. The amounts of culture water, rehydration water, and TPP required to achieve this 0.025% starter culture concentration was determined based on a plant-based burger (PBB) recipe provided by Solina.

In a 5-liter metal bowl, dry TPP, distilled water, and culture water were weighed according to the end application recipe. The ingredients were gently mixed to ensure all TPP was fully moistened. After 15 minutes, once the liquid was fully absorbed, the TPP was transferred to a sterile, airtight glass container and incubated at 30°C for 48 hours. A separate container of each culture was prepared for the 24-hour samples for sensory evaluation, vacuum-packed (Multivac), and frozen immediately after fermentation.

During fermentation, samples were withdrawn at 6, 12, 24, and 48 hours using a sterile metal spoon. Approximately 20 g of each sample was collected for pH and acidity measurements. At 24 and 48 hours, an additional approximately 70 g was withdrawn, placed in aluminium trays covered with aluminium foil, then frozen and subsequently freeze-dried (Heto Drywinner). The remaining TPP after 48 hours of fermentation was vacuum-packed and frozen for sensory evaluation. An unfermented sample, representing the 0-hour time point, was prepared without adding starter culture. After freeze-drying, the samples were crushed into a fine powder using a pestle and mortar, vacuum-packed, and stored under refrigerated conditions for further analysis.

3.2.2 pH and titratable acidity

For pH measurement, 10 g of each fresh sample was suspended in 50 mL of distilled water and stirred. The pH was then measured using a pH meter (Thermo Scientific™ Orion Lab Star PH111).

To determine titratable acidity, the procedure of Tyl and Sadler (2017) was followed, with lactic acid assumed as the predominant acid. Approximately 5 g of each sample was suspended in 50 mL of distilled water, stirred for 60 seconds, and then left to sit for 30 minutes. The acidity was then measured by titrating 20 mL of the prepared sample with 0.1 N NaOH, using phenolphthalein as an indicator. Titration continued until a light pink colour persisted for 30 seconds. The total percentage of acidity as lactic acid was calculated using the following equation (1):

$$\text{Acidity as lactic acid [\%]} = N \times V \times Eqwt \times \left(\frac{1}{W}\right) \times \left(\frac{1}{1000}\right) \times 100 \quad (1)$$

Where:
N= normality of NaOH = 0.1 N [mEq/mL]
V= volume of titrant [mL]
Eqwt = equivalent weight of lactic acid = 90.08 mg/mEq
W= mass of sample [g]

While lactic acid was used for the calculation, other organic acids, such as acetic acid, particularly produced by the heterofermentative strains *L. sakei* and *L. carnosum*, may also have been present. Given the lower equivalent weight of acetic acid (60.05 mg/mEq), this could lead to a slight overestimation of total acidity if present in significant amounts.

3.2.3 Phytic acid analysis

To determine the PA content in the unfermented and fermented samples, the commercial phytic acid assay kit from Neogen® Megazyme was used with slight modifications (McKie & McCleary, 2016). The kit measures phosphorus released through the enzymatic hydrolysis of myo-inositol phosphates by phytase and alkaline phosphatase, under the assumption that other phosphates are negligible.

Briefly, 1 g of freeze-dried sample was dissolved in 20 mL 0.66 M HCl, stirred for 3 hours and then left to stand overnight for convenience. To enhance the extraction of the unfermented sample, continuous stirring was carried out for 24 hours in a water bath set at 30 °C. Following extraction, 1 mL of the solution was centrifuged (Eppendorf MiniSpin®) at 13,000 rpm for 10 minutes, and 0.5 mL of the supernatant was neutralized with 0.5 mL of 0.75 M NaOH. The enzymatic dephosphorylation was performed in two parallel reactions: one measuring free phosphorus (no enzymes) and one for total phosphorus (with enzymes). For the free phosphorus assay, 0.05 mL of sample extract was mixed with 0.2 mL of 200 mM sodium acetate buffer (pH 5.5) and 0.62 mL of distilled water, vortexed, and incubated at 40 °C for 10 minutes. In the total phosphorus assay, 0.02 mL of phytase replaced the same volume of

water. After incubation, both reactions were treated with 0.2 mL of buffer (400 mM glycine, 4 mM $MgCl_2$, 0.4 mM $ZnSO_4$, pH 10.4), followed by 0.02 mL alkaline phosphatase (total phosphorus) or 0.02 mL water (free phosphorus). After vortexing, samples were incubated at 40 °C for 15 minutes, stopped with 0.3 mL of 50% (w/v) trichloroacetic acid, and centrifuged at 13,000 rpm for 10 minutes. The supernatant was collected for colorimetric phosphorus quantification.

The colour reagent was freshly prepared, consisting of 10% (w/v) ascorbic acid in 1 M sulfuric acid and 5% (w/v) ammonium molybdate, mixed in a 5:1 ratio. For analysis, 1 mL of supernatant was mixed with 0.5 mL of the colour reagent, vortexed, and incubated at 40 °C for 1 hour alongside standards. Absorbance was read at 655 nm (Varian Cary 50 UV spectrophotometer, Australia), and PA content was calculated using equations (2, 3).

$$\text{Phosphorus [g/100 g FDW]} = M \times 20 \times F \times v \times \left(\frac{1}{10{,}000 \times 1.0 \times W}\right) \times \Delta A \quad (2)$$

Where:
M = mean value of phosphorus standards [µg/ΔA]
20 = original sample extract volume [mL]
F = dilution factor
v = sample volume used in colorimetric determination [mL]
W = weight of original sample material [g]
ΔA = absorbance change of sample

$$\text{Phytic acid [g/100 g FDW]} = Phosphorus\ [\text{g}/100\ \text{g}] \times \left(\frac{1}{0.282}\right) \quad (3)$$

Where:
0.282 = Assuming that the amount of phosphorus measured is exclusively released from PA and that this comprises 28.2% of PA.

3.2.4 *In vitro* protein digestibility

To investigate how fermentation affected the protein digestibility of TPP, the INFOGEST protocol was followed with slight modifications (Brodkorb et al., 2019; Minekus et al., 2014). This method aims to mimic the upper gastrointestinal tract, specifically the salivary, gastric, and small intestinal phases of human digestion, and maintains a constant ratio of food to enzymes and electrolytes, as well as a fixed pH for each digestive phase. For analysis, the unfermented control and 48-hour fermented TPP were selected based on a pre-assessment of their sensory characteristics, along with water blanks.

In preparation, 400 mL of simulated digestion fluids were prepared at a 1.25× concentration: simulated salivary fluid (SSF), simulated gastric fluid (SGF), and simulated intestinal fluid (SIF), following the electrolyte stock solutions listed in Table 2. The prepared fluids were stored at 5°C until use. The enzyme and bile extract solutions were freshly prepared on the day of experiment, following the activities, concentrations, and volumes specified in Table 3. These solutions were kept on ice until use. Although carbohydrate digestion was beyond the scope of

this study, α-amylase was included in the salivary phase, as it was readily available during the experiment and represents a natural component of the initial stage of human digestion. Gastric lipase, however, was excluded from the protocol, as lipid digestion was not within the study's scope.

Table 2. Volumes of electrolyte stock solutions used for each digestion fluid, prepared at a 1.25× concentration and diluted to a final volume of 400 mL with Milli-Q water.

Chemicals	Stock concentration [g/L]	Molarity	Amount added for each fluid [mL]		
			SSF (pH 7)	SGF (pH 3)	SIF (pH 7)
KCL	37.3	0.5	15.1	6.9	6.8
KH_2PO_4	68	0.5	3.7	0.9	0.8
$NaHCO_3$	84	1	6.8	12.5	42.5
NaCl	117	2	-	11.8	9.6
$MgCl_2(H_2O)_6$	30.5	0.15	0.5	0.4	1.1
$(NH_4)_2CO_3$	48	0.5	0.06	0.5	-
HCl		6	0.09	1.3	0.7
$CaCl_2(H_2O)_2$*	44.1	0.3	0.025	0.005	0.04

*To prevent precipitation, $CaCl_2(H_2O)_2$ was added separately during each phase of digestion.

Table 3. Enzymes and bile extract used for IVPD, along with their final and specific activities, concentrations, and volumes. Each solution was prepared using the corresponding simulated digestion fluid.

Enzymes & Bile salts	Final enzyme activity or Bile concentration	Specific activity or Bile concentration	Concentration	Volume added per sample (5g)
α-Amylase	75 U/mL	1000 U/mg	1.5 mg/mL	0.5 mL*
Pepsin	2000 U/mL	11976 U/mg	2.09 mg/mL	1.6 mL**
Trypsin in pancreatin	50 U/mL	100 U/mg	4 mg/mL	5.0 mL***
Bile extract	10 mM	2.447 mmol/g	6.54 g/100 mL	2.5 mL***

*Prepared with SSF; **Prepared with SGF; ***Prepared with SIF to achieve the final volume.

To begin the digestion, 5 g of sample was weighed into a 250 mL conical flask and mixed with pre-warmed SSF at 37°C, $CaCl_2(H_2O)_2$, α-Amylase solution and water to achieve a 1× concentration of the SSF. The protocol recommends a swallowable, paste-like consistency for the oral bolus. Thus, an additional 10 mL of water was added. The flasks were sealed and placed in a shaking water bath (Lauda A120S/A100, Germany) at 37 °C for 2 minutes.

To initiate the gastric phase, flasks were removed from the water bath and mixed with pre-warmed SGF at 37 °C. The pH was adjusted to 3 using 5 M HCl, followed by the addition of $CaCl_2(H_2O)_2$, pepsin solution and water to reach a 1× SGF concentration. The flasks were returned to the shaking water bath (37 °C) for 2 hours. The timer was started upon pepsin addition, and pH was monitored every 30 minutes and adjusted as needed to maintain a stable level of 3. The intestinal phase was initiated by adding pre-warmed SIF at 37°C and adjusting the pH to 7 using 5 M NaOH. Bile extract, pancreatin, and $CaCl_2(H_2O)_2$ were added, followed by water to reach a 1× SIF concentration. Flasks were then incubated under the same conditions

for another 2 hours, with pH monitored every 30 minutes to ensure a stable level of 7. The timer was started upon pancreatin addition. Reagent volumes added for all phases are listed in Table 4.

Digestion was halted by raising the pH to 7.5 using 5 M NaOH and placing the samples on ice for 10 minutes to stop enzymatic activity. Samples were transferred to Falcon tubes and centrifuged (Beckman Coulter Allegra is X-15R, USA) at 4000 g for 20 minutes at 20 °C, and the pellet (insoluble fraction) and supernatant (soluble fraction) were separated and prepared for protein analysis using the Dumas method. Specifically, 20 mL of supernatant was mixed with 1.0 g of soluble starch in aluminium trays, while the pellets were separately placed in similar trays. All trays were covered with aluminium foil, frozen, and freeze-dried for 88 hours and 40 minutes.

Table 4. Amounts of simulated digestion fluids, $CaCl_2(H_2O)_2$, enzyme and bile solutions, and total volumes of pH-adjusting agents and water added in each digestion phase, to achieve the final target volume.

Digestion phase	**Simulated fluid [mL]**	**$CaCl_2(H_2O)_2$ [mL]**	**Enzyme & bile solution [mL]**		**Total volume of pH adjustments and H_2O [mL]**	**Final volume [mL]**
Salivary	3.5	0.025	α-Amylase	0.5	0.975	10
Gastric	7.5	0.005	Pepsin	1.6	0.895	20
Intestinal	11	0.04	Pancreatin	5	1.46	40
			Bile extract	2.5		

3.2.5 Protein analysis

The protein content of the freeze-dried samples, before and after IVPD, were analysed using Dumas analysis (Thermo Scientific™ FlashEA 1112 N/Protein Analyzer, USA). For the analysis, 25 mg of each sample was combusted, and the total nitrogen content was measured. A protein conversion factor of 6.25 was used to determine the final protein content of the samples (Pelgrom et al., 2013).

3.2.6 Formulation of plant-based burgers

The PBBs were formulated at Solina's facilities in Malmö. The previously frozen TPP (unfermented and fermented for 24 and 48 hours) were thawed overnight in a refrigerator and then mixed (KitchenAid stand mixer) with an emulsion of ice water, neutral rapeseed oil, methylcellulose, vegetable fibre, and starch, which had been pre-blended (Vorwerk Thermomix) according to a recipe provided by Solina. Additionally, 1% salt (wet w/w) was added to the mixture. No additional colouring or flavouring agents were included to maintain a product as neutral as possible. The mixture was vacuum-treated to reduce air incorporation and then divided into 80-gram portions and shaped into PBBs using a patty press. The patties were placed on metal trays, covered with plastic foil, and frozen overnight. Once fully frozen, the PBBs were vacuum-sealed and stored in the freezer until sensory evaluation.

3.2.7 Sensory analysis

To evaluate the effect of fermentation on the sensory properties of the PBBs, a two-step descriptive sensory analysis was applied, where the intensity of selected attributes in smell, texture, and taste were assessed.

A preliminary internal assessment of the formulations was conducted to determine the setup for the main evaluation. Since the intensity differences were perceived as more pronounced after 48 hours of fermentation compared to 24 hours, the 48-hour fermented samples and the unfermented control were selected for further analysis. Ten sensory attributes (pea-like smell, yeast-like smell, hay-like smell, firmness, chewiness, juiciness, pea-like taste, yeast-like taste, sweet taste, umami taste) with descriptors, listed in Appendix A, were defined to create a reference framework for the panel, as descriptive analysis requires precise terminology (Lawless & Heymann, 2010). The selected attributes were based on prior studies of PBMA and pea protein characteristics (Kaleda et al., 2020; Valtonen et al., 2023; Bakhsh et al., 2021; Elhalis et al., 2023a).

The sensory evaluation was conducted on May 5th at Solina's facilities with a panel of 17 untrained participants, all Solina employees. PBB samples were prepared from frozen using sous-vide cooking at 72°C for 20 minutes and served as quarters on paper plates labelled with randomized three-digit codes (Figure 3). All samples were presented simultaneously, allowing participants to choose their tasting order. A Google Forms questionnaire was used, with each attribute rated on a 9-point intensity scale (1 = no intensity, 9 = extreme intensity) (Lawless & Heymann, 2010). Between samples, participants were instructed to cleanse their palate and neutralize taste perception with water and plain wheat crackers ("smörgåsrån"). Participation was voluntary, anonymous, and based on informed consent, with all participants informed of ingredients and their right to withdraw at any time.

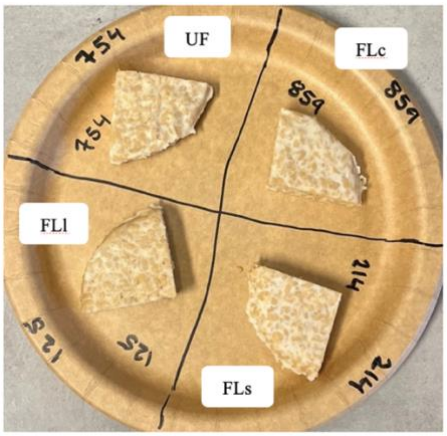

Figure 3. PBB prepared for sensory analysis, formulated with either unfermented or fermented TPP for 48 hours, each sample labelled with a three-digit code. **UF** (Unfermented), **FLs** (Fermented, *L. sakei)*, **FLc** (Fermented, *L. carnosum)*, **FLl** (Fermented, *L. lactis).*

3.3 Statistical analysis

All data were analysed using Microsoft® Excel (Version 2502, Build 18526.20168), supplemented with the Real Statistics Resource Pack add-in. Statistical differences between and within samples were assessed using one-way or two-way ANOVA, depending on the dataset, followed by Tukey's HSD post hoc test for pairwise comparisons. The data obtained from the sensory analysis were log-transformed prior to statistical analysis (Curran-Everett, 2018). A significance level of $p < 0.05$ was applied.

4. RESULTS

4.1 pH and titratable acidity

The effect of fermentation on pH and titratable acidity is shown in Figure 4. After 48 hours of fermentation, pH significantly ($p<0.0001$) decreased in all samples compared to the initial value (7.88 ± 0.00). However, by the end of fermentation, differences between samples emerged, with FLl (6.54 ± 0.12) having a significantly ($p<0.0001$) lower pH than both FLs (6.87 ± 0.02) and FLc (6.88 ± 0.02). No significant difference was found between FLs and FLc.

The rate of pH change varied among the samples. During the first 6 hours, only FLc showed a significant decrease ($p<0.0002$) compared to the initial value. At this time, FLc also had a significantly lower pH compared to both FLs ($p<0.05$) and FLl ($p<0.002$). No significant difference was observed between FLs and FLl. By 12 hours, FLs exhibited a significant ($p<0.0001$) drop compared to the initial value. Additionally, both FLs and FLc had continued to decrease compared to their values at 6 hours ($p<0.0001$) and exhibited lower pH values than FLl ($p<0.0001$). However, no significant difference between each other.

After 24 hours, FLl (7.65 ± 0.01) had significantly ($P<0.0005$) decreased in pH compared to the initial value. Yet, both FLs (6.97 ± 0.01) and FLc (7.10 ± 0.01) had a lower pH compared to FLl ($p<0.0001$). Additionally at 24 hours, the pH had dropped further in FLs ($p<0.0001$) and FLl ($p<0.0007$) compared to its 12 hours value, while FLc showed no significant change during this period. Between 24 and 48 hours, a significant decrease in pH was observed for FLc ($p<0.0007$) and FLl ($p<0.0001$), whereas FLs showed no further significant change.

All starter cultures exhibited a significant increase ($p<0.0002$) in acidity as lactic acid after 48 hours of fermentation compared to the initial value (0.029 ± 0.000%). By the end of fermentation, acidity levels differed among the samples, were FLl (0.234 ± 0.026%) showed significantly higher ($p<0.0001$) acidity compared to both FLs (0.121 ± 0.019%) and FLc (0.090 ± 0.000%).

Similar to the pH development, the rate of acidity change varied between starter cultures. During the first 12 hours, no significant differences were observed within any sample compared to the initial value, nor between the samples. By 24 hours, acidity had increased significantly in FLs (0.072 ± 0.000%; $p<0.008$) and FLc (0.066 ± 0.006%; $p<0.03$), while the slight increase in FLl (0.049 ± 0.002%) was not statistically significant. Between 24 and 48 hours, acidity continued to rise significantly in FLs ($p<0.002$) and FLl ($p<0.0001$), whereas FLc showed no further significant change during this time period.

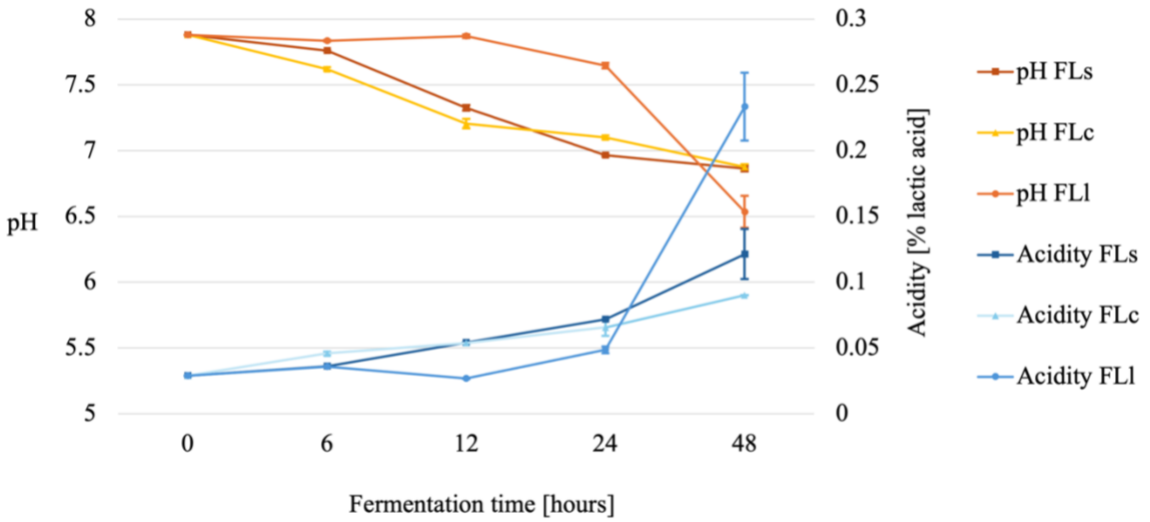

Figure 4. Mean effect of fermentation time at 30°C on the pH and titratable acidity of TPP. Error bars indicate standard deviation (n=4). **FLs** (Fermented, *L. sakei)*, **FLc** (Fermented, *L. carnosum)*, **FLl** (Fermented, *L. lactis).*

4.2 Phytic acid

The PA content of the unfermented and fermented TPP for 24 and 48 hours is presented in Figure 5. A slight reduction in PA content was observed in all fermented samples compared to the unfermented control (2.12 ± 0.07 g/100 g). However, statistically significant reductions ($p<0.008$) were only shown for FLs at 24 hours (1.81 ± 0.07 g/100 g) and FLc at both 24 (1.82 ± 0.05 g/100 g) and 48 hours (1.81 ± 0.03 g/100 g). These samples were also significantly lower ($p\leq0.02$) than FLl at 48 hours (2.07 ± 0.10 g/100 g). No significant differences were observed for FLl at 24 hours (1.90 ± 0.19 g/100 g) and FLs at 48 hours (2.01 ± 0.05 g/100 g) compared to any of the treatment.

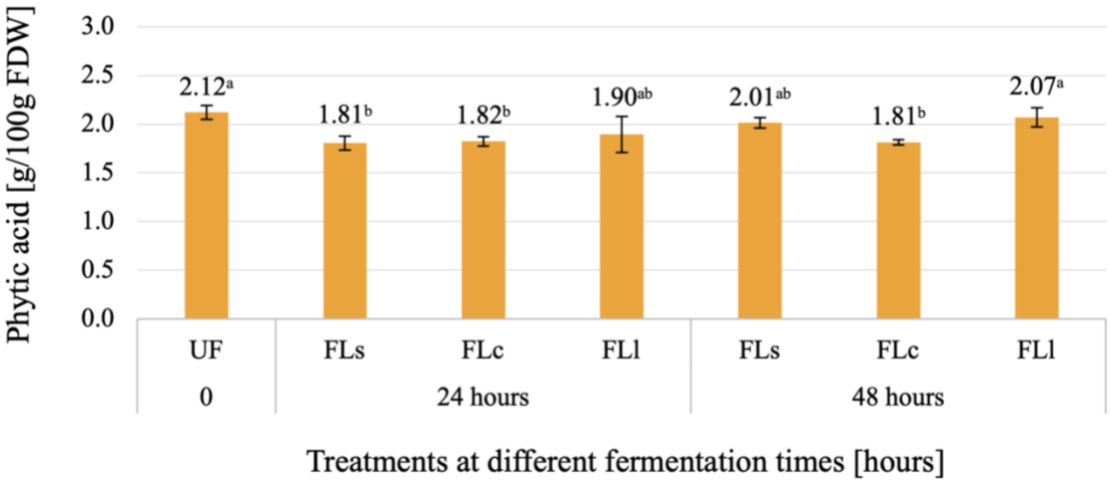

Figure 5. Mean phytic acid content in TPP before fermentation and after 24 and 48 hours, measured using the Megazyme phytic acid assay kit. Error bars represent standard deviation (n=4). Different letters indicate statistically significant differences ($p<0.05$). **UF** (Unfermented), **FLs** (Fermented, *L. sakei)*, **FLc** (Fermented, *L. carnosum)*, **FLl** (Fermented, *L. lactis).*

4.3 Protein content and *in vitro* protein digestibility

The protein content of unfermented and 24- and 48-hour fermented TPP ranged from 74.67% to 75.53%, with no statistically significant differences observed among treatments (Figure 6).

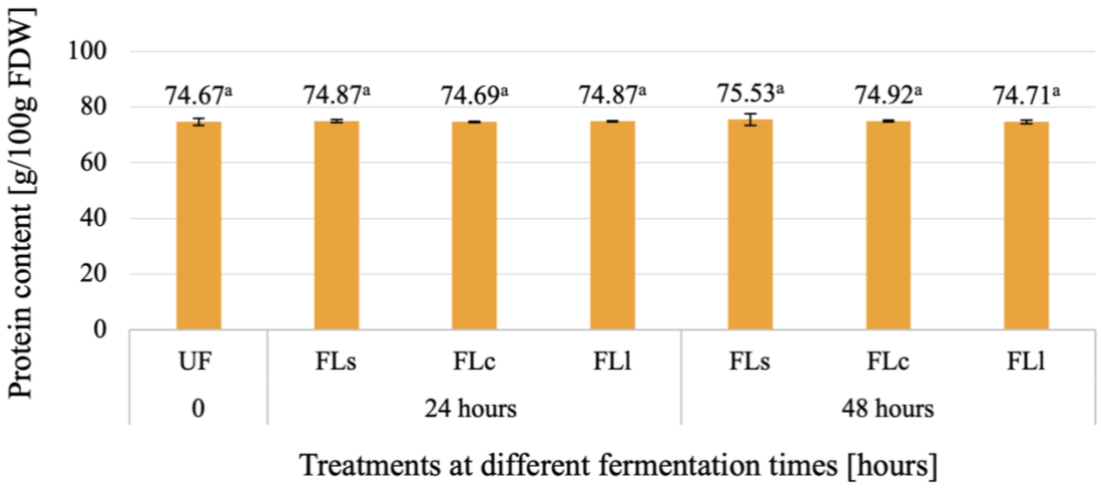

Figure 6. Mean protein content in TPP before fermentation and after 24 and 48 hours, measured using the Dumas method. Error bars represent standard deviation (n=4). Different letters indicate statistically significant differences ($p<0.05$). **UF** (Unfermented), **FLs** (Fermented, *L. sakei)*, **FLc** (Fermented, *L. carnosum)*, **FLl** (Fermented, *L. lactis).*

Following IVPD, no significant differences were observed in the percentages of digested and undigested protein among the unfermented and 48-hour fermented TPP samples (Figure 7). Digestibility values ranged from 34.0% to 34.7%, while undigested protein levels ranged from 55.4% to 57.6%. Total protein recovery after IVPD ranged from 90.0% to 91.6%.

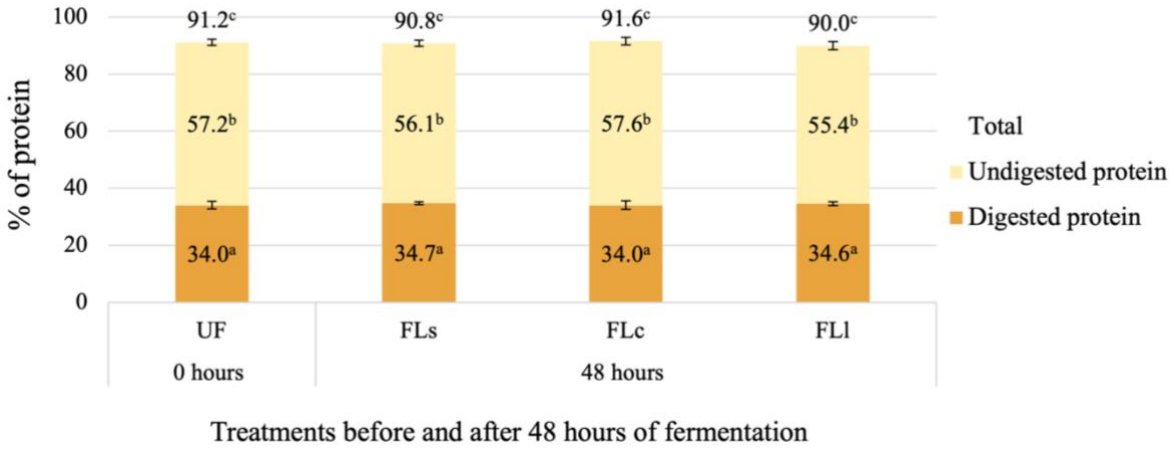

Figure 7. Mean percentages of digested and undigested protein after IVPD in unfermented and 48-hour fermented TPP, measured using the Dumas method. Error bars represent standard deviation (n=4). Different letters indicate statistically significant differences ($p<0.05$). **UF** (Unfermented), **FLs** (Fermented, *L. sakei)*, **FLc** (Fermented, *L. carnosum)*, **FLl** (Fermented, *L. lactis).*

4.4 Sensory analysis

The sensory profiles of the formulated PBB are shown in Figure 8. FLl was rated significantly more intense for yeast-like taste ($p<0.03$) and smell ($p<0.01$) than the control, and more intense in yeast-like taste than FLs ($p<0.02$). FLc also showed significantly higher intensity in yeast-like smell compared to the control ($p<0.05$). No other attributes differed significantly. However, the fermented samples indicated a trend towards higher intensity in sweet and umami taste, as well as firmness, compared to the unfermented control. In addition, FLc exhibited a slight trend toward greater chewiness and lower juiciness. For hay-like smell, FLc and FLs appeared slightly more intense than FLl and the control. Pea-like smell and taste were rated similar across samples, though the control showed a slight tendency toward lower intensity in pea-like taste. An overview of the statistical data, including mean log-transformed values and standard deviations, are presented in Appendix B.

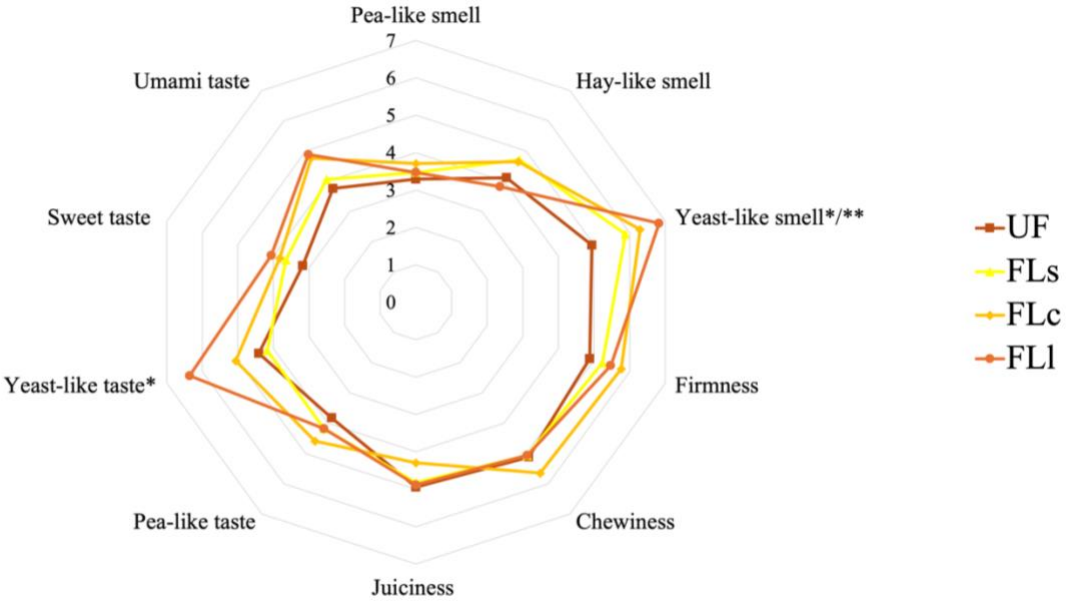

Figure 8. Mean sensory profile of PBB formulated with unfermented and fermented TPP for 48 hours, based on a 9-point intensity scale (n=17). Attributes marked with an asterisk indicate statistically significant differences (*: $p<0.05$, **: $p<0.01$). **UF** (Unfermented), **FLs** (Fermented, *L. sakei)*, **FLc** (Fermented, *L. carnosum)*, **FLl** (Fermented, *L. lactis).*

5. DISCUSSION

pH and titratable acidity

All starter cultures were able to grow in TPP, as indicated by a significant acidification after 48 hours of fermentation compared to initial values (Figure 4). The largest pH change occurred in TPP fermented with *L. lactis* showing a 1.4-unit drop, from 7.9 to 6.5, followed by *L. sakei* and *L. carnosum*, each showing a drop of 1.0, from 7.9 to 6.9. The pH decline was accompanied by a corresponding increase in acidity, consistent with previous findings (Bartkiene et al., 2015; Valtonen et al., 2023).

The observed pH reductions, ranging from 0.2 to 0.9 units after 24 hours and 1.0 to 1.4 units after 48 hours of fermentation, are broadly consistent, though not identical, with previous findings. Kaleda et al. (2020) reported a 0.7-1.0 unit decrease in a pea–oat protein blend (76% protein) after 24 hours of fermentation at 30 °C. In contrast, Maung et al. (2020) observed a non-significant 0.2-unit reduction in HME TVP (78% protein) after 48 hours of fermentation with *Bacillus subtilis* at 37 °C, which is lower than the reductions found in the present study. Despite differences in substrate composition and microbial strains, these studies support the observation that pH reductions in high-protein materials such as TPP tend to be less pronounced than in lower-protein material. For instance, Çabuk et al. (2018b) reported a 3.1-unit drop (from 7.5 to 4.3) in 40% pea flour after 11 hours of fermentation with *L. plantarum* at 32 °C. Similarly, Valtonen et al. (2023) found a 2.1-unit decrease (from 6.5 to 4.3) in 55% pea protein concentrate fermented with *L. lactis* at 30 °C. A likely explanation for the smaller pH shifts observed in higher-protein substrates (>70%) is their lower carbohydrate content. Since LAB primarily metabolize carbohydrates to produce lactic acid, limited carbohydrate availability reduces acid production and, consequently, leads to smaller pH declines (Akpoghelie et al., 2025).

Interestingly, *L. lactis* exhibited delayed acidification compared to *L. sakei* and *L. carnosum*. No significant drop in pH was observed for *L. lactis* until after 24 hours, with a noticeable increase in acidity occurring only after 48 hours (Figure 4). Most changes took place between 24 and 48 hours. In contrast, *L. sakei* and *L. carnosum* showed significant pH reductions within the first 12 hours and reached significantly lower pH levels than *L. lactis* after 24 hours. These findings suggest that *L. lactis* required more time to initiate acidification in TPP at 30 °C. However, this does not necessarily indicate slower growth. Kaleda et al. (2020) reported a similar observation with *L. plantarum*, where no early pH change was detected despite rapid initial growth and a short lag phase, as shown by heat-flow analysis. This underscores the importance of assessing bacterial growth in parallel with acidification in future studies.

Another plausible explanation for the differing acidification patterns observed is variation in lag phase duration among the cultures when exposed to protein-rich, low-carbohydrate substrates such as TPP. *L. lactis*, a homofermentative species well-adapted to dairy environments rich in sugars like lactose, may require more time to adjust to TPP's low-

carbohydrate composition. In contrast, *L. sakei* and *L. carnosum*, both facultative heterofermenters commonly associated with meat fermentation, are likely better suited to such conditions. Their ability to shift metabolic pathways in response to sugar availability and environmental factors provides greater metabolic flexibility, potentially giving them an advantage during the early stages of fermentation (Barbieri et al., 2022; Candeliere et al., 2021). However, this hypothesis requires further validation through studies specifically investigating bacterial growth under these conditions.

Phytic acid

The PA content in the unfermented TPP was 2.12 g/100 g FDW, which is higher than previous findings by Kaleda et al. (2020), reporting 1.42 g/100 g DW for an unfermented pea–oat protein powder (76% protein, 59% pea-derived), using the same analytical method. This discrepancy may partly be explained by the extended acid extraction procedure employed in the present study, which involved shaking the unfermented samples at 30 °C for 24 hours, a modification not mentioned by Kaleda et al. The modified extraction likely improved PA solubilization, thereby contributing to the higher measured values. However, varietal differences may also account for the observed variation. Carnovale et al. (1988), using an anion-exchange method, reported PA levels ranging from 1.34 to 1.90 g/100 g DW depending on pea variety, with their highest value, 1.90 g/100 g in a 50% protein sample, closely aligning with the present findings. However, the slightly elevated PA levels observed in this study may also be partly attributed to the higher protein content of the TPP (74.7%), given that PA is stored in globoid crystals within protein bodies and its concentration tends to increase with protein content (Carnovale et al., 1988; Gupta et al., 2015).

A significant reduction in PA content was observed in TPP fermented with *L. carnosum*, showing decreases of 14.2% and 14.6% after 24 and 48 hours, respectively. Additionally, a 14.6% reduction was observed in TPP fermented with *L. sakei* after 24 hours, compared to the unfermented control (Figure 5). Although not identical, these reductions are similar to those reported by Ojokoh and Wei (2011), who found an approximately 10% decrease in PA after 24 hours of fermenting LME soy protein with *Bacillus natto* at 25 °C, using an acidic iron (III) precipitation method. The reductions observed in this study suggest that the starter cultures, particularly *L. carnosum* and *L. sakei*, exhibited phytase activity, facilitating the hydrolysis of PA into lower myo-inositol phosphate forms and free phosphorus, mitigating the anti-nutritional effects of PA (Gupta et al., 2015).

However, no significant PA reduction was seen for TPP fermented with *L. sakei* and *L. lactis* after 48 hours, nor for *L. lactis* at 24 hours. Moreover, a trend toward increased PA levels between 24 and 48 hours in these treatments was observed (Figure 5). Similar results have been reported by Kaleda et al. (2020), who found significantly increased PA levels following fermentation using the same method as in this study. Ojokoh and Wei (2011) also noted a tendency toward increased PA content after fermentation of medium- and HME soy protein, compared to unfermented controls. Despite PA levels not exceeding unfermented values, the

inconsistent reductions and upward trends at 48 hours may reflect limitations in assay specificity. As noted by Kaleda et al. (2020) and further discussed by McKie and McCleary (2016), the Megazyme PA Assay Kit may overestimate PA content, particularly in processed food, potentially explaining this outcome.

The Megazyme PA Kit quantifies total phosphorus and PA via enzymatic hydrolysis, but lacks specificity, as it cannot differentiate intact PA (IP_6) from its lower myo-inositol phosphate forms (IP_5–IP_1) (McKie & McCleary, 2016). Since the TPP was subjected to both extrusion and fermentation, processes known to hydrolyse PA in legumes, it is likely that some PA was converted to lower forms without affecting the total measured PA content (Nikmaram et al., 2017; Samtiya et al., 2020). As these lower forms have reduced mineral-binding capacity, this likely improved mineral bioavailability in TPP (Gupta et al., 2015). However, given these uncertainties, future studies should include more specific methods, such as HPLC or LC-MS, to differentiate IP_6 from its derivatives and better evaluate PA degradation and nutritional impact in processed foods like TPP (Raboy et al., 2020; Tur et al., 2013).

Another plausible explanation for the inconsistent PA reductions following fermentation is the pH range, which remained between 7.9 and 6.5 across all treatments (Figure 4). Phytase activity in LAB have been observed being highly pH-dependent, with optimal activity reported between pH 4 and 6 (Emkani et al., 2022; De Angelis et al., 2003). Suboptimal pH conditions may therefore have impaired phytase efficiency and limited PA hydrolysis. Hence, a modification for future studies could be the addition of sucrose or another fermentable sugar prior to fermentation, to stimulate acid production, lower the pH, and potentially enhance phytase activity (Akpoghelie et al., 2025). Alternatively, the starter cultures may lack sufficient phytase activity, as previously suggested (Kaleda et al., 2020). Nevertheless, to validate these hypotheses, further analyses using more targeted methods or optimized protocols are needed.

Protein content and *in vitro* protein digestibility

The protein content of unfermented TPP was 74.7% FDW, slightly higher than the manufacturer's estimate of 70% DW. This discrepancy may be due to the higher accuracy of direct analytical methods compared to calculated estimates, as well as potential batch-to-batch variation. The standard nitrogen-to-protein conversion factor of 6.25 may also have slightly overestimated protein content (Hayes, 2020).

The protein content did not change significantly after fermentation compared to the unfermented control (Figure 6). Similar results have been reported in previous studies, where the lack of significant changes in protein content have been attributed to fermentation primarily affecting the molecular size of proteins, breaking them down into polypeptides, peptides and free amino acids, rather than altering the total protein content (Chandra-Hioe et al., 2016; Thompson et al., 2020). Notably, Li et al. (2021) showed that lactic acid fermentation altered the amino acid composition of yellow pea flour, particularly increasing levels of certain essential amino acids, thereby enhancing protein quality without significantly changing total

protein content. Although amino acid profiles were not assessed in this study, this highlights a valuable direction for future research to examine how fermentation affects TPP protein quality.

Prior to fermentation, the IVPD was 34%, closely comparable to the 37% reported by Reynaud et al. (2020) for commercial pea protein isolate using a slightly modified INFOGEST protocol. However, most studies report significantly higher IVPD values for both textured and non-textured legume proteins. For instance, Jiménez-Muñoz et al. (2023) found IVPD values exceeding 70% for pea protein isolates and concentrates using a similar protocol, while Chen et al. (2021) reported 85.5% for unfermented HME TPP, and Ojokoh and Wei (2011) observed approximately 83% for unfermented LME soy-based TVP. These findings reveal both similarities and notable discrepancies in IVPD across studies, underscoring the complexity of legume protein matrices.

Fermentation did not affect IVPD in this study, contrasting prior findings where lactic acid fermentation enhanced *in vitro* digestibility due to proteolytic activity and a reduction in ANFs, such as TIs (Çabuk et al., 2018a; Emkani et al., 2022). Ojokoh and Wei (2011) reported a 6% increase in IVPD after 24 hours of fermentation of LME soy protein, while De Pasquale et al. (2020) observed a 13% increase after fermenting raw pea flour at 30 °C for 24 hours using a 1:1 ratio of the LAB strains *Lactobacillus plantarum* MRS1 and *Lactobacillus brevis* MRS4. The limited effect observed in this study may reflect both low proteolytic activity of the starter cultures and methodological limitations, such as overestimation of undigested residue during centrifugation (Aguirre et al., 2008; Orlien et al., 2023; Jiménez-Muñoz et al, 2023). Furthermore, some loss of protein, both soluble and insoluble, can be assumed to have occurred during analysis, as indicated by total protein recovery rates after IVPD ranging from 90.0% to 91.6%.

During the extrusion of TPP, high temperatures may have altered the food matrix and induced protein aggregation by denaturing proteins and exposing hydrophobic groups, which promotes disulfide bond formation (Drulyte & Orlien, 2019). This aggregation can reduce the accessibility of both proteolytic enzymes from fermentation and the added pepsin and pancreatin used in the IVPD assay, limiting protein hydrolysis. Similarly, heat-induced structural changes of proteins may have facilitated aggregation with smaller soluble peptides during digestion (Yan et al., 2024; Jiménez-Muñoz et al., 2023). These aggregates likely contributed to the undigested fraction during centrifugation, potentially leading to overestimation. Additionally, partially digested proteins forming larger peptides may have precipitated, further contributing to this effect. To deepen the assessment of protein digestibility, techniques such as SDS-PAGE could be employed to evaluate the molecular weight distribution of peptides and proteins, offering greater insight into the digestion progression (Orlien et al., 2023).

Another factor potentially affecting the extent of IVPD in the present study is the interaction between proteins and PA at different pH levels during digestion. During the gastric phase, when the pH was adjusted to 3, PA may have interacted directly with proteins via electrostatic forces to form insoluble complexes, limiting the accessibility of digestive enzymes to hydrolyse the

proteins. During the intestinal phase, indirect interactions may also have occurred through the formation of ternary complexes via cation bridging at higher pH levels. Additionally, more complex indirect interactions involving PA, digestive enzymes, and proteins may have further inhibited enzymatic activity and overall digestibility *in vitro* (Sarwar Gilani et al., 2012).

Although PA levels were significantly reduced by both *L. sakei* and *L. carnosum* in this study, this decrease was not reflected in the IVPD results, where no significant differences were observed (Figure 5; Figure 7). Therefore, the influence of protein–PA interactions remain unclear. Moreover, a reduction in PA does not necessarily improve digestibility, as the food matrix and other ANFs also influence these interactions (Amat et al., 2023). Phenolic compounds may have contributed to poorly soluble protein–phenolic complexes, particularly under the alkaline conditions of the intestinal phase (Günal-Köroğlu et al., 2023). Nonetheless, this hypothesis requires further investigation, as phenolics were not analysed. The same applies to the fermentation's potential to reduce trypsin- and chymotrypsin inhibitors, which could also influence IVPD outcomes (Singh et al., 2023).

Methodological challenges were encountered during the IVPD assay, particularly in maintaining stable pH levels throughout the gastric and intestinal phases. Despite accounting for the slower pH response to HCl and NaOH additions in solid foods (≥5 minutes), pH stabilization remained difficult, likely due to the particle size and buffering capacity of the TPP (Brodkorb et al., 2019). Therefore, improved and more precise pH control is recommended for future studies to ensure static and consistent *in vitro* digestion conditions. Additionally, including a verification step for proteolytic enzyme activity is recommended to enhance reliability. Due to time constraints, enzyme and bile activities were not experimentally verified in this study, with activities assumed based on manufacturer data. Experimental confirmation of enzyme activity is thus recommended to ensure desired enzyme activity also after storage. Furthermore, to better mimic real meal conditions, IVPD should also be assessed in cooked PBMA formulated with TPP in future work. However, it is important to highlight that, although static *in vitro* models like the INFOGEST protocol provide valuable initial insights into food digestibility, the complexity of the human gastrointestinal tract and individual variability in digestive capacity limit its accuracy in quantifying absolute protein digestibility in the human body (Brodkorb et al., 2019).

Sensory analysis

Fermentation with *L. lactis* and *L. carnosum* significantly increased yeast-like taste in the formulated PBB. In addition, *L. lactis* also contributed to a notable enhancement of the yeast-like smell. This aligns with findings by Valtonen et al. (2023), who observed similar effects in fermented pea-based sausages. The intensification of yeast-like attributes may be explained by the production of volatile precursors during fermentation. During carbohydrate metabolism, LAB can produce volatiles such as 3-methyl-2-buten-1-ol, associated with yeasty odours (Ferrocino et al., 2018). In addition, volatiles may also derive from fermentation-driven lipid and protein hydrolysis, producing free fatty acids and amino acids (Flores & Piornos, 2021).

However, yeast contamination during sample collection throughout fermentation, or the presence of yeast on the TPP prior to fermentation, may also have contributed to the enhanced yeast flavour and aroma observed. To reduce the risk of contamination in future studies, a separate container could be used for the sensory samples instead of relying on the remaining TPP after sample collection. This approach would help minimize the likelihood of contamination during sampling.

Yeast-like flavours are often associated with meat-like characteristics due to the presence of naturally occurring compounds such as glutamic acid, which is also found in meat and is strongly linked to the umami taste. Consequently, yeast extract is commonly used as a flavouring agent in PBMA to enhance their sensory resemblance to meat (Dinali et al., 2024; Raza et al., 2020). In the present study, an indication of increased umami intensity, particularly following fermentation with *L. lactis* and *L. carnosum*, was observed, highlighting the described association between yeast-like and umami flavours. Previous studies have shown that LAB can release umami-related compounds, such as glutamic and aspartic acid, through proteolysis and glutaminase activity (Valtonen et al., 2023). This mechanism may have occurred in this study as well, contributing to the observed tendency toward enhanced umami intensity. Although the increase in umami was not statistically significant, the pronounced yeast-like flavours and umami enhancement suggest fermentation may improve meat-like flavour profiles in PBMAs. This has potential for improving consumer acceptance, especially among those resistant to plant-based alternatives due to sensory differences from conventional meat (Giacalone et al., 2022).

The results indicated a slight tendency toward increased sweet taste intensity following fermentation, a trend that has also been observed in previous studies (Valtonen et al., 2023). This may result from incomplete sugar consumption during fermentation, or sweet-tasting peptides released during proteolysis (Yamamoto et al., 2014). Sweetness has been linked to a reduction in the perception of other, less desirable flavours such as bitterness, which can be particularly beneficial in legume-based products where bitter notes are often considered unpleasant (Valtonen et al., 2023). However, since the observed increase in sweetness was not statistically significant, definitive conclusions regarding the effect of fermentation on sweet flavour perception cannot be drawn.

No significant changes were observed in pea-like taste, pea-like smell, or hay-like smell after fermentation (Figure 8). This contrasts with previous studies, where fermentation has been shown to reduce aldehydes like hexanal, strongly associated with 'hay-like' and 'beany' notes in peas, via aldehyde and alcohol dehydrogenase activity (El Youssef et al., 2020; Fischer et al., 2022; Kaleda et al., 2023). One possible explanation is that the starter cultures used were not effective in degrading these volatile compounds (Fischer et al., 2022). For instance, El Youssef et al. (2020) found that the inclusion of yeast strains alongside LAB significantly reduced 'leguminous plant' and 'green/vegetal' flavour intensities compared to LAB alone. Therefore, future studies could explore whether co-fermentation with yeast strains may more effectively influence these sensory attributes of TPP in plant-based formulations. Another

possible explanation is that structural changes during extrusion may have encapsulated 'beany' compounds within the TPP matrix reducing their accessibility to microbial degradation during fermentation (Yang et al., 2023). Additionally, the high protein content of the TPP (>70%) may have promoted the retention of beany volatiles through gel network formation, further limiting their availability for degradation. Alternatively, these attributes may have been challenging for the untrained panel to identify. Future studies could benefit from including a training session prior to sensory evaluation to calibrate panellists on the selected attributes.

No significant effects of fermentation on textural properties were observed, though a trend toward increased firmness was noted across all starter cultures compared to the unfermented control (Figure 8). This may be attributed to protein gelation induced by fermentation-driven proteolysis and pH reduction (Valtonen et al., 2023; Ren & Li, 2022). However, the inclusion of methylcellulose in the PBB formulation may also have influenced these results. As a synthetic hydrocolloid, methylcellulose is widely used in PBMAs to create firm, cohesive textures, which may have masked more subtle changes induced by fermentation (Peñaranda et al., 2025). Hence, to better isolate fermentation effects, future studies should assess TPP texture independently of the final formulation. Nonetheless, the observed trend indicates that fermentation could contribute to slightly firmer textures, potentially reducing the need for methylcellulose and supporting clean-label product development efforts (Peñaranda et al., 2025; Bakhsh et al., 2021).

Finally, while this study focused on descriptive sensory analysis, future research should include consumer liking and acceptance tests to assess the overall flavour and texture appeal of PBMAs formulated with fermented TPP. It would also be valuable to evaluate samples fermented for 24 hours to determine whether fermentation duration influences consumer preference.

6. CONCLUSION

This study demonstrated the potential of lactic acid fermentation as a pre-treatment for dry TPP to enhance the nutritional and sensory properties of PBMAs. Three commercial starter cultures, *L. sakei*, *L. carnosum*, and *L. lactis*, were successfully applied to TPP, each resulting in significant acidification by the end of fermentation. Fermentation reduced PA levels by up to 14.6%, particularly with *L. sakei* and *L. carnosum*, showing potential for improved mineral bioavailability in plant-based products. The protein content remained stable throughout fermentation; however, *in vitro* digestion analysis showed no improvement in protein digestibility. Sensory evaluation showed that fermented TPP could be successfully incorporated into a plant-based burger formulation, with a marked enhancement of yeast-like aroma and flavour, especially in TPP fermented with *L. lactis* and *L. carnosum*. Additionally, consistent trends across all fermented samples indicated potential enhancements in sweetness, firmness, and umami. However, the fermentation process did not affect the perceived pea-like and hay-like notes. Overall, this study represents an initial exploration of lactic acid fermentation to modify the nutritional profile and sensory attributes of TPP. The results highlight fermentation's potential as a strategy to enhance the quality and consumer appeal of PBMAs, while supporting clean-label product development. Nonetheless, further research is needed to thoroughly explore and optimise the use of fermented TPP to improve sensory properties, nutritional value, and overall consumer acceptance of PBMAs.

6.1 Future perspectives

Future research on the fermentation of TPP for use in PBMAs should focus on optimising fermentation conditions, particularly for industrial applications, and on refining and expanding analytical methodologies. It is recommended that future studies investigate how fermentation influences the protein quality of TPP through amino acid profiling, and assess additional ANFs, such as trypsin and chymotrypsin inhibitors, to gain a more comprehensive understanding of factors affecting TPP digestibility. Furthermore, examining the growth dynamics and metabolic activity of the starter cultures could provide deeper insights into the mechanisms behind fermentation-induced changes in TPP properties. Exploring combinations of different commercial starter cultures, as well as introducing additional species or strains, may also provide valuable insight into synergistic interactions that can enhance both nutritional and sensory properties. Finally, incorporating consumer liking and acceptance tests into future studies is advised to better evaluate the overall sensory appeal of PBMAs when formulated with fermented TPP.

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APPENDICES

A. Sensory attributes and descriptors

Table 5. Sensory attributes and corresponding descriptors used in the sensory analysis.

Attribute	**Descriptor**
Pea-like smell	The smell reminiscent of cooked neutral legumes, such as beans, yellow peas, lentils, and soybeans.
Yeast-like smell	The smell reminiscent of baker's yeast and freshly proofing bread.
Hay-like smell	The smell reminiscent of hay and dried grass
Firmness	How much resistance the burger gives when pressed or bitten into. 1 = Very soft, no noticeable resistance; 9 = Extremely firm/compact, distinct resistance
Chewiness	The number of chewings needed before swallowing. 1 = Mushy, soft, no chewing needed; 9 = Extremely tough, hard, requires extensive chewing.
Juiciness	The amount of moisture present in the burger and how much is released during chewing. 1 = Lack of moisture, dry; 9 = Extremely juicy/moist, wet
Pea-like taste	The taste reminiscent of cooked neutral legumes, such as beans, yellow peas, lentils, and soybeans.
Yeast-like taste	The taste reminiscent of baker's yeast and freshly proofing bread.
Sweet taste	The taste reminiscent of sugar, syrup, honey, or other sweet substances.
Umami taste	Full-bodied, rich, and deep flavour characterized as 'meaty' or 'brothy' taste.

B. Statistical data - sensory analysis

Table 6. Mean log-transformed values and standard deviations of sensory attributes across treatments. Asterisks indicate statistically significant differences, and different letters within that row denote significant differences between samples ($p<0.05$). **UF** (Unfermented), **FLs** (Fermented, *L. sakei)*, **FLc** (Fermented, *L. carnosum)*, **FLl** (Fermented, *L. lactis).*

Attribute ⬇ Sample ➡	**UF**	**FLs**	**FLc**	**FLl**	***P-value***
	Mean ± stdv	**Mean ± stdv**	**Mean ± stdv**	**Mean ± stdv**	
Pea-like smell	0.481 ± 0.194	0.470 ± 0.272	0.505 ± 0.255	0.495 ± 0.213	0.956
Hay-like smell	0.545 ± 0.277	0.644 ± 0.165	0.606 ± 0.257	0.552 ± 0.168	0.766
Yeast-like smell*	0.633 ± 0.260^{a}	0.745 ± 0.158^{ab}	0.788 ± 0.105^{b}	0.824 ± 0.098^{b}	0.012
Firmness	0.671 ± 0.126	0.704 ± 0.120	0.735 ± 0.170	0.719 ± 0.139	0.509
Chewiness	0.690 ± 0.132	0.693 ± 0.101	0.731 ± 0.152	0.692 ± 0.107	0.723
Juiciness	0.668 ± 0.159	0.651 ± 0.175	0.605 ± 0.165	0.667 ± 0.149	0.465
Pea-like taste	0.543 ± 0.206	0.569 ± 0.235	0.619 ± 0.204	0.597 ± 0.149	0.686
Yeast-like taste*	0.603 ± 0.212^{a}	0.592 ± 0.167^{a}	0.676 ± 0.173^{ab}	0.785 ± 0.135^{b}	0.013
Sweet taste	0.450 ± 0.219	0.516 ± 0.218	0.537 ± 0.218	0.548 ± 0.252	0.124
Umami taste	0.521 ± 0.233	0.578 ± 0.186	0.647 ± 0.176	0.656 ± 0.177	0.123