Impact of lactic fermentation on pea protein composition and their aromatic profile in a modified isoelectric precipitation extraction method

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Impact de la fermentation lactique sur la composition des protéines de pois et leur profil aromatique dans une méthode modifiée d'extraction par précipitation isoélectrique.

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Titre : Impact de la fermentation lactique sur la composition des protéines de pois et leur profil aromatique dans une méthode modifiée d'extraction par précipitation isoélectrique

Mots clés : Protéine de pois ; Extraction ; Fermentation ; Bactéries lactiques ; Protéolyse ; Composés volatils

Résumé : Malgré sa popularité, la consommation de protéines de pois reste limitée dans l'industrie agroalimentaire en raison de ses propriétés fonctionnelles imparfaites, de sa faible solubilité et de son profil sensoriel mal accepté. Cependant, ces propriétés peuvent être modifiées par différents procédés tels que l'extraction. Face à cette situation, c'est nécessaire de modifier et/ou d'hybrider les méthodes conventionnelles afin de développer des méthodes innovantes, rentables et écologiques pour la production de protéines végétales tout en respectant la pureté et la qualité des protéines. L'objectif de cette étude était d'explorer une alternative à la méthode d'extraction conventionnelle de solubilisation alcaline/précipitation isoélectrique dans laquelle la diminution du pH était obtenue par la fermentation lactique au lieu de l'ajout d'acide minéral. Pour étudier la faisabilité de la méthode, différentes souches commerciales (*Streptococcus thermophilus* (S), *Streptococcus thermophilus* et *Lactobacillus acidophilus* (SL), *Streptococcus thermophilus*, *Lactobacillus acidophilus* et *Bifidobacterium lactis* (SLB)) ont été ajoutées à des extraits de protéines obtenus à partir de farine de pois commerciale. L'acidification naturelle jusqu'à un pH de 4,8 a conduit à la précipitation des globulines qui ont été séparées de la fraction soluble (albumines). Les résultats ont montré une teneur plus élevée en protéines pour la fraction d'albumine obtenue par fermentation par rapport à la méthode Différentes bactéries lactiques (*Streptococcus thermophilus* (ST), *Lactipluntibacillus plantarum* (LP)) et leur co-culture ont été sélectionnées pour leur activité protéolytique et leur contribution aux propriétés organoleptiques des protéines de légumineuses. Les résultats soutiennent l'idée que la fermentation peut améliorer la qualité nutritionnelle de la fraction d'albumine (diminution des phytates, des inhibiteurs de trypsine, de la teneur en α-galactosides et l'amélioration de l'activité antoxydante) sans altérer le profil protéique de la fraction de globuline. Cependant, l'adaptation des propriétés de la fraction albumine dépend de la souche de bactéries lactiques. Par exemple, le niveau le plus élevé de protéolyse et de production de peptides a été observé pour les bactéries en co-culture par rapport à la mono-culture. La troisième partie de l'étude a porté sur l'effet de la fermentation lactique sur le profil aromatique des fractions de protéines de pois. L'analyse des composés volatiles et des composés odorants actifs a montré que les échantillons obtenus par fermentation (en fonction des souches) étaient associés à des arômes plus agréables tels que fruités, floraux et lactiques, par rapport aux échantillons obtenus par acidification conventionnelle. Les résultats montrent que la solubilisation alcaline/précipitation isoélectrique assistée par fermentation est une méthode prometteuse pour la production de protéines de légumineuses avec une valeur nutritionnelle accrue et un profil aromatique amélioré. À l'avenir, il serait

conventionnelle. La fraction albumine est généralement éliminée par l'industrie en raison de la présence de composés antinutritionnels. La deuxième question était donc d'étudier l'effet de l'extraction assistée par fermentation sur le profil protéique et les propriétés nutritionnelles de la fraction d'albumine. intéressant de déterminer les propriétés techno-fonctionnelles des protéines de pois obtenues par cette méthode, ainsi que le profil sensoriel des aliments contenant ces protéines comme ingrédients. L'application industrielle de cette méthode nécessite une optimisation plus poussée en termes de sélection des bactéries, de rentabilité et de mise à l'échelle.

Title : Impact of lactic fermentation on pea protein composition and their aromatic profile in a modified isoelectric precipitation extraction method

Keywords : Pea protein ; Extraction ; Fermentation ; Lactic acid bacteria ; Proteolysis ; volatile compounds

Abstract : Despite its popularity, pea protein consumption is still limited in industry due to its imperfect functional properties, low solubility and low accepted sensorial profile. However, these properties can be modified through different food processing methods such as extraction. Face with this situation, it has been highlighted the urge to modify and/or hybridize conventional methods in order to develop innovative, cost-effective, green methods for production of protein with respect to the purity and quality of the protein. The purpose of this study was to explore an alternative to the conventional alkaline solubilization/isoelectric precipitation method in which the decrease of pH was achieved by lactic acid fermentation instead of mineral acid addition. To study the feasibility of the method, different commercial starters (i.e. *S. thermophilus* (S), *S. thermophilus* and *Lactobacillus acidophilus* (SL), *S. thermophilus*, *Lactobacillus acidophilus* and *Bifidobacterium lactis* (SLB)) were added to protein extracts obtained from commercial pea flour and the natural acidification up to pH 4.8 led to the precipitation of globulins separated from the soluble fraction (albumins). The results showed Different lactic acid bacteria (*Streptococcus thermophilus* (ST), *Lactipluntibacillus plantarum* (LP)) and their co-culture were selected for their proteolytic activity and their contribution to organoleptic properties of legume protein. The results supported the idea that fermentation can improve the nutritional quality of albumin fraction (decrease of phytate, trypsin inhibitor, α-galactosides content and improve antoxidant activity) without impairing the protein profile of the globulin fraction. However, tailoring the properties of albumin fraction depended on the strain of lactic acid bacteria. For instance, the highest level of proteolysis and production of peptides was observed for the bacteria in co-culture compared to the mono-cultures. The third part of the study investigated the effect of lactic acid fermentation on aromatic profile of the pea protein fractions. The analysis of volatile compounds and odor active compounds evidenced that the samples obtained by fermentation (depending on the strains) were associated with more pleasant aroma such as fruity, floral and lactic, compared to the samples obtained by conventional acidification. The result represented that alkaline

a higher content of protein for albumin fraction obtained by fermentation compared to the conventional method. Albumin fraction is usually discarded in industry due to the presence of anti-nutritional compounds. Hence, the second question was to study the effect of the extraction assisted by fermentation on protein profile and nutritional properties of albumin fraction.

solubilization/isoelectric precipitation method assisted by fermentation is promising for production of legume protein with increased nutritional value, and improved aromatic profile. In the future, it would be interesting to determine the techno-functional properties of pea proteins obtained by this method, and the sensory profile of food including these proteins as ingredients. The industrial application of this method requires further optimization in terms of bacteria selection, cost-effectiveness and scale-up.

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Scientific publications

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Emkani, M.; Oliete, B.; Saurel, R. **How fermentation with lactic acid bacteria impact pea protein.** 21st IUFoST World Congress of Food Science & Technology (Singapore, 31 October-3 November 2022)

Emkani, M.; Oliete, B.; Saurel, R. **Pea protein extraction assisted by lactic fermentation: impact on protein profile.** ICEF14 International congress on engineering and food (Nantes, France, 20-23 June 2021)

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Abbreviations

GHG: Greenhouse gas

AEIEP: Alkaline extraction-isoelectric precipitation

ANF: Antinutritional factors

TIA: Trypsin inhibitor activity

TIU: Trypsin inhibitor unit

RFO: Raffinose family of oligosaccharides

HPL: Hydroperoxydes lyase

ADH: Alcohol dehydrogenase

ALDH: Aldehyde dehydrogenase

-OOH: –hydroperoxides

LOX: Lipoxygenase

ISO: Isomerase

β-OX: β-oxidation

Auto: Auto-oxidation.

FA: Fatty acid,

AA: Amino acids

SBSE: Stir bar sorptive extraction

SPME: Headspace solid-phase microextraction

SAFE: Solvent assisted flavor evaporation

GRAS: Generally recognized as safe

LAB: Lactic acid bacteria

S: *Streptococcus thermophilus* (commercial starter)

SL: *Streptococcus thermophilus* + *Lactobacillus acidophilus*

SLB: *Streptococcus thermophilus* + *Lactobacillus acidophilus* + *Bifidobacterium lactis*

ST: *Streptococcus thermophilus*

LP: *Lactipluntibacillus plantarum*

STLP: *Streptococcus thermophilus* + *Lactipluntibacillus plantarum*

LH: *Lactobacillus helveticus*

LR: *Lacticaseibacillus rhamnosus*

Lb: *Lactobacillus*

L.: *Lactococcus*

Lc.: *Leuconostoc*

P.: *Pediococcus*

S.: *Streptococcus*

W.: *Weissella*

EMP: Embden-Meyerhof-Parnas pathway

PP: Phosphogluconate pathway

CEP: Cell-envelope proteinase

DSC: Differential scanning calorimetry

SDS-PAGE: SDS polyacrylamide gel electrophoresis

HPLC-SEC: Seize exclusion chromatography

TEAC: Trolox equivalent antioxidant capacity

GC-MS: Gas chromatography–mass spectrometry

GC-O: Gas chromatography olfactometry:

MRS: De Man, Rogosa and Sharpe agar

CFU: Colony forming units

DCU: Danisco culture unit

AOAC: the association of official agricultural chemists

OPA: O-phthaldialdehyde

TNBS: Trinitrobenzene Sulfonic acid

FAS: Ferric ammonium sulphate

LRI: Liner retention index

OAC: Odor active compounds

ANOVA: One-way analysis of variance

PCA: Principal component analysis

AHC: Agglomerative hierarchical clustering

Mw: Molecular weight

PA: Phytic acid

CAR: Cartouche pea cultivars

AC : Ascension pea cultivars

AS : Assas pea cultivars

Introduction

Over the past decade, there has been challenges regarding the consumption of animal-based proteins. The concerns are growing with the increase of global population by more than one billion people within the next 15 years (reaching to 8.5 billion in 2030 according to the projection by United Nations) which leads to a drastic increase in food demands (Goldstein & Reifen, 2022). While, the consumption of animal-based protein is already associated with many environmental issue and human health problems. Indeed, livestock production is responsible for at least 14.5% of greenhouse gas (GHG) emissions (Lemken et al., 2019), air pollution, high water consumption, decrease of soil and water quality, damage to biodiversity, and degradation of grazing area (Leip et al., 2015). Furthermore, from the human health standpoint, the consumption of animal-based proteins increases the risk of coronary heart disease, type 2 diabetes and some type of cancers (Pihlanto et al., 2017). Therefore, it is urgent to find alternative sources of protein which are available, cost-effective and more promising for both human and environment health.

Legumes are the possible solution that contribute toward sustainable and healthier diet. In fact, legumes are able to fix nitrogen as they have biological interaction with nitrogen-fixing soil bacteria (rhizobia) which results in the formation of root nodules (Wang et al., 2018). The production of legumes, does not require additional nitrogen fertilizer for growth. Indeed, legumes provide other plants with excess nitrogen in crop rotation, hence, they can indirectly reduce GHG emissions (Foyer et al., 2016). Apart from this, legumes are easy to grow and highly dry-tolerant thanks to their deep-root system (Didinger & Thompson, 2020). Therefore, the production of legumes can prevent ecosystem damages, it needs lower water consumption and it can free-up cropland (Henn et al., 2022). These environmental benefits are getting more and more value in consumer's choice for consumption of legumes.

Legumes possess numerous positive health benefits for human including weight maintenance, gut health and reduced the risk of cardiovascular disease and cancers. They belong to the Fabaceae or Leguminosae families and represent the second most commonly produced food crop after cereals. Legumes comprise oil-seed legumes (i.e., soybean) and pulses such as lentils, chickpeas, peas, lupines, and beans (Semba et al., 2021). Among which pea is of great interest for its high nutritional value which can serve as a motivator for its consumption. Pea is rich in proteins and carbohydrates, low in fat and contain some of important vitamins and minerals. Pea consists of approximately 20–30% protein of its total dry seed. Pea protein contains high quality amino acids with high levels of lysine and lower level of methionine and

tryptophan (Lam et al., 2018). It has lower allergenicity and better digestibility compared to other pulses. It can be used either as grain components (e.g., flour milled from grains) or as enriched protein ingredients such as protein concentrate (50–55%) and protein isolate (80–90%) (Barać et al., 2015; Boye et al., 2010). The two principal protein of pea proteins are, albumins (10-20%) and globulins (70-80%). Albumin and globulin fractions can be purified, since they have different solubility. Globulins are salt soluble, while albumins are water soluble. Albumins possess better nutritional properties compared to globulins since they contain higher concentration of essential amino acids such as tryptophan, lysine, threonine, and methionine (Kumari et al., 2022).

Despite the promising properties of pea proteins, its application in food industry is still challenging for its low desirable techno-functional properties specially low solubility and emulsifying properties, imperfect sensorial properties, and the presence of some antinutritional factors (Emkani et al., 2021). All these deficiencies depend on protein structure and composition, which are influenced by different parameters such as cultivar, extraction process, and biological process such as fermentation. Genetic and environmental conditions of growth are the first step of controlling pea protein structure since they can affect the composition and content of protein, amino acids, volatile compounds and antinutritional compounds (Daba & Morris, 2022; Jakobsen et al., 1998; Nikolopoulou et al., 2007).

Different methods have been proposed for the extraction of protein from pea flour each of which can impact the yield and quality of proteins. On the other hand, there has been a great interest recently on green production of commercial valuable products which is partly for the environmental concerns and climate change, and partly for the consumers demands for the improvement of nutritional and health-promoting value of the products (Abdel-Rahman et al., 2013). These challenges highlight the need to modify and hybridize conventional methods in order to develop an innovative, easy to operate, green, environmental-friendly and cost-effective extraction method which at the same time increase the purity and quality of proteins (Eze et al., 2022; Wen et al., 2022).

The conventional wet extraction methods are alkaline extraction-isoelectric precipitation (AEIEP), salt extraction-dialysis, micellar precipitation, and aqueous extraction (pH > 7) (Lam et al., 2018; Owusu-Ansah & McCurdy, 1991). AEIEP is the most popular technique with high yield for producing pea protein isolates in the food industry. The extraction process uses pH manipulation to valorise globulin in form of isolate (Tanger et al., 2020). The method separates

albumins and globulins since globulin fraction has high solubility at alkaline pH, and minimal solubility at their pI, while albumin fractions remain soluble in almost all the pH values (Djemaoune et al., 2019). Currently, globulin fraction obtained by this method is exploited in the form of isolates by industry, while, to the best of our knowledge, albumin fraction is generally discarded for lower protein content compared to globulins and the presence of some antinutritional compounds. There are also some draw backs with the conventional methods of protein extraction, such as high demand for chemicals, the environmental food prints of the solvents, denaturation of protein in some cases, and most importantly the present technics have not been able to reduce the content of antinutritional compounds and off-flavors (Eze et al., 2022).

Fermentation, is one of the oldest food processing techniques, which has been used traditionally in some countries (Knez et al., 2023). There are different types of fermentation, among which lactic acid fermentation is getting more and more popularity and it is widely used in dairy and food industry since it is considered as safe and it contributes to the flavor, texture and nutritional value of the fermented foods. Indeed, the enzymatic activity of the bacteria during fermentation lead to the production of aroma components and/or reduction of off-flavors; production of exopolysaccharides; hydrolysis of proteins to the smaller peptides and amino acid with bioactive properties; modification of techno-functional properties; modification of protein digestibility by reduction of antinutritional compounds, and production of nutritional components such as vitamins; food preservation by production of bacteriocines; or promoting health benefits when used as probiotics (Fischer et al., 2022b; Harlé et al., 2020; Reuben et al., 2020; Xing et al., 2020). However, the efficiency and functionality of lactic acid fermentation depends greatly on the type of lactic acid bacteria (LAB) strain, the fermentation technique, the composition of the protein ingredient, and slightly on the genetic variety (Bartkiene et al., 2015; Rui et al., 2017).

Considering the positive effects of lactic acid fermentation on legume properties and the drop in pH due to lactic acid formation, the main objective of this thesis was to explore an alternative extraction method based on alkaline solubilization/isoelectric precipitation, in which the decrease in pH was achieved by lactic acid fermentation instead of mineral acid addition. The first attempt was performed to validate the method and the possibility to obtain pea protein isolates. The extraction method was performed on commercial pea flour by application of commercial starters (*Streptococcus thermophilus* (S), *Streptococcus thermophilus* +

Lactobacillus acidophilus (SL) and *Streptococcus thermophilus* + *Lactobacillus acidophilus* + *Bifidobacterium lactis* (SLB)). Further in this study, different LAB (*Streptococcus thermophilus* (ST), *Lactipluntibacillus plantarum* (LP)), were selected for their aptitude for acidification, proteolysis and/or their recognized positive effect on legume protein properties, and used in the study of protein characteristics, the antinutritional compounds, and the volatile profile of proteins.

This work starts with a bibliography study (Chapter I) on pea protein composition, antinutritional compounds, off-flavors and the impact of different parameter such as cultivars, extraction method and fermentation on protein profile and its properties. It is then followed by research strategy (Chapter II) and presentation of different materials and methods (Chapter III). Results and discussions were divided into three chapters: (i) pea protein extraction assisted by lactic fermentation: impact on protein profile and thermal properties (Chapter IV); (ii) impact of fermentation on the protein composition and nutritional aspects of pea protein fractions obtained by a modified isoelectric precipitation method (Chapter V); and (iii) identification of volatile compounds and odor-active compounds in pea protein fractions (Chapter VI). This study finishes with a general conclusion and discussion on perspectives of possible future research.

I. Literature review

I.1. Peas

Although pea seed (*Pisum sativum* L.) exists in the traditional diet of some countries, only recently its consumption as human food gained popularity. It is ranked as the second most important legume food after soybean. This is partly for its availability and massive worldwide production, and partly because of its high nutritional quality and protein content (Ratnayake et al., 2002). Depending on the cultivars, pea seeds contains 20–30% protein (of its total dry seed), 1.5-2.0% fat, and minor components such as vitamins, minerals, phytic acid, polyphenols, saponins and oxalates. Carbohydrate in pea, accounts for 60-65% and it consists of starch, dietary fiber and non-starch carbohydrate such as sucrose and oligosaccharides (Gueguen, 1983). Pea can be consumed either as a part of grain components (e.g., flour milled from grains) or as enriched protein ingredients such as protein concentrate (50–55%) and protein isolate (80–90%) (Barać et al., 2015; Boye et al., 2010).

I.2. Pea protein composition

The majority of pea proteins consists of albumins (15–20%) and globulins (60–70%) (Crévieu et al., 1996). These two fractions can be purified, since they have different solubility. Albumins are water soluble, while globulins are salt soluble. Fig I-1. shows the proteins of pea and their subdivisions.

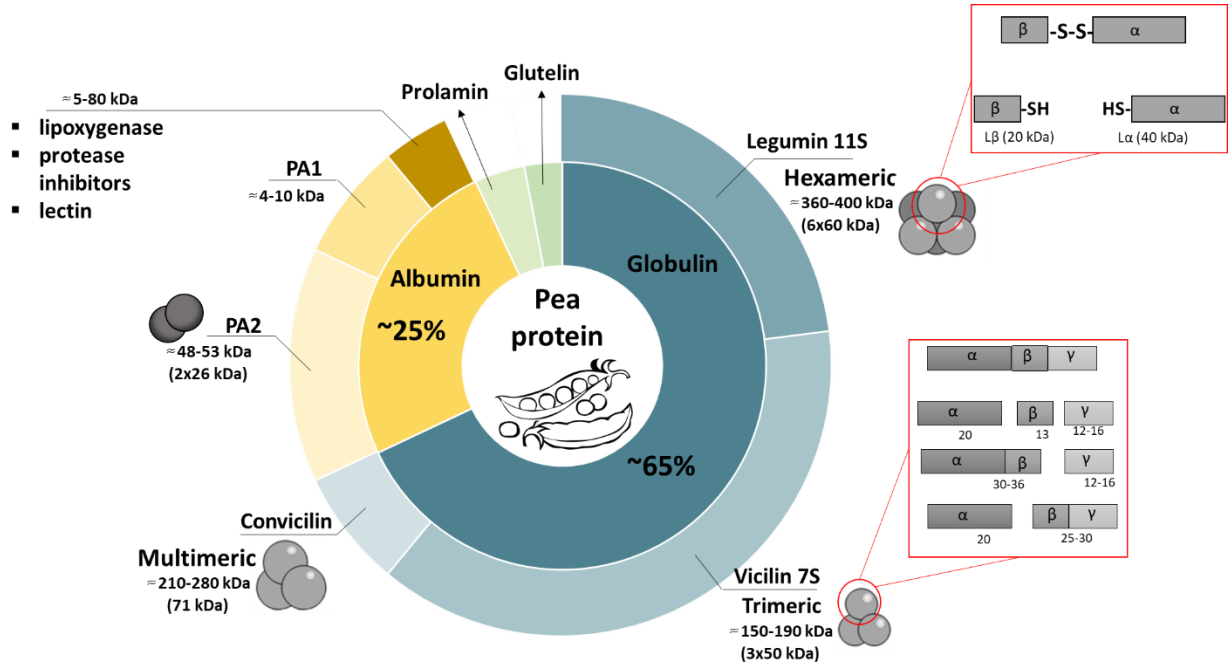


Figure I-1. Schematic composition of main pea protein fractions.

I.2.1. Albumins

The two major abundant albumin proteins in pea are named PA1 and PA2 belonging to the 2S group according to their sedimentation coefficient (Gruen et al., 1987). These two proteins differ in molecular weight (Mw) and hydrophobicity. PA1 comprises about 7% of the total pea seed protein and contributes about 50% of the seed's sulphur amino acids. In the mature grain, PA1 consists of two components, PA1a (~6 kDa) and PA1b (~4 kDa) (Lu et al., 2000). The PA1 polypeptide has a isoelectric point of ~7.2 (Bérot et al., 2007). PA2, contributes approximately 16% of the seed's sulphur amino acids. It has a Mw of approximately 26 kDa and it consists of two subunits of PA2a and PA2b which are combined by non-covalent bond form homodimers of Mw 53 kDa and 48 kDa (Lu et al., 2000). The isoelectric point of PA2 is between 5 to 5.6 (Crévieu et al., 1996). The polypeptide chain in PA2 is more flexible and is able to change its conformation easily. This is for the presence of only three cysteine residues out of 230 residues which yields one intrachain disulphide bridge and one free sulphhydryl group. While PA1 albumins are rich in cysteine residues and their conformation is covalently blocked by more than one disulphide bonds and their structure is more stable (Lu et al., 2000). That is why PA2 represents better techno-functional properties compared to PA1.

I.2.2. Globulins

The three abundant pea globulins are legumin, vicilin and convicilin with an isoelectric point (pI) close to pH 4.8. Legumins belong to the 11S group and have a hexameric structure with a high Mw of 360–400 kDa. A mature legumin consists of six subunit pairs (~60 kDa) that interact non-covalently. Each of these subunit pairs consists, in turn, of an acidic subunit of ~40 kDa (Lα) and a basic subunit of ~20 kDa (Lβ), linked by a single disulfide bridge (Fig I-1). Legumin has higher content of sulphur amino acids (approximately seven cysteine residues and four methionine per monomer) compared to the two other globulin proteins (Croy et al., 1980; Tzitzikas et al., 2006). Vicilin is a trimer of 150–200 kDa and it belongs to the 7S group. It lacks cysteine, so it cannot form a disulfide bond. Each monomer (~50 kDa) has two cleavage sites, which make possible the post-translational formation of various polypeptide fragments of about 20 kDa (α), 13 kDa (β) and 12–16 kDa (γ), 30–36 kDa (α + β) and 25–30 kDa (β + γ) (Gatehouse et al., 1982). Finally, convicilin with its trimeric structure belongs to the 7S group. It consists of three subunits (~70 kDa) which are thought to associate non-covalently in a multimer of 210–280 kDa (Barac et al., 2010). It represents only 6% of total seed proteins, containing higher levels of sulfur aminoacids than vicilins (Tzitzikas et al., 2006).

I.2.3. Pea amino acid composition

The amino acid composition of pea globulin fractions (legumin, vicilin) and albumin is shown in Table I-1. Pea protein is characterized by high content of essential amino acids, especially arginine, isoleucine, lysine, leucine, phenylalanine and valine. On the contrary to globulin proteins, pea albumin has high content of sulfur containing amino acids (methionine and cysteine) and tryptophane (Saurel, 2020).

Table I-1. Amino acid composition of pea protein fractions (g/16 g N) (modified from Saurel (2020))

Amino acids	Albumin	Legumin	Vicilin
Essential and semi-essential			
Arginine	5.7	1.25±0.2	6.9±0.8
Histidine	2.6	2.6±0.2	2.4±0.5
Isoleucine	3.9	4.8±2.2	5.1±0.1
Leucine	4.9	7.8±0.3	9.1±0.1
Lysine	9.3	4.8±0.3	7.3±1.1
Methionine	1.3	0.6±0.1	0.5±0.2
Phenylalanine	4.5	4.1±0.7	6.1±0.1
Threonine	5.7	3.4±0.1	3.4±0.6
Tryptophan	1.5	0.8±0.4	0.1±0.0
Valine	4.4	4.7±0.3	5.1±0.8
Non-essential			
Alanine	5.9	5.5±1.2	3.7±1.2
Aspartic acid (+asparagine)	11.9	12.1±0.6	12.0±0.0
Cysteine	3.2	0.7±0.1	0.1±0.1
Glutamic acid (+glutamine)	15	19.6±2.1	18.5±1.3
Glycine	6	6.2±2.0	3.7±1.1
Proline	4.5	5.0±0.6	4.0±0.9
Serine	5	5.9±1	6.2±0.7
Tyrosine	4.7	2.5±0.7	2.7±0.6

I.3. Antinutritional compounds in pea

Despite the popularity of pea protein, its application is still limited due to the presence of antinutritional compounds. In general, pea seeds contain certain non-nutritive compounds some

of which could interfere with nutrient availabilities. These compounds are known as antinutritional factors (ANF) (Nikolopoulou et al., 2007). Examples of these ANFs are lectin; α-galactosides; phenolic compounds including tannins; trypsin, and chymotrypsin inhibitors; phytic acid; saponins; isoflavones; and biogenic amines. Some of these compounds are discussed here.

I.3.1. Trypsin inhibitors

Trypsin inhibitors belong to albumin fractions. They are low molecular weight proteins capable to bind to digestive enzyme trypsin and inactivate it. The presence of these proteins in human and animal diets could lead to pancreatic enlargement, reduced digestibility of protein, reduced absorption of amino acids and reduced availability of minerals (Bacon et al., 1995a). Trypsin inhibitor activity (TIA) is expressed by trypsin inhibitor unit (TIU) per dry matter. In general, peas have 5-20 times lower TIA compared to soybean (Avilés-Gaxiola et al., 2018). Still this value could be significant depending on the cultivars (Vidal-Valverde et al., 2003). Gueguen and Cerletti (1994) reported the value of trypsin inhibitor activity in pea flour, was around 4.4 to 9.3 TIU/mg dry matter.

I.3.2. α-galactosides

The α-galactosides of sucrose, also known as the raffinose family of oligosaccharides (RFOs) are the most abundant soluble carbohydrates in legumes (Jones et al., 1999). α-galactosides are soluble low-molecular weight oligosaccharides such as raffinose (trisaccharide), stachyose (tetrasaccharide), and verbascose (pentasaccharide) (Fig I-2). Verbascose has three molecules of α-D-galactose attached to sucrose; stachyose has two, and raffinose one. All these carbohydrates are nonreducing. While the release of fructose by invertase can induce the reducing saccharides. Fig I-2 showed that the treatment of raffinose with invertase (β-fructosidase) gives fructose and melibiose, whereas α-galactosidase gives sucrose and galactose (Tester & Karkalas, 2003).

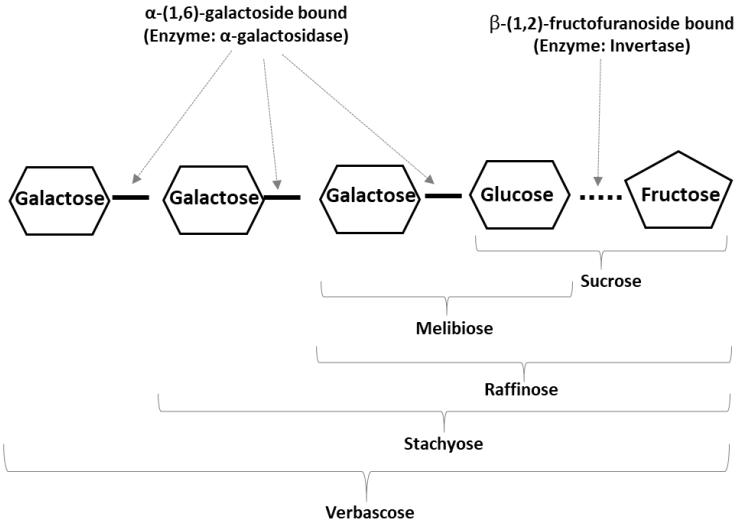


Figure I-2. Structure of raffinose family of oligosaccharides (RFOs) (modified from Martins et al. (2019)).

They are responsible for digestive discomfort and flatulence due to their fermentation by gut bacteria in the large intestine. However, moderate doses of α-galactosides favour the metabolism of beneficial intestinal microorganisms such as *Bifidobacteria* (Teixeira et al., 2012).

I.3.3. Phytates

Phytic acid (myo-inositol hexaphosphoric acid (IP6)) consists of a cyclic ring ($C_6H_6O_6$) where each oxygen is connected to a phosphate group $P(OH)_3$ (Amat et al., 2022). Figure I-3 shows the structure of phytic acid and its fully ionized form which is known as phytate.

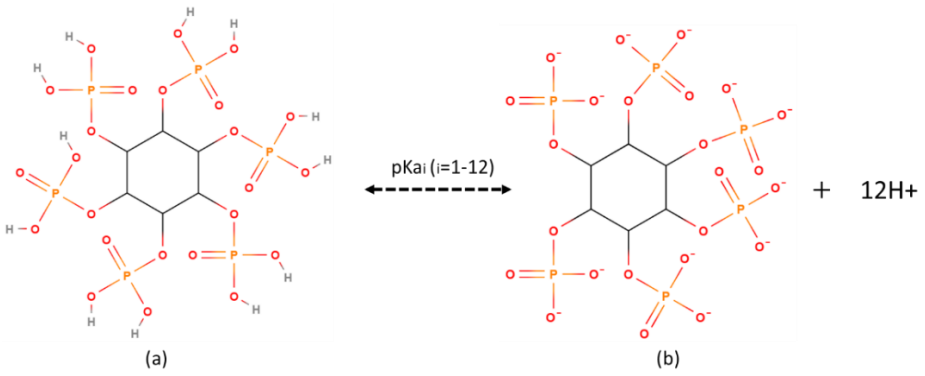


Figure I-3. Phytic acid structure (a), and formation of phytate anion (b) (modified from Amat et al. (2022)).

The particular structure of phytic acid allows this molecule to bind with different molecules (e.g. proteins) through different type of interactions (bridge formation, complex formation and electrostatics). Phytic acid can interact with protein through ionic interaction and it decrease the solubility of protein (Urbano et al., 2000). The interaction of phytic acid and protein is pH dependent. At pH below the isoelectric point of protein, phytate anion interacts strongly to protein. The complex of phytate-protein is resistant to proteolysis so it interferes with protein digestion. Above the isoelectric point of protein, both phytate and protein are negatively charged. However, in presence of cations, they can make a soluble protein-cation-phytate complex. This complex can be disrupted by high ionic strength, high pH and high concentration of phytate (Nissar et al., 2017). Phytate can also bind with enzymes such as proteases and amylases and decrease the protein digestibility (Samtiya et al., 2020; Tiwari & Singh, 2012). Additionally, phytic acid forms complexes with certain minerals, such as calcium, copper, magnesium, iron, manganese, zinc, and amino group derivatives in protein moieties, and thus decreases their absorption in the gastrointestinal tract. For people with high daily pulse consumption, this can result in anemia due to iron deficiency (Tiwari & Singh, 2012). In legume seeds, phytic acid is mainly stored in protein bodies under the form of phytin. In peas, it accounts for 0.2 to 1.2% of seeds dry matter (Gupta et al., 2015). The amount of phytic acid depends on many factors such as cultivars, growing condition, harvest, processing such as heat treatment, soaking, germination and fermentation (Urbano et al., 2000).

I.4. Off-flavors in peas

The words flavor, aroma, odor and taste are often used interchangeably which may lead to a confusion. Taste is caused by non-volatile compounds and is perceived by the receptors in the oral cavity (Chandrashekar et al., 2006). Odor refers to volatile compounds that can be perceived by the sense of smell (orthonasal olfaction), whereas aroma is used to describe chemical compounds that are detected via the back of the throat when tasting a food (retro-nasal olfaction) (y Lillo et al., 2005). Flavor is mainly composed of taste and aroma. There are also other effects in mouth influencing flavor perception such as astringent. The terms off-flavor refers to the perception of an unpleasant taste, aroma and other effect such as astringency.

Pea protein is facing a major challenge regarding the off-flavors. The off-flavors in pea protein are mainly described as beany, earthy and grassy (Klein & Raidl, 1986). The majority of the off-flavors can be originated from the pea seed itself or they can be developed during different

steps of pea preparation such as harvesting, storage, physical treatment and chemical process. The off-flavors that are inherent to pea can be removed or masked but they cannot be prevented other than breeding new cultivars (Roland et al., 2017). Whereas, the development of off-flavors during processing can be controlled by physical, chemical or biological process. For instance, avoiding or controlling the conditions of storage such as temperature and light. Physical treatments such as removing the shells which contains a high number of off-flavors; also grinding or heat treatment which can activate or inactivate the enzymes involved in the development of off-flavors; by chemical treatment such as delipidation and controlling the condition of extraction; or by biological process such as fermentation which can reduce or mask off-flavors to a great stand (Fischer et al., 2022b). Controlling the off-flavors, requires a good knowledge on the origin and formation pathways of the volatile compounds. Indeed, the three main pathways for the production of volatile compounds are lipid oxidation, amino acid degradation and carotenoid degradation.

I.4.1. Origin of off-flavors in peas and pea protein ingredients

I.4.1.1. Lipid oxidation

Despite a small quantity of lipids in pea (approximately 2% of its total dry seed) (Coxon & Wright, 1985; Hoover et al., 1988), lipid oxidation is generally the main pathway for production of off-flavors in pea protein. The volatile compounds produced from this mechanism are mainly responsible for green and beany off-notes (Wang et al., 2021). Lipid oxidation can be enzymatic by lipoxygenase pathway or nonenzymatic in the presence of oxygen (autoxidation) (Fig I-4). The oxidation pathway starts with decomposition of the triglyceride to fatty acids by lipase activity. The unsaturated fatty acid (RH) then lose hydrogen in presence of initiator (e.g. lipoxygenase, metal ion, light, heat) and it forms alkyl radicals (R•). This radical then reacts with oxygen and form peroxyl radicals (ROO•). The peroxyl radicals then react with another unsaturated fatty acid to form hydroperoxide (ROOH). Hydroperoxides are the primary products of lipid oxidation (Frankel, 1983). Thereafter, the hydroperoxydes decomposed by hydroperoxydes lyase (HPL) to volatile aldehyde or ketone. The aldehyde and ketones can further transfer to alcohol by the activity of alcohol dehydrogenase (ADH) or to acid by aldehyde dehydrogenase (ALDH) (Fischer et al., 2022b).

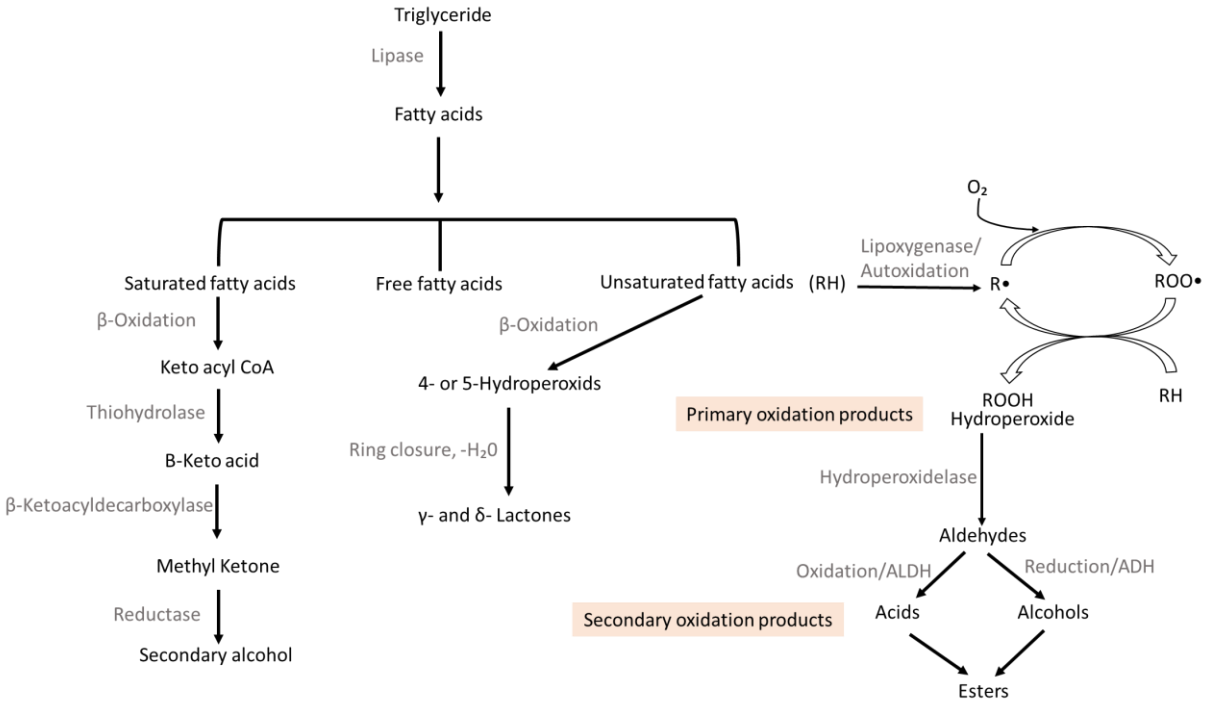


Figure I-4. General pathway for degradation of lipid (modified from Cheng (2010), McSweeney and Sousa (2000), Frankel (1983))

The production of different volatile compounds depends on the unsaturated fatty acid and the oxidation. There are different types of unsaturated fatty acids. Figure I-5 shows the production of some volatile compounds in pea protein from different unsaturated fatty acids. Linoleic acid is the major unsaturated fatty acid in legume protein and it has a principal role in the formation of volatile compounds especially hexanal (Frankel, 1983). It can be converted to 9-, 10- or 13-hydroperoxides (OOH) by lipoxygenase (LOX). Deca-2,4-dienal is formed by autoxidation. Generally, at high temperature the formation of "dienals" become more significant (Frankel, 1983). 10-OOH is transferred to oct-1-en-3-one by HPL and then further to oct-1-en-3-ol by ADH. 13-OOH transferred to hexanal by HPL and then to hexanol by ADH. Linolenic acid can be decomposed to 9- and 13-OOH. The former, lead to the production of (*Z*)-nonen-3-al by HPL and then to its isomer (*E*)-nonen-2-al which subsequently can form (*E*)-nonen-2-ol by ADH. On the other hand, 13-OOH can be cleaved to (*Z*)-hexen-3-al and its isomer (*E*)-hexen-2-al by isomerase, hexanal, pent-1-en-3-one. These compounds can further convert to their alcohols by ADH. 13-OOH can also undergo the β-oxidation and form jasmonate. Oleic acid can be converted to 8-, 9-, 10- and 11-OOH. The oxidation of these fatty acid lead to the production of shorter chain aldehydes such as octanal which can be further converted to octanoic acid by ALDH or to octanol by ADH.

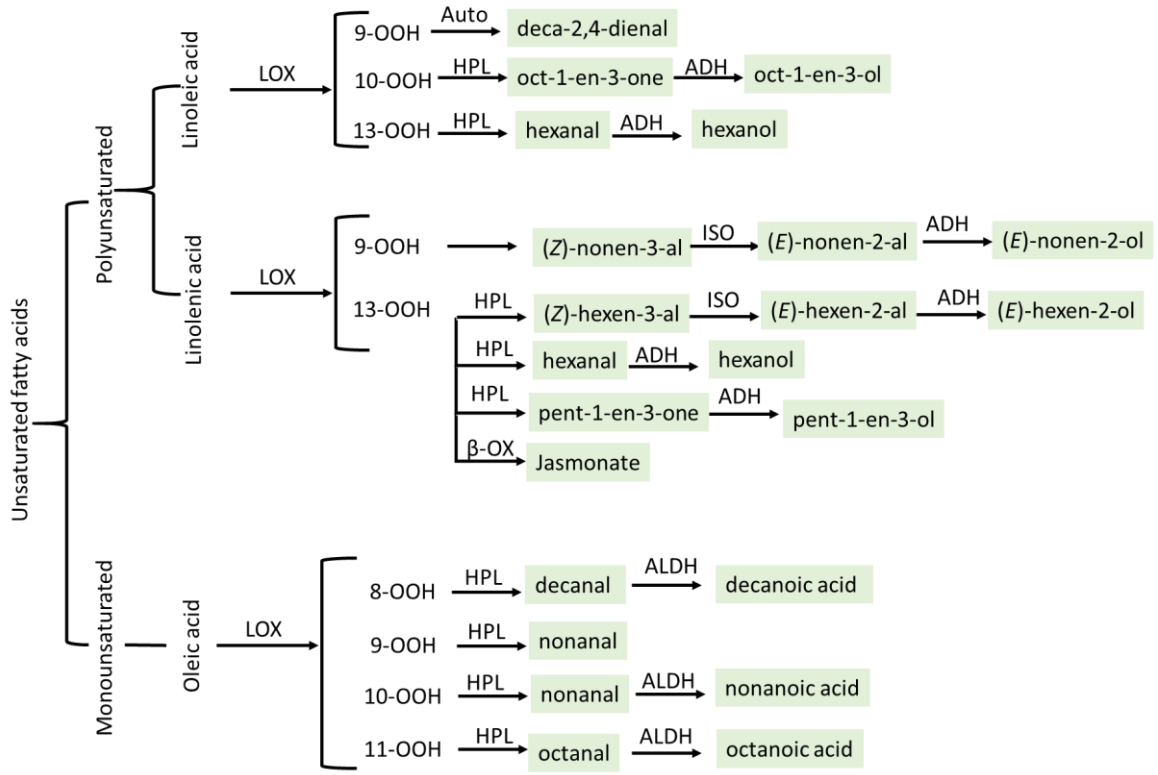


Figure I-5. Formation of volatile compounds originated from different unsaturated fatty acids (linoleic acid, linolenic acid, oleic acid) (inspired and modified from Dudareva et al. (2013), Frankel (1983), Fischer et al. (2022b)). Arrows illustrated the involvement of multiple enzymatic reactions. Volatile compounds are highlighted with a green background. LOX, lipoxygenase; HPL, hydroperoxidelyase; ISO, isomerase; ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; β-OX, β-oxidation; Auto, auto-oxidation.

I.4.1.2. Amino acid degradation

Degradation of amino acids is another important pathway producing volatile compounds in pea protein. The degradation of amino acids could be induced biologically or by Maillard reactions which lead to the production of branched aldehydes, sulphides, pyrazines, alcohols and acids.

I.4.1.2.1. Biological reaction

In biological pathway, the proteins degrade to oligopeptides by enzymatic activity of proteases (Fig I-6). The next step is the transportation of di- tri- and oligopeptides into the cell. These peptides further hydrolysed to amino acids by peptidase (Chen et al., 2017). The produced free amino acids convert to their corresponding α-keto acids through a transamination reaction. The α-keto acids can further go through the different enzymatic reactions, either a reduction

generating flavourless α-hydroxy acids, or a decarboxylation forming aldehydes (Mayo et al., 2010). Thereafter, aldehydes could be reduced to alcohols by ADH or oxidize to carboxylic acid by ALDH (Fischer et al., 2022b). Ester and thio-ester can be formed also in reaction between alcohols and carboxylic acids by esterase activity (Smit et al., 2005). Another conversion pathway of amino acids is the elimination reaction, by which the side chain of amino acids could be cleaved through the enzymatic activity of lyase. This reaction is generally associated with off-flavors in pea protein and production of compounds such as indole and sulphides (Ianni et al., 2020).

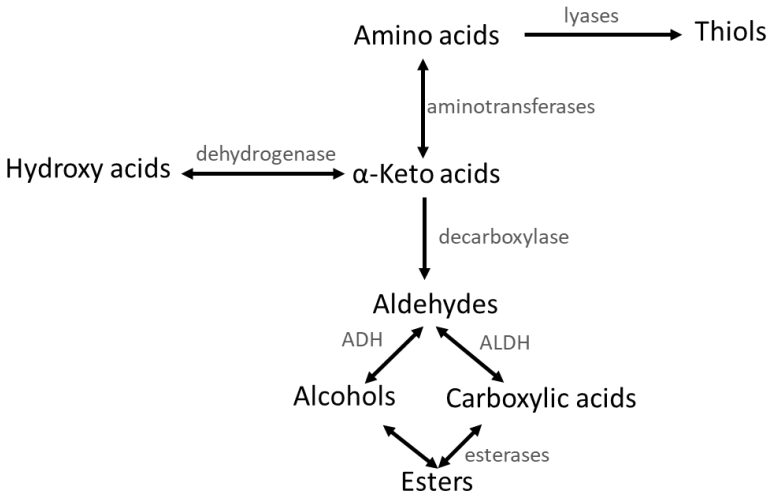


Figure I-6. Overview representing the biological degradation of amino acid in production of volatile compounds (modified from Smit et al. (2005))

Different group of free amino acids including branched-chain amino acids (e.g. valine, leucine, isoleucine), aromatic amino acids (e.g. phenylalanine, tryptophan, tyrosine) and sulphur amino acids (e.g. cysteine, methionine), could be metabolized (Chen et al., 2017). Methyl aldehydes generally originate from branched amino acids. For instance, the production of 3-methylbutanal, 2-methylbutanal and 2-methylpropanal from leucine, isoleucine and valine, respectively, through the decarboxylation (Fischer et al., 2022b). Methionine also can be convert to methanethiol through the elimination reaction and can further convert to dimethyl sulphide, dimethyl disulphide and dimethyl trisulphide (Ianni et al., 2020).

I.4.1.2.2. Maillard reaction

Maillard reaction is the nonenzymatic pathway for production of amino acid origin volatile compounds. The type of volatile produced, depends on the type of sugar and amino acid, and

the condition sof reaction such as temperature and pH. The first step in Maillard reaction is the reaction between a reducing sugar and the amino group of an amino acid which leads to the production of Amadori (if the reducing sugar is aldose) and Heyns (if the reducing sugar is ketose) products (FigI-7). The next step is the rearrangement of the sugars and release of an amino group. Finally, the rearranged sugar could go through different reaction and produce different volatile compounds (Van Boekel, 2006). For instance, amino acid could participate again in the reaction and it could be degraded by carbonyls which lead to the decarboxylation and deamination of the amino acid. This is called the Strecker degradation which is one of the utmost reactions in flavour formation. The most important compounds originated from Maillard/Strecker reaction in pea protein are pyrazines and Strecker's aldehydes such as benzaldehyde which is originated from phenylalanine. 3-isopropyl-2-methoxypyrazines, 3-s-butyl-2methoxypyrazines, and 3-isobutyl-2-methoxypyrazines are of the common pyrazine volatile compounds in pea protein which originated from leucine, isoleucine, and valine, respectively (Murray et al., 1976). They are also involved in the production of 3-isopropyl-2-methoxypyrazines, 3-s-butyl-2methoxypyrazines, and 3-isobutyl-2-methoxypyrazines, respectively.

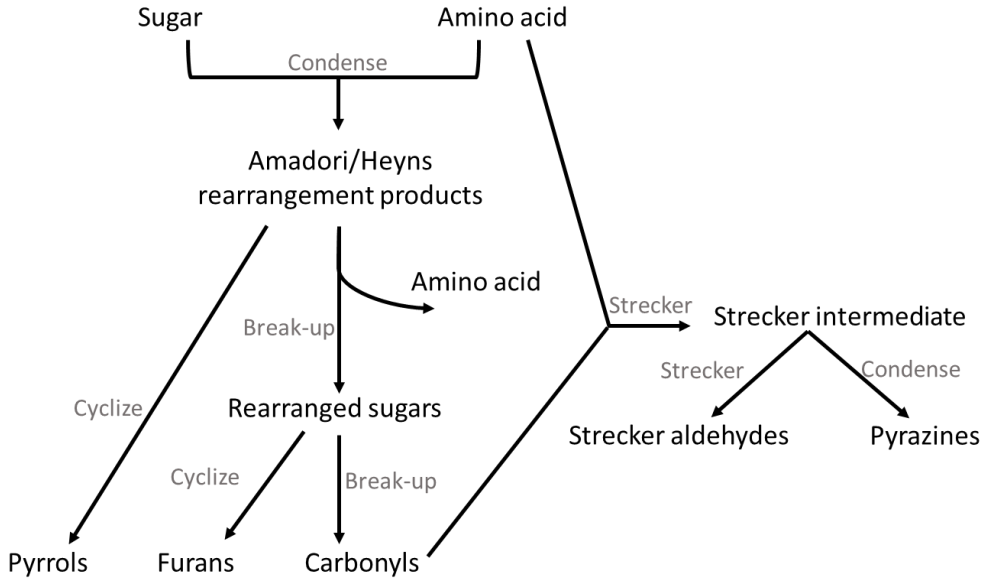


Figure I-7. Schematic of flavor formation by Maillard reaction (modified from Jousse et al. (2002))

I.4.1.4. Carotenoids degradation

Carotenoids can be oxidized by LOX (Karolkowski et al., 2021). Terpenes are considered to be the main volatile compounds derived from carotenoids breakdown. However, these volatile compounds have low concentration in pea protein which could suggest that the degradation of carotenoids has less impact on pea flavor (Murray et al., 1976).

I.4.2. Volatile compounds responsible for off-flavors in pea protein

Numerous studies have reported the volatile compounds responsible for unpleasant perception in pea protein. Several molecules are considered to be responsible for pea off-flavors. Table I-2 summarizes some of these volatile compounds in peas. As it was mentioned earlier, the off-flavors in pea are mainly described as beany and green. However, the beany character seems to be the combination of different sensory attributes. Vara-Ubol et al. (2004) reported that beany odor attribute is the combination of musty/earthy with green/pea pod or with nuts/brown.

Various compounds are usually defined as beany including, 3-methylbutan-1-ol, pentan-1-ol, oct-1-en-3-ol, (2*E*,4*E*)-hepta-2,4-dienal, acetophenone, oct-1-en-3-one and 3-isopropyl-2-methoxypyrazine (Vara-Ubol et al., 2004). However, the beany characteristics of these compounds depends on their concentration. For instance, oct-1-en-3-one and oct-1-en-3-ol were considered beany although they were described as musty, earthy, and mushroom-like (Badenhop & Wilkens, 1969; Boatright & Lei, 1999). Vara-Ubol et al. (2004) shown that oct-1-en-3-ol was musty/earthy at 1 to 10 ppm, and was beany at 100 to 1,000 ppm and lost the beany characteristic at higher concentration. Also, oct-1-en-3-one was perceived as beany at 1 to 10 ppm, as musty/earthy at 100 ppm, and as green from 100 to 5,000 ppm.

On the other hand, some volatile compounds such as hexanal, (*E*)-hexen-2-al, (*E*)-octen-2-al, pentanal, (2*E*,4*E*)-deca-2,4-dienal, (2*E*,4*E*)-nona-2,4-dienal and 2-pentylfuran which are often reported as beany off-flavor in pea, are not considered as beany at any concentration (Vara-Ubol et al., 2004). Indeed, they can lead to an increase in the intensity of beany odor once combined with other volatiles (Bott & Chambers IV, 2006). For instance, hexanal is the most abundant volatile in peas and it is referred as the main volatile compound responsible for peas off-flavor (Ben-Harb et al., 2019; Böttcher et al., 2015). The odor characteristic of hexanal is usually described as grassy and green (Bi et al., 2020). Bott and Chambers IV (2006) reported that the addition of hexanal to oct-1-en-3-one, increased the overall beany intensity of the

sample compare to the oct-1-en-3-one alone. It means, an off-flavor cannot be explained by the presence of single molecules alone (Murray et al., 1976).

Aldehydes are of the main contributors to pea protein off-flavor (Xiang et al., 2022). They are mainly originated from fatty acid oxidation. (*E*)-hexen-2-al, (*E*)-octen-2-al are usually sited as beany, while their odor is mainly associated with fatty (Brown er al. 1973). Xiang et al. (2022) have reported that di-unsaturated aldehydes such as (2*E*,4*E*)-deca-2,4-dienal, (2*E*,4*E*)-nona-2,4-dienal and (2*E*,4*E*)-hepta-2,4-dienal are the key volatiles in pea protein off-flavor due to their low odor threshold. (2*E*,4*E*)-deca-2,4-dienal, (2*E*,4*E*)-nona-2,4-dienal are described as green and cucumber, while (2*E*,4*E*)-hepta-2,4-dienal have beany characteristics. Benzaldehyde is originated from amino acid degradation (phenylalanine) during Maillard reaction (Fischer et al., 2022b). It is typically described as bitter almond and is reported to be impacting in overall pea off-flavor (Fischer et al., 2022a).

3-methylbutan-1-ol, hexan-1-ol, oct-1-en-3-ol and octan-1-ol, are reported regularly in the literature as peas off-flavors. oct-1-en-3-ol, hexan-1-ol and octan-1-ol seem to be abundant in pea protein and are originated from the degradation of fatty acid (Jakobsen et al., 1998; Murray et al., 1976). 3-methylbutan-1-ol, on the other hand, is originated from the degradation of leucine (Murray et al., 1976). These alcohols, could be produced from the reduction of the corresponding ketones or aldehydes by ADH activity (Pei et al., 2022). hexan-1-ol and octan-1-ol have green characteristics while 3-methylbutan-1-ol and oct-1-en-3-ol have beany attributes. 2-ethylhexan-1-ol, with its earthy and slightly fresh cut grass odor attribute, is reported to be the main compound released from protein isolate fractions i.e. legumin and vicilin (Heng et al., 2004; Roland et al., 2017).

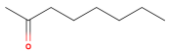
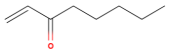
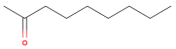
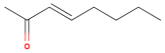
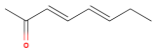
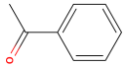
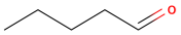
The most important ketones contributing to pea off-flavors include, oct-1-en-3-one, oct-3-en-2-one and (3*E*,5*E*)-octa-3,5-dien-2-one. They are mainly described as earthy and mushroom-like notes. Utz et al. (Utz et al., 2022) reported that (3*E*,5*E*)-octa-3,5-dien-2-one had the highest concentration in pea protein isolate compare to the other odor active compounds. Murat et al. (2013) also showed that oct-1-en-3-ol and (3*E*,5*E*)-octa-3,5-dien-2-one with mushroom-like note and (2*E*,4*E*)-deca-2,4-dienal with grilled and nut note, had high quantity and high odour intensity in both pea flour and pea protein. Ebert et al. (2022) reported that hexanal, 2-pentylfuran, (*E*)-octen-2-al, (2*E*,4*E*)-deca-2,4-dienal and (2*E*,4*E*)-nona-2,4-dienal were the highest abundances with intensive green odor attribute in pea protein isolate. 2-pentylfuran seems to be the typical off-flavor in pea protein since it has been reported in many studies. It

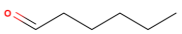
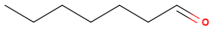
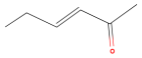
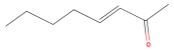
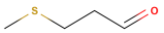
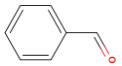
is originated from degradation of fatty acids and is described as earthy (Ebert et al., 2022; Jakobsen et al., 1998).

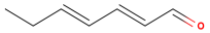
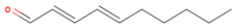
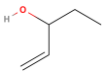
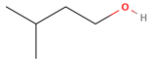
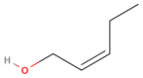
Despite the low concentration of pyrazines in pea, they are reported to be impacting in pea protein off-flavors due to their low odor thersholds (Trindler et al., 2021). These compounds are mainly present naturally in pea and they are mainly originated from amino acid degradation (Murray et al., 1976). 3-isopropyl-2-methoxypyrazine is known for having the odor descriptive of bell pepper. 2,5-dimethylpyrazine and 2-isopropyl-3-methoxypyrazine are reported to attribute to nutty and pea-like notes, respectively.

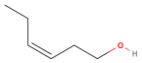
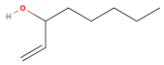
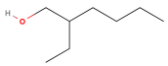
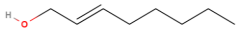
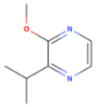
Sulphides compounds are mainly responsible for sulfurous onion like odor. They are the products of amino acid degradation. Although these volatile compounds not responsible of beany note, they can impact strongly the off-flavor profile of the products (Fischer et al., 2022b; Schindler et al., 2012).

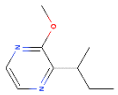
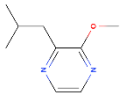
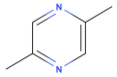
Table I-2. Overview of the main volatile compounds responsible for the off-flavors in pea ingredients.

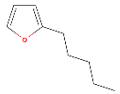
Chemical class	Volatile compounds	CAS*	Origin	Structure**	Detection threshold	Odor descriptors***	References
Ketones	octan-2-one	111-13-7	x		248ppm[8]	Earthy, green, woody	2, 3, 5, 8, 7, 10
	1-octen-3-one	4312-99-6	FA[2]		0.005-10ppb[7]	Earthy, mushroom-like	8
	nonan-2-one	821-55-6	x		0.01ppm	Green, earthy	2, 8, 7, 9, 10, 7
	3-octen-2-one	1669-44-9	FA[1]		0.03-1.12ppb	Earthy, mushroom-like	2, 3, 4, 8
	(3*E*,5*E*)-octa-3,5-dien-2-one	30086-02-3	FA[1]		150ppb[7]	Green, grassy, mushroom-like	3, 4, 5, 8, 7, 9, 10, 11
	acetophenone	98-86-2	AA[1]		0.36-0.6ppm	Beany, musty, earthy	7, 10
Aldehydes	pentanal	110-62-3	FA[1]		12 ppb[6]	Fermented	8

Chemical class	Volatile compounds	CAS*	Origin	Structure**	Detection threshold	Odor descriptors***	References
	hexanal	66-25-1	FA[1]		4.5 ppb[6]	Green, vegetal, grass	2, 3, 4, 5, 7, 8, 9, 10, 11,13
	heptanal	111-71-7	FA[1]		3ppb[6]	Green	2, 3, 5, 8, 11 13
	(*E*)-hexen-2-al	6728-26-3	FA[1]		17ppb[6]	Green, fatty	2, 3, 5, 6, 7, 8, 10
	(*E*)-octen-2-al	2548-87-0	FA[1]		3ppb[5]	Fresh cucumber, fatty, green	3,5, 4, 7, 8, 10, 11, 13
	methional	3268-49-3	AA[5]		0.2ppb[5]	Cooked potato, earthy, vegetal	2,3, 11, 13
	benzaldehyde	100-52-7	AA[1]		35ppb[6]	Bitter almond	2,3, 5, 7, 8, 9, 10, 11, 12
	(2*E*,4*E*)-nona-2,4-dienal	6750-03-4	FA[1]		0.09ppb[5]	Green, cucumber	2,5, 4, 7, 8, 11, 12, 13

Chemical class	Volatile compounds	CAS*	Origin	Structure**	Detection threshold	Odor descriptors***	References
	(2*E*,4*E*)-hepta-2,4-dienal	5910-85-0	x		x	Musty, earthy	8
	(2*E*,4*E*)-deca-2,4-dienal	25152-84-5	FA[1]		0.07ppb[5]	Green, cucumber, fatty	2,3, 4, 5, 7, 11, 13
Alcohols	pent-1-en-3-ol	616-25-1	FA[1]		0.4ppm[7]	Green	2, 3, 5, 8, 10
	3-methylbutan-1-ol	123-51-3	AA[1]		100-300ppb[7]	Beany	3, 10
	pentan-1-ol	71-41-0	FA[1]		4ppm[5]	Beany	2,3, 5, 7, 8, 9, 10, 13
	(*Z*)-pent-2-en-1-ol	1576-95-0	FA[1]		x	Green, phenolic	2,3, 5
	hexan-1-ol	111-27-3	FA[1]		0.09-5.2ppm[7]	Green	2, 3, 5, 7, 8, 9, 10, 12

Chemical class	Volatile compounds	CAS*	Origin	Structure**	Detection threshold	Odor descriptors***	References
	(*Z*)-hex-3-en-1-ol	928-96-1	FA[1]		x	Fresh cut grass, green	2, 3, 5
	oct-1-en-3-ol	3391-86-4	FA[1]		1ppb[5]	Beany	2, 3,5, 7, 8, 9, 10, 13
	2-ethylhexan-1-ol	104-76-7	x		0.4-0.73ppb	Fresh cut greass, green	2, 5, 7, 12
	octan-1-ol	111-87-5	FA[1]		0.69ppb[8]	Waxy, green	2,3,7,8,10,12
	(*E*)-oct-2-en-1-ol	18409-17-1	FA[1]		40-840ppb	Green, fatty	3, 5, 7, 8
Pyrazines	2-isopropyl-3-methoxypyrazine	25773-40-4	AA[1]		15ppb[7]	Beany, green, bell pepper	1,3

Chemical class	Volatile compounds	CAS*	Origin	Structure**	Detection threshold	Odor descriptors***	References
	3-sec-butyl-2-methoxypyrazine	24168-70-5	AA[1]		1ppm	Green, pepper	1,3
	2-isobutyl-3-methoxypyrazine	24683-00-9	AA[1]		0.002ppb[5]	Green, pea, bell pepper	1,2
	2,5-dimethylpyrazine	123-32-0	AA[1]		80ppb-1ppm[5]	Roasted, musty	5
	dimethylsulfide	75-18-3	AA[4]		0.3ppb[6]	Sulfury	14, 15
Sulfides	dimethyldisulfide	624-92-0	AA[4]		0.16-12ppb[7]	Sulfurous vegetable	5, 6
	dimethyltrisulfide	3658-80-8	AA[4]		0.005-0.01ppb[7]	Sulfurous cooked onion	3, 5, 7

Chemical class	Volatile compounds	CAS*	Origin	Structure**	Detection threshold	Odor descriptors***	References
Furans	2-pentylfuran	3777-69-3	FA[3]		6ppb[5]	Earthy, mushroom-like	1, 4

FA: fatty acid, AA: amino acids. *CAS from database (NIST, 2023); **Structure from (https://molview.org/?cid=8063) (February, 2023), *** Odor descriptors from (http://www.thegoodscentscompany.com) (February, 2023). [1](Murray et al., 1976), [2](Jakobsen et al., 1998), [3](Ho & Chen, 1994), [4](Ianni et al., 2020), [5](Trikusuma et al., 2020), [6](Guadagni et al., 1963), [7](Fischer et al., 2022b), [8](https://pubchem.ncbi.nlm.nih.gov)

1(Jakobsen et al., 1998), 2 (Benavides-Paz et al., 2022a), 3(Murat et al., 2013), 4(Ebert et al., 2022), 5(Schindler et al., 2012), 6(Ben-Harb et al., 2019), 7(Xiang et al., 2022), 8(Fischer et al., 2022a), 9(Shi et al., 2021), 10(El Youssef et al., 2020), 11(Utz et al., 2022), 12(Pei et al., 2022), 13(Trikusuma et al., 2020), 14(Malcolmson et al., 2014), 15(Bi et al., 2020)

I.4.3. Protein-volatile compound interactions

The interaction of volatile compounds with protein is of importance since it can affect the intensity and perceptions of aroma. Proteins are known to interact with different chemical classes of volatile compounds such as aldehydes, ketones, alcohols and esters. Depending on the type and strength of the binding, the release of aroma compounds can be decreased which can change the perception of volatiles (Guichard, 2002). The protein-volatile interaction can be reversible hydrophobic, reversible hydrogen bonding, Van Der Waals forces, ionic bonds and irreversible covalent linkage (Fig I-8) (Guichard, 2006). Many factors can affect the protein-volatile interaction including, nature of the volatile compound (composition, structure and length chain), composition and concentration of protein and protein processing.

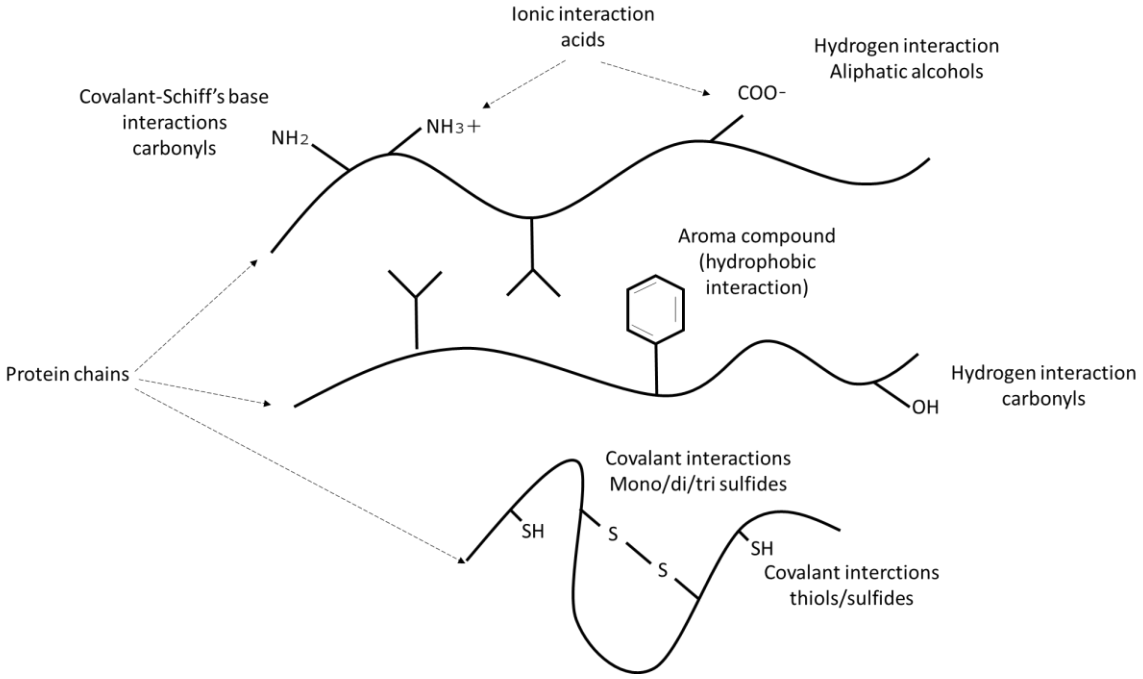


Figure I-8. Opportunities for volatile compounds to interact with protein molecules (adapted and modified from Reineccius (2005))

Volatile compounds, depending on their functional groups, could establish different interactions with protein. In aqueous systems, the interaction between protein and volatile compounds is mainly through hydrophobic binding (Damodaran & Parkin, 2017). Non-polar volatile such as aldehydes and ketones might primary interact through the non-polar sites on protein surface (Heng, 2005). Volatile acids could interact with the charged/polar group on protein through the ionic interaction. The greater binding of butyric acid compared to aldehydes and ketones with soy protein could be related to these type of interaction (Zhou et al., 2006).

Van der Waals forces are responsible for binding hydrocarbon volatiles. Hydrogen bond could occur between alcohols and carbonyls in this interaction (Guichard, 2006). Covalent binding is through specific groups on protein (e.g. -NH_2, -SS-, -SH). This type of interaction is more efficient and lead to the greater binding with volatile compounds (Wang & Arntfield, 2017). Aldehydes which bind covalently have been found to retained stronger by protein than ketones who bind only through non-covalant interactions (Heng et al., 2004). Chain length of the volatile compounds is also defining for the protein-volatile interaction. Long chain volatile compounds possess higher affinities to protein than the short chain ones. Heng et al. (2004) reported that ketones and aldehydes with longer chain had higher affinities to pea vicilin. They also showed that aldehydes have higher affinities for protein than ketones.

The affinity of a volatile compound to protein, depends also on the protein composition, structure and its concentration. For instance, protein fractions containing higher content of sulfur containing amino acids, exhibit higher binding capacities through the covalent bond (Wang & Arntfield, 2017). Different protein fractions can also have different affinities. Heng et al. (2004) showed that at pH 7.6 pea vicilin bind to both aldehydes and ketones while legumin bind only with aldehydes. Moreover, increasing the protein concentration can increase the interaction of protein-volatile. Ng et al. (1989) reported an overall reduction of the number of binding sites available for vanillin as the concentration of faba bean protein increased. Probably it could be said that the intensity of off-flavors can be reduced from protein isolate and concentrate to flour. Cowan et al. (1973) showed that beany was detected at 300-400 ppm in soy flour, at 2,000 ppm for protein concentrate and at 12,500 ppm for protein isolate.

Any factor that modify the composition and conformation of protein, could disrupt protein-volatile interactions (Heng, 2005). Changes in pH can affect the conformation of protein and provoke different aggregation states (Wang & Arntfield, 2017). Wang and Arntfield (2015b) found that binding in pea protein increased at their isoelectric point due to the physical sorption of the volatile compound on the aggregated protein surface. While, the denaturation of protein at extreme pH (pH 3 and 11) reduced the access to hydrophobic sites and subsequently reduced the protein-volatile interaction. Changes in the temperature is also another important factor, since high temperature can induce structural changes in protein. However, the effect of heating on protein-volatile interaction seems to be debating. Some believe that heating cause protein denaturation which could be followed by aggregation. This aggregation might reduce the available binding area of protein and subsequently reduce the protein-volatile binding (Heng

et al., 2004). However other studies reported that unfolding protein by heating, can increase the chance of volatile binding by exposing the interior hydrophobic sites (Chung & Villota, 1989; Damodaran & Kinsella, 1981). Ionic strength, the concentration and type of salt can also affect the conformation of protein and its interaction with volatile compounds. Chaotropic salts like CH_6CIN_3, destabilizing protein structure and decrease the protein-volatile binding capacity. While non-chaotropic salts such as NaCl have stabilizing effect on hydrophobic bonds and enhances the interaction between volatile compound and protein (Wang & Arntfield, 2015b). Other factors such as proteolysis and enzymatic modification of protein side chains could alter protein structure and accessibility of aroma compounds to the protein active sites (Wang & Arntfield, 2017). Proteolysis, eliminates hydrophobic binding with aroma compounds and it decrease the aroma binding to protein. However, the hydrolysis of protein would increase the accessibility to the terminal amino groups of initially blocked amino acid residues. Hence, protein would react with carbonyls through sulfur interaction (Reineccius, 2005).

I.4.4. Extraction of volatile compounds

Selection of the appropriate extraction technique is extremely important in the identification of the volatile compounds. Several considerations must be consided when choosing a technique. The most important thing to consider is the analytical object. There are numerous methods available for the extraction of volatile compounds (Reineccius, 2005) each of which has its own strength/weakness. Statistic headspace is a direct analysis for the equilibrium headspace above the food products. The method is easy but it lacks sensitivity especially for low volatility. It has been previously used by Heng et al. (2004) for studying pea protein-flavor interaction; and by Wang and Arntfield (2015a, 2015b) for the study on the interactions of carbonyl-containing flavors with salt- and alkaline-extracted pea protein isolate. Otherwise in dynamic headspace (purge and trap) method, a Tenax trap is used for capturing volatiles purged form the food sample. Although, the method reduces matrix effects and increase the sensitivity compared to statistic headspace, it has low surface area and low sorption capacity. This method has been used by Murray and Whitfield (1975) to study the effect of 3-alkyl-2-methoxypyrazines in raw pea; and by Jakobsen et al. (1998) to study the volatile compounds in blanched green peas. Headspace solid-phase microextraction (HS-SPME) is the adsorption of the volatile compounds in the vapour phase on a fiber. This technique is simple, fast and solvent-free. However, the technique has some limitations, including competition of volatile compounds for the adsorption sites and low surface area of the fiber which limit the adsorption

capacity. The technique has been widely used for the extraction of volatile compounds in pea by Fischer et al. (2022a) for the study of the impact of aging on pea protein volatile; by Azarnia et al. (2011a); Azarnia et al. (2011b) to study the volatile compounds in field pea cultivars; or by Shi et al. (2021) to study the elimination of off-flavors in pea protein isolate. Stir bar sorptive extraction (SBSE) technique is known for being fast, simple and it requires small amount of sample. This method was used by Ebert et al. (2022) in studying the effect of extraction and texturization on odor-active compounds of pea protein. Finally, solvent assisted flavor evaporation (SAFE) is the combination of vacuum distillation and solvent extraction. This technique is a gentle isolation as it uses low extraction temperature and it preserves the native aroma profile of the samples (Pico et al., 2018). This technique allows maximum isolation of volatile compounds and a quantification by a standard. However, it requires a long extraction time, intensive work, a significant amount of sample and the use of organic solvent. The technique was used by Benavides-Paz et al. (2022a, 2022b) to study the aroma profile of pea protein isolate during the extraction by alkaline solubilization/isoelectric precipitation and salt solubilization coupled with membrane filtration; also by Trikusuma et al. (2020) to evaluate the impact of processing and aging on aroma profile of pea protein. Murat et al. (2013) showed, among various extraction methods, SAFE was the most suitable technique since it has a good extraction capacity and high representatively of the global odor of pea flour.

I.5. Parameters controlling the composition and nutritional and aromatic properties of pea protein

The variety in structure and conformation of pea protein can affect its techno-functional, nutritional and sensorial properties. For instance, vicilin has less compact structure, thus, it is more readily extractable and offers better functional properties than legumin (Dagorn-Scaviner et al., 1986). However, the low content of sulfur amino acids in vicilin make it less interesting from a nutritional point of view (Tzitzikas et al., 2006). On the other hand, the nutritional quality of pea protein is generally defined by the content of sulphur amino acids which have higher value in albumin fraction (Schroeder, 1982). Therefore, pea with a specific protein composition would be desirable for food industry. Various parameter could control the composition, nutritional and aromatic profile of pea protein such as genetic variation, extraction condition and biological process such as fermentation. These parameters were discussed below.

I.5.1. Pea cultivars

Cultivars, location and environmental condition of growth, can affect the content, quality and composition of pea protein (Wang & Daun, 2004). Numerous studies have reported the variation in pea protein content affected by genetic factors (Gueguen & Barbot, 1988; Martínez-Villaluenga et al., 2008). Tzitzikas et al. (2006) showed the pea protein content ranged from 13.7 to 30.7% among cultivars and globulins corresponded from 49.2 to 81.8% from total protein. Barac et al. (2010) found that the protein content of six pea cultivars varied between 22.3-31.8% and the isolate approximately 84-89%. The protein content seems to be quite sensitive to environmental stress (Gueguen & Barbot, 1988). Al-Karaki and Ereifej (1999) reported that water deficit and high temperature are often responsible for elevated protein content. Nikolopoulou et al. (2007), and Wang and Daun (2004) reported a wide variation in protein content due to a combination of genetic factors and environmental conditions.

Protein composition is also affected by both genetic characteristics and environmental factors. The vicilin/legumin ratio which generally determines the techno-functional properties of pea protein, appeared to be significantly different between genetic varieties in different studies. Schroeder (1982) reported that the vicilin/legumin ratio ranged from 0.5 to 1.7. This ratio was quite similar to the one reported by Gueguen and Barbot (1988) ranging from 1.2 and 3.7. However, Tzitzikas et al. (2006) showed the ratio of vicilin/legumin ranged from 1.3 to 8.2 and vicilin/convicilin varied from 3.5 to 11.4. Gatehouse et al. (1980) suggested that the large variation in vicilin/legumin ratio could be related to the stage of development and maturity of the seeds as well as the effect of environment since legumin predominantly accumulates late in development. The ratio of albumin and globulin defines the nutritional quality of pea protein. Schroeder (1982) observed the variation in albumin content ranging from 14 to 40% between pea genotypes. Gueguen and Barbot (1988) have shown in their study that some cultivars had inverse albumin/globulin ratio with globulin having the less proportion. They have explained this by the environmental conditions rather than genetics. Sell et al. (2005) also suggested that the variation in albumin and globulin content between the cultivars could be related to different stage of seed maturation.

Amino acid composition is closely associated with nutritional and techno-functional properties of proteins (Stone et al., 2015a). Apart from their contribution to nutritional properties of pea protein, the presence of amino acids with different characters (polar, non-polar, acidic, basic)

can modify the protein surface and consequently the functional properties and aromatic profile (protein-volatile binding). Tang and Sun (2011) reported the differences in the content and composition of charged (acidic, basic) and uncharged (polar, non-polar) amino acids of three different beans, altered the surface chemistry of protein. Stone et al. (2015b) observed only a slight difference in the amino acid content of pea cultivars. Martínez-Villaluenga et al. (2008) have shown that the content of essential amino acids varied between the cultivars. They also reported a higher content of sulfur amino acids for albumin fraction compare to globulin in all the cultivars.

The content of ANFs can be varied between pea cultivars. Previous studies reported that year and cultivation location of varieties had significant impact on the content of phytic acid (Nikolopoulou et al., 2007). Wang et al. (2010) stated the highest value of phytic acid content in pea cultivars could be attributed to the higher phosphorous content of the soil. They reported that phytic acid content ranged from 0.4 to 1.1%. Maharjan et al. (2019) observed no difference in phytic acid content between pea genotypes while environment had a significant effect. The value of phytic acid in their study changed from 0.4 to 0.8%. TIA in pea is also cultivar dependent and generally it ranges from 1 to 15 TIU/mg (Bacon et al., 1995b). Griffiths (1984) showed the content of TIA varied from 0.1 to 4.6 TIU/mg between pea cultivars which were grown in the same year. Vidal-Valverde et al. (2003) showed that TIA ranged from 0.8 to 8.4 TIU/mg between 18 pea cultivars. The authors suggested that the variations could be explained by the different climate and soil conditions of cultivation. Bacon et al. (1995a) showed that the combination of genotype and environment was important in TI activity and the value ranged from 0.5-4 TIU/mg. Jones et al. (1999) showed the total content of α-galactosides ranged from 4.9 to 10.7% in different pea genotypes. Maharjan et al. (2019) showed genotype and environment both had significant effect on α-galactoside content in pea cultivars. The environment did not affect significantly on verbascose. Stachyose and verbascose were the most abundant soluble carbohydrate in pea cultivars and their content ranged from 2.6-3.9% and 1.3-2.6%, respectively. Gdala and Buraczewska (1997) also showed that stachyose and verbascose were the major α-galactosides. Raffinose accounted for less than 0.07%. The total α-galactosides in their study ranged from 6.3-5.2% in pea cultivars.

The off-flavors of peas also depend on the cultivar, the location, and the growth and storage conditions (Bishnoi & Khetarpaul, 1994; Daveby et al., 1997). This could be partly due to the variation in protein composition which change the protein-volatile interactions (see I-4.3), and

to the differences in the chemical composition (protein, lipid and carbohydrate) of the cultivars (see I-4.1). Jakobsen et al. (1998) reported the concentration of hexanal and hexanol varied significantly among pea genotypes. They also observed that the concentration level of aroma compounds at different harvest date in the same field were different. The increase in (*Z*)-3-hexenylacetate and (*Z*)-hexan-3-ol concentration between the varieties reflects the effect of differences in the average development stage or age of the maturing seed. Therefore, they suggested that the concentration of (*Z*)-3-hexenylacetate and (*Z*)-hexan-3-ol increased when harvest was delayed. Kaneko et al. (2011) studied the effect of soybean cultivars on soy milk off-flavors. They observed that almost all the key aromas (such as hexanal, (*E*)-nonen-2-al) were common in cultivars, while the intensity of these aroma was different among the cultivars. They suggested that it could be for the differences in the content of fatty acids between the cultivars. They also reported the remarkable difference was found in the content of 2-isopropyl-3-methoxypyrazine between the cultivars. Azarnia et al. (2011b) found the content of volatile compounds were affected by pea cultivars (yellow, green and dun) and crop year. For instance, green pea had higher content of hexanal while yellow pea had the highest content of (*E*)-octenal. Comparing the crop year, the ones were grown in 2006 had higher content of alkenes, while the one in 2005 had higher content of alcohols. Malcolmson et al. (2014) reported that the combination of cultivars by crop year had significant impact on pea-like and metal flavor attributes. They also showed the intensity and content of volatile compounds were different between the cultivars. The highest and lowest intensity of volatile compounds was related to the yellow and dun pea respectively.

I.5.2. Pea protein extraction method

Various methods have been proposed for the extraction of protein from legume flour, each of which have significant effect on protein yield, quality, purity and functional properties. Hence, choosing the right extraction method is an important parameter for food industry (Stone et al., 2015b). In the same time, the environmental concerns and consumer's demand for improvement of nutritional value of the products have convinced researchers to seek for a green production of protein from legumes (Abdel-Rahman et al., 2013).

The common methods for pea protein production include alkaline extraction-isoelectric precipitation, salt extraction-dialysis, micellar precipitation and aqueous extraction (pH> 7) (Saurel, 2020; Stone et al., 2015b). The selection of methods might be varied in industry for different protein types which in turn influence the final composition and functionality of the

isolate product (Stone et al., 2015b). For instance, isoelectric precipitation produces isolates containing mostly globulins, whereas salt extraction generally results in a mixture of both globulins and albumins (Kiosseoglou et al., 2021; Liu et al., 2008). Micellar precipitation results in a protein isolate having a micelle-type form, likely stabilized by hydrogen bonds (Arntfield et al., 1985). The isolate obtained by this method, may contain both globulins and albumins and there might be less protein denaturation compared to isoelectric precipitation (Cordero-De-Los-Santos et al., 2005). The conventional extraction methods are often associated with some draw backs such as significant use of water and chemicals, high energy requirements and noticeable environmental food prints of the chemical solvents (Hewage et al., 2022). Also, none of the common techniques are able to address the presence of antinutritional factors in legume proteins. However, many industries might still rely on the conventional methods due to their techno-economic feasibility. These challenges necessitate to develop an innovative, selective and eco-efficient technique with high protein yield and high contribution to the nutritional value and quality of the protein (Eze et al., 2022; Wen et al., 2022).

Among the conventional extraction techniques, alkaline extraction followed by isoelectric precipitation (AEIEP) is a common technique with high yield for producing pea protein isolate in food industry. Stone et al. (2015b) observed that pea isolate obtained by AEIEP (83.3-86.9%) had significantly higher content of protein compared to salt extracted (71.5-79.3%). Karaca et al. (Karaca et al., 2011) also reported that AEIEP (85.6%) had higher content of protein than salt extraction (78.4%) for legume protein isolate including pea, chickpea, faba bean, lentil, soybean. The method aims at separate albumins and globulins thanks to differential solubility. The reason is the high solubility of both legumin and vicilin at alkaline pH, but minimal solubility at their pI while the albumin part remains soluble (Gao et al., 2020). The extraction process starts with the solubilization of protein by suspending pea flour in water at alkaline pH (~8) (Fig 1-9). It then follows by the isoelectric precipitation step in which the pH of the protein-rich extract is lowered up to ~4.5–4.8 by the addition of mineral acid in order to reach pI of the globulins. Then, the precipitated globulins can be neutralized and dried to produce protein isolate as powder (Emkani et al., 2021).

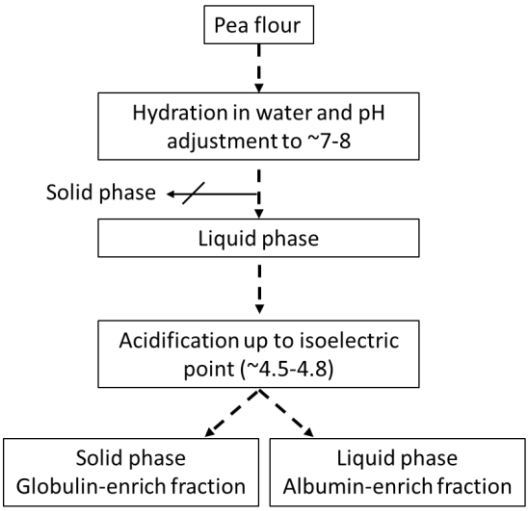


Figure I-9. Schematic of alkaline extraction/isoelectric precipitation of pea protein isolate (modified from Lam et al. (2018))

The isolate yield in this method can be 60-70% (Hansen et al., 2022). However, controlling the condition of extraction such as pH could affect the solubility of protein and the extraction yield and modifying their nutritional properties and aromatic (Lam et al., 2018). Salcedo-Chávez et al. (2002) showed a significant variation in protein content of protein isolate obtained at different alkaline and precipitation pH values. Gao et al. (2020) reported that increasing the alkaline pH from 8.5 to 9.5 in extraction of pea protein isolate, increased the extraction yield, promoted the aggregation of protein and subsequently decreased the solubility of protein (at pH 3 and 7). They also have reported that, increasing the alkaline extraction pH would increase the content of beany off-flavor in pea protein isolate. Indeed, modification of pH is known to alter protein structure and hydrophobicity, which in turn it could either create additional binding site and increase the retention of volatile compounds (protein folding), or could loss the binding site (protein unfolding or high denaturation) leading to the release of the volatile compounds (see I-4.3) (Heng et al., 2004; Wang & Arntfield, 2015b, 2017). Benavides-Paz et al. (2022a) studied the content of volatile compounds during the different step of alkaline solubilisation and isoelectric precipitation. They stated that the increase in the level of some volatile compounds such as oct-1-3-ol and (*E*)-nonen-2-al after alkaline solubilization could be related to the release of these compounds from the solid cellular structure of pea flour. They also explained the significant changes (increase or decrease) in the content of some volatile compounds after isoelectric precipitation at pH 4.5, by the hydrophobic association of proteins at isoelectric point which can impact the protein-volatile interaction. Murat et al. (2013) studied the volatile compounds and the odour active compounds along extraction process. They

reported that aldehydes and ketones were the most represented group in pea protein isolate compared to other fractions (pea flour, protein solubilized and soluble fraction (albumin)). This could be explained by the hydrophobic nature of aldehydes and ketones. Hexanal, heptanal and (3*E*,5*E*)-octa-3,5-dien-2-one were identified in every step in the extraction but in lower quantity compare to protein isolate. The content of pent-1-en-3-ol, pentan-1-ol, hexan-1-ol decreased for solubilized and extracted products compare to pea flour. Cui et al. (2020) observed the evolution of volatile compounds during the different steps of AEIEP. They reported that the beany off-flavors were more abundant in solubilized samples compared to pea flour. They also reported the variation of volatile compounds in different steps is related to the changes in pH and protein structure.

The ratio of globulin proteins is less affected by extraction method since their isoelectric point are very similar. The isoelectric point of legumin is at pH 4.8 and for vicilin at pH 5.5 (Derbyshire et al., 1976). Also, these proteins are reported to be located in the protein bodies. Hence, any mechanical treatments such as milling or screening does not alter the ratio of vicilin/legumin (Gueguen, 1983).

I.5.3. Lactic acid fermentation

Biological processing such as fermentation and germination are also determinant in controlling the protein composition and properties. Fermentation is a traditional technique that serves as a practical method for food preservation (Holzapfel, 2002; Matejčeková et al., 2019). It has been used to enhance the bioaccessibility and bioavailability of nutrients (Cabuk et al., 2018a; Rui, 2019; Stanisavljevic et al., 2015; Vermeirssen et al., 2003; Xing et al., 2020) or to improve the organoleptic properties (Schindler et al., 2011; Schlegel et al., 2019; Yousseef et al., 2016) and shelf life of various legume proteins. Fermentation consists of modifying food by microorganisms (bacteria, molds and yeasts) that grow and consume part of the substrates and enrich the medium with the products of their metabolism (Fernandez-Orozco et al., 2007). However, selection of the right microorganism is necessary, since some microorganisms including yeasts and molds might concern food safety (Nkhata et al., 2018). Lactic acid bacteria (LAB) with the generally recognized as safe (GRAS) status are of great interest in food fermentation (Klupsaite et al., 2017). The growing interest in the lactic acid fermentation of legumes is clear from the increasing number of scientific studies about the effects of lactic acid fermentation on the nutritional, physicochemical, and sensorial properties of various sources of legume protein ingredients, including soybean, chickpea, lupine, pea, faba bean, lentil, bean,

etc. Lactic acid fermentation can be applied to legume seed, flour-, or protein-enriched ingredients. However, the efficiency and functionality of lactic acid fermentation depends greatly on the type of LAB strain, the fermentation technique (solid-state and liquid-state fermentation), the type of legume, the composition of the protein ingredient, and slightly on the genetic variety (Bartkiene et al., 2015; Rui et al., 2017). Previous reviews studied the effect of fermentation on legumes such as Boeck et al. (2021) studied the effect of fermentation on nutritional and antinutritional properties of pulses; Licandro et al. (2020) discussed the effect of lactic acid fermentation on microbiological and chemical safety of Asian legume based fermented products; Garrido-Galand et al. (2021) studied the potential of fermentation on nutritional and technological improvement of cereal and legume flours. However, the review published recently in our team (Emkani et al., 2022), focused particularly on the effect of fermentation with LAB on composition, nutritional, techno-functional and sensorial profile of legume proteins. Fig I-10. summarizes the effect of lactic acid fermentation on legume protein.

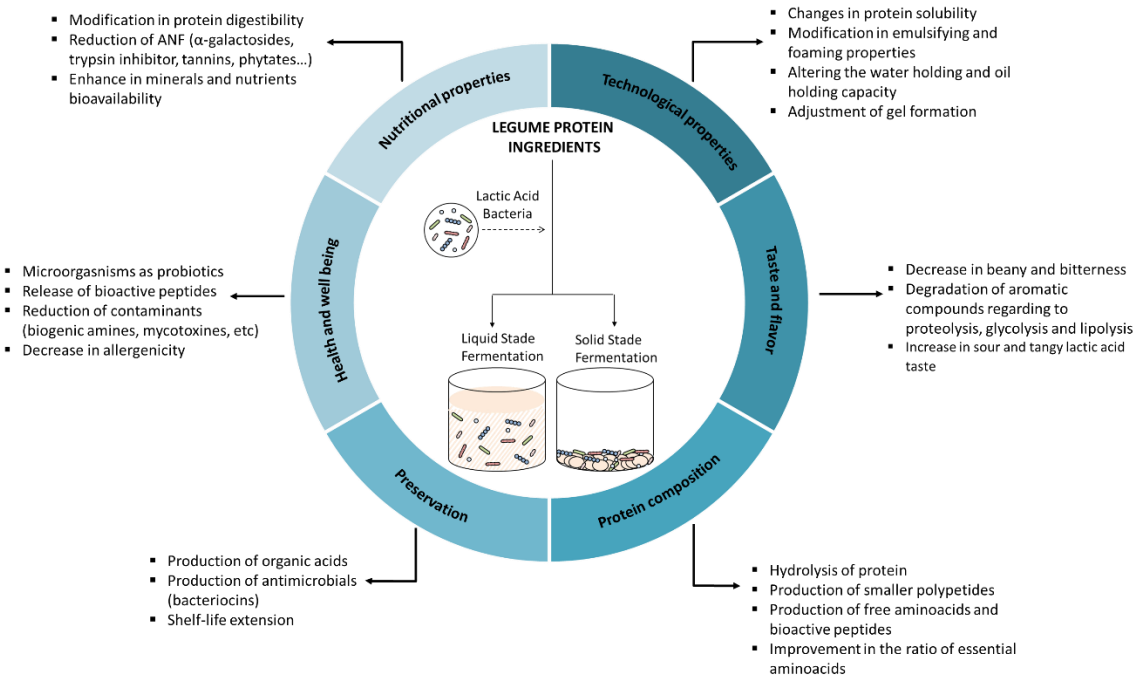


Figure I-10. Schematic depicting the effects of lactic acid fermentation on legume protein (adapted from Emkani et al. (2022)).

I.5.3.1. Lactic acid bacteria

LAB are acid-tolerant, aero-tolerant, and non-spore-forming bacteria that can adapt to different food matrices. LAB genera include *Lactobacillus* (Lb.), *Lactococcus* (L.), *Leuconostoc* (Lc.),

Pediococcus, *Streptococcus* (S.), *Aerococcus*, *Alloiococcus*, *Carnobacterium*, *Dolosigranulum*, *Enterococcus*, *Oenococcus*, *Tetragenococcus*, *Vagococcu*s, and *Weissell*a (W.), with Lactobacillus being the largest genus. The genus *Lactobacillus* has been recently reclassified into 25 genera. The new genera are *Lactobacillus* and *Paralactobacillus*, and the 23 novel genera are *Amylolactobacillus*, *Acetilactobacillus*, *Agrilactobacillus*, *Apilactobacillus*, *Bombilactobacillus*, *Companilactobacillus*, *Dellaglioa*, *Fructilactobacillus*, *Furfurilactobacillus*, *Holzapfelia*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Lapidilactobacillus*, *Latilactobacillus*, *Lentilactobacillus*, *Levilactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, *Liquorilactobacillus*, *Loigolactobacilus*, *Paucilactobacillus*, *Schleiferilactobacillus*, and *Secundilactobacillus* (Ayivi et al., 2020). The selection criteria for LAB are decisive in terms of the properties of the final products (Vinicius De Melo Pereira et al., 2020).

LAB require energy to grow, and they obtain this energy through the metabolism and consumption of different compounds, such as minerals, amino acids and sugar. The two main metabolic pathways of lactic acid fermentation are glycolysis (fermentation of sugars and acid formation) and proteolysis (degradation of proteins) (Bintsis, 2018). Glycolysis is the breakage of glucose into two molecules of three-carbon pyruvate, called pyruvic acid, which can alternatively be reduced to lactic acid. Lactic acid has two optical isomers, L-(+)-lactic acid and D-(–)-lactic acid (Datta et al., 1995). The L-(+) isomer can be produced by LAB of the genera *Streptococcus*, *Pediococcus*, *Lactococcus*, and *Lactobacillus*, whereas the D-(–) isomer can be produced only by particular strains of the genus *Lactobacillus* (Trontel et al., 2011).

Depending on the type of fermented sugar and fermentation products, LAB are divided to homofermentative, obligate heterofermentative and facultative heterofermentative. Fig I-11. shows the catabolism of glucose by LAB with three different pathways. Homofermentative, ferment hexose exclusively to lactic acid through the Embden-Meyerhof-Parnas pathway (EMP) or glycolysis (Salvetti et al., 2012). Indeed, pentose are not fermented by homofermentative LAB. Homofermentative produce more acids than heterofermentative since they produce two moles of lactic acid from one mole of glucose. This is the case for species such as *Lactobacillus acidophilus*, *Lactobacillus amylophilus*, *Lactobacillus bulgaricus*, and *Lactobacillus helveticus*. A mixture of two lactic acid stereoisomers is usually produced by homofermentative LAB that belong to the genera *Pediococcus* and *Lactobacillus* (Trontel et al., 2011). At slow growth or substrate limitation, homofermentative strains could change to mixed acid fermentation in which they produce formate, acetate, ethanol, and lactate (Nuryana

et al., 2019). Facultative heterofermentative bacteria, ferment hexoses to lactic acid via EMP. They are also able to degrade pentoses under glucose limitation by phosphoketolase (an enzyme of the phosphate pathway), which results in the production of acetic acid, ethanol and formic acid (Salvetti et al., 2012). *Lactiplantibacillus plantarum* is a facultative heterofermentative LAB. Finally, the obligate heterofermentative bacteria do not have phosphoketolase, and they metabolize pentoses and hexoses exclusively by phosphogluconate pathway (corresponding to the first part of the PP) and produce lactic acid, ethanol (or acetic acid) and CO_2 (Salvetti et al., 2012). An example of obligate heterofermentative is *Limosilactobacillus fermentum.* There are hexoses other than glucose, such as fructose, mannose, and galactose, which can be consumed by LAB. LAB are also able to hydrolyze disaccharides such as maltose, sucrose, and lactose and more complex sugars such as α-galactosides, depending on their enzymatic capacity (Gänzle & Follador, 2012).

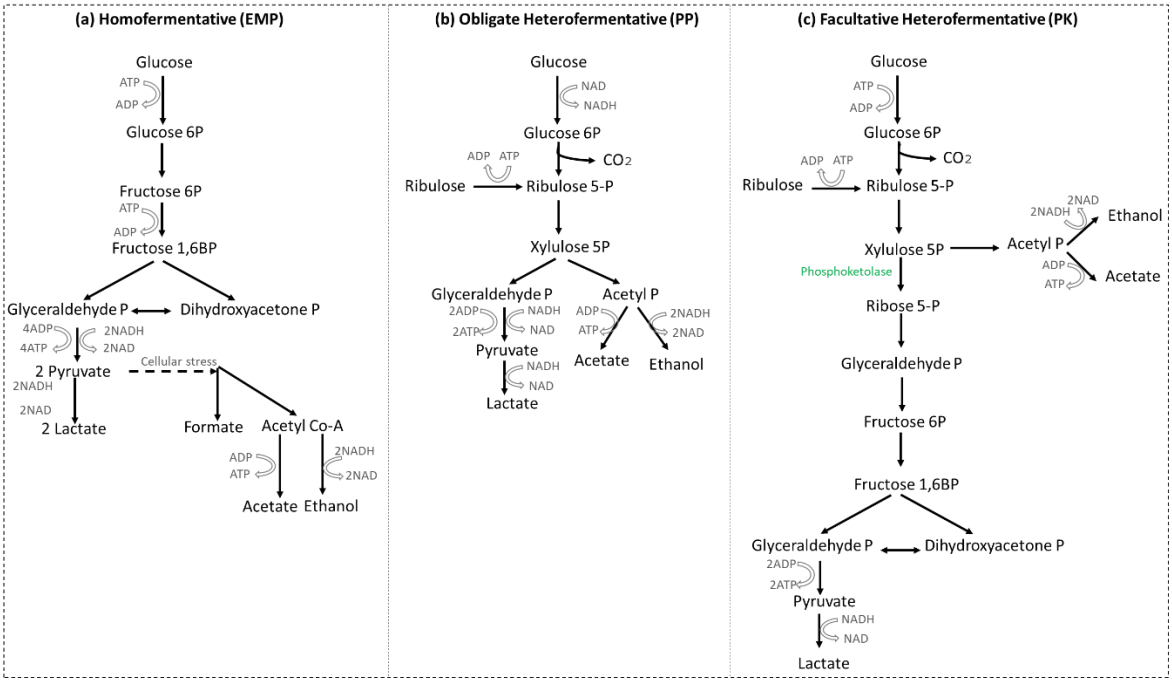


Figure I-11. Glucose fermentation pathway. (a) homofermentative metabolism (Embden-Meyerhoff pathway (EMP)), (b) obligate heterofermentative (phosphogluconate pathway (PP)), (c) facultative heterofermentative (phosphoketolase pathway (PK)) (modified from Teleky et al. (2020) and Romo-Buchelly et al. (2019)).

Another important metabolic activity of LAB is proteolysis. In fact, LAB are unable to synthesize many amino acids, vitamins, or nucleic acids, so they need to hydrolyze proteins and peptides in food matrices in order to liberate amino acids and subsequently utilize them

(Bintsis, 2018). The proteolytic system of LAB is a well-designed system which initiates with the degradation of protein to oligopeptides by cell-envelope proteinase (CEP) (Kunji et al., 1996). The majority of LAB have CEP, while some of them do not contain this enzyme. Hence, they rely on other LAB for the production of peptides and amino acids. Different type of CEP has been characterized from LAB, including PrtP from *Lactococcus lactis,* PrtH from *Lb. helveticus,* PrtR *Lacticaseibacillus rhamnosus* and *Lb. plantarum,* PrtS *S. thermophilus,* PrtB from *Lb. bulgaricus* (Savijoki et al., 2006). These enzymes have some differences which affect their activity. For instance, the isoelectric point of PrtS (pI=4.6) is lower than PrtP (Pi=4.8), which leads to the precipitation and subsequent inactivation of PrtS (Chang et al., 2014). Ren and Li (2022) reported the effect of some extracellular protease activity on the gel characteristics of soybean protein. The produced peptides by CEP, are then taken up by the cells through the specific peptide transporters. Once transported to the cell, the oligopeptides are broken down to the smaller peptides by intracellular endopeptidase and aminopeptidase. Smaller peptides are further broken up by dipeptidases and tripeptidases to individual amino acids. The generated amino acids are then broken down to different volatile compounds (Harper et al., 2022; Kunji et al., 1996). The proteolysis pathway is of importance for industry since the produced peptides, amino acids and their derivatives are known to contribute to the texture, flavor and nutrition of the fermented products (Savijoki et al., 2006).

I.5.3.2. Effect of lactic fermentation on legume protein composition

Lactic acid fermentation modifies the content and the composition of proteins. This can happen either through the enzymatic proteolysis or acid-induced hydrolysis which implied by bacteria metabolism. Indeed, during lactic acid fermentation, bacteria produce organic acid as a side product (Meinlschmidt et al., 2016). The produced organic acids such as lactic acid and acetic acid may disrupt the ionic interaction between protein side chains that stabilize the secondary structure, and thus, the presence of acid may lead to the loss of the secondary and tertiary structures of the protein (Lampart-Szczapa et al., 2006; Meinlschmidt et al., 2016). Ma et al. (2017) reported that fermented pea flours with a mixed culture containing *S. thermophiles*, *Lb. bulgaricus,* and *Lb. acidophilus* exhibited severe damage in the legume cell wall structure compared to unfermented flours, as determined through the use of scanning electron microscopy. These changes led to smaller peaks in the differential scanning calorimetry (DSC) thermograms of fermented samples, indicating less energy for the breakage of intermolecular bonds within protein bodies to achieve protein denaturation.

The hydrolysis of legume protein depends highly on the type of LAB strains (Raveschot et al., 2018). Aguirre et al. (2008) observed that the proteolytic enzymes of twelve different lactic acid bacteria (*Lacticaseibacillus paracasei, Lb. fermentum, Lactobacillus lactis, Lb. plantarum, Lb. helveticus, Lactobacillus reuteri*, and *Pediococcus pentosaceus*), were able to hydrolize differently the soy protein isolate fractions (7S Beta-conglycinin, 11S-glycinin). Moreover, the initial differences in the polypeptide profiles of the initial legume proteins may determine their sensitivity to proteolysis. This could be the reason that Lampart-Szczapa et al. (2006) observed differences in the proteolytic effects of lactic acid fermentation with *Leuconostoc mesenteroides, Lb. plantarum*, and *Levilactobacillus brevis* co-cultures when comparing flours from different lupine cultivars.

Studies on the effect of fermentation on protein content have shown different results, depending on the legume substrate and the lactic acid bacteria used for fermentation. Changes in the protein structure and hydrolysis of proteins up to the formation of amino acids may decrease the extraction yield of proteins, as indicated in a study of faba bean flour fermentation with different LAB strains (Xu et al., 2019) and in a study of lupine flour fermentation with a mixture of *Lc. mesenteroides, Lb. plantarum*, and *Lb. brevis* (Lampart-Szczapa et al., 2006). Other authors, however, did not observe a diminution in protein content after fermentation, indicating that fermentation only changes the molecular size of proteins, such as in the fermentation of lyophilized chickpea and faba bean flours with a yogurt starter (*Lb. bulgaricus* and *S. thermophilus*) (Chandra-Hioe et al., 2016). Other authors have indicated an increase in the protein content after fermentation. In a study of the fermentation of pea-protein-enriched flour using *Lb. plantarum* (Cabuk et al., 2018a), the authors observed an increase in the percentage of protein, fat, and ashes, presumably due to an increase in the bacterial biomass and the loss of carbohydrates during the fermentation process. When the condition of proteolysis is adequate, fermentation may modify the free amino acid profile. In the fermentation of pea-protein-enriched flours with *Lb. plantarum* (Cabuk et al., 2018a), all amino acid mass fractions (mg amino acids/100 g sample) increased with fermentation time, except for arginine and tryptophan, which remained similar.

I.5.3.3. Effect of lactic acid fermentation on nutritional properties of legume ingredients

Lactic acid fermentation can improve the digestibility and the quality of protein either by proteolysis and production of smaller peptides, or by reducing the antinutritional compounds (Chandra-Hioe et al., 2016; Ma et al., 2018). Only some of these ANFs were discussed here.

Certain LAB species, such as *Streptococcus sp., Leuconostoc sp.,* and *Lactobacillus sp.,* show α-galactosidase activity, which gives them the ability to transform α-galactosides into absorbable mono- and di-saccharides (Egounlety & Aworh, 2003; Harlé et al., 2020; Holzapfel, 2002). Lactic acid fermentation of legumes such as chickpeas fermented with *Pediococcus* strains (Xing et al., 2020); yellow and red lentil, white and black bean, chickpea, and pea flours fermented with *Lb. plantarum* and *Lb. brevis* (De Pasquale et al., 2020); faba beans and field peas fermented with *Lb. reuteri* (Meinlschmidt et al., 2016); and faba beans fermented with *P. pentosaceus* (Coda et al., 2017); showed a decrease in the content of raffinose. However, the diminution depended on the kind of legume, as indicated in a study of fermentation of different varieties of chickpeas, lentils, and peas using *Lb. plantarum* and *Lb. brevis* (Curiel et al., 2015).

The enzymatic hydrolysis of trypsin inhibitors by LAB permitted the reduction in trypsin inhibitor activity (Cabuk et al., 2018a). In general, trypsin inhibitor activity was decreased in fermented yellow pea flour by *S. thermophilus, Lb. bulgaricus,* and *Lb. acidophilus* (Ma et al., 2018); in pea protein concentrate by *Lb. plantarum* (Cabuk et al., 2018a); in red and yellow lentil, white and black bean, chickpea, and pea flours by *Lb. plantarum, Lactobacillus rossiae, Lb. brevis, P. acidilactici, and Lc. mesenteroides* (De Pasquale et al., 2020); in grass pea flour by *Lb. plantarum* (Starzyńska-Janiszewska & Stodolak, 2011); and in faba bean flour *by Lb. plantarum* (Coda et al., 2015). However, Chandra-Hioe et al. (2016) did not observe significant differences due to fermentation in chickpea (desi/kabuli) and faba bean flour after 16 h of fermentation with a freeze-dried yogurt culture.

Some LAB are able to degrade phytic acid by producing a phytase enzyme (Gupta et al., 2015; Sharma et al., 2020). For instance, phytic acid decreased during the fermentation of soymilk with *S. thermophilus* (Lai et al., 2013), *Lb. fermentum, Lb. plantarum, Lacticaseibacillus casei, Lb. bulgaricus,* and *Lb. acidophilus* (Rekha & Vijayalakshmi, 2010). Fermentation of faba, chickpea, lentil, and pea flours with *Lb. plantarum* and a mix of *Lb. fermentum* and *Lactobacillus pontis* also reduced the phytic acid content as a result of bacterial phytases (Curiel et al., 2015; De Pasquale et al., 2020; Gabriele et al., 2019). Lupine flour also showed lower phytic acid content after fermentation with lactic acid bacteria (Van Vo et al., 2015). Microorganism-inherent phytases, dissociate non soluble organic complexes with minerals (Chu et al., 2009; Olukomaiya et al., 2020). Studies have indicated that the effect on phytates was closely dependent on the microbial strain (Kasprowicz-Potocka et al., 2016). It has also been observed that phytic acid degradation is pH-dependent (De Angelis et al., 2003; Zhao et

al., 2017). The optimal pH for most phytases ranges between 4.0 and 6.0 (Vats & Banerjee, 2004).

I.5.3.4. Effect of lactic acid fermentation on aromatic profile of legume ingredients

Numerous studies have confirmed the improvement in sensorial properties and aromatic profile of legume protein after lactic acid fermentation. In general, the flavor of unfermented samples are associated with green and beany note for the presence of different odorant compounds (Murat et al., 2013). Indeed, lactic acid bacteria show a set of enzymatic activity which can improve the aroma perception of legume protein either by reducing the undesirable volatile compounds, or by formation of new compounds which mask the undesirable ones (Ben-Harb et al., 2019; Schindler et al., 2012). Modifications are closely related to the kind of legume, the fermenting microorganisms, and the fermentation metabolism. Blagden and Gilliland (2005) indicated that fermentation with *Lactobacillus* and *Streptococcus* strains decreased the beany flavor of soymilk due to the partial or total elimination of hexanal. Arteaga et al. (2021) showed the aroma attribute of pea protein isolate fermented with six different LAB (*L. Lactis Subsp. Cremoris, Lb. fermentum, Lactobacillus perolens, Lb. casei, P. pentosaceus, Lb. plantarum*) was significantly different from the unfermented one, since fermentation decreased the typical aroma attributes, such as pea-like, green, and earthy aromas. Xiang et al. (2022) observed a significant elimination of key volatile aldehydes originated from lipid degradation such as (*E*)-nonen-2-al, (*E*)-octen-2-al, (2*E*,4*E*) deca-2,4-dienal, (2*E*,4*E*)-nona-2,4-dienal, hexanal and nonanal for pea protein fermented with *Lb. plantarum.* They stated that these aldehydes were reduced to their corresponding alcohols. Schindler et al. (2011) stated that the more pleasant odor for fermented lupine protein with *Lb. plantarum* and *P. pentosaceus* was not specifically for the reduction of compounds responsible for off-flavor such as hexanal and pentanal. It was indeed, related to the presence of additional perceptible volatiles which may mask the off-flavors. Zheng et al. (2020) also reported the improvement of the sensory attributes of soymilk after fermentation with *Lactobacillus harbinensis* was related not only to the reduction in the hexanal content but also to the formation of desirable compounds such as acetoin and butane-2,3-dione. The main enzymatic pathways in LAB metabolism which leads to the formation of volatile compounds are the fermentation of sugar (glycolysis), the degradation of protein (proteolysis) and the degradation of fat (lipolysis) (Grujović et al., 2022) (Fig I-12).

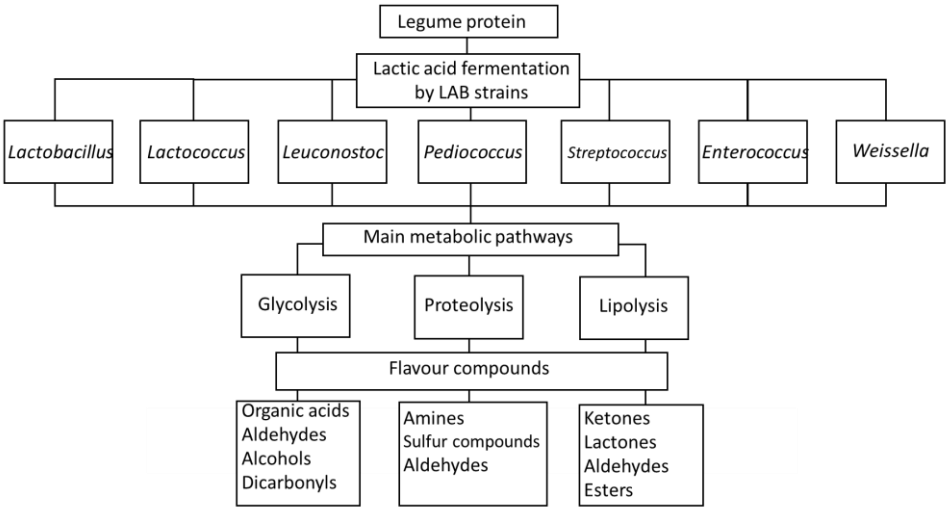


Figure I-12. Schematic of the main enzymatic pathway in lactic acid metabolism leading to the production of aromatic compounds (adapted from Emkani et al. (2022))

Lactate is the main product generated from glucose metabolism, and the intermediate fraction, pyruvate, can alternatively be converted by oxidative decarboxylation of α-acetolactate to diacetyl, acetoin, acetaldehyde, or acetic acid, which are considered important molecules in determining the flavor of legume products. Diacetyl (butane-2,3-dione) is a volatile compound, identified as belonging to the group of ketones, that positively contributes to the perception of buttery and creamy flavors in butter and some fermented milk products (Cogan & Hill, 1993; Hugenholtz et al., 2000). Ben-Harb et al. (2020) reported the presence of certain volatile compounds such as, butane-2,3-dione, only for pea protein isolate fermented with LAB and not for those fermented by the other bacteria and yeasts. Arteaga et al. (2021) studied that in fermentation of pea protein isolate with six different LAB (*L. Lactis Subsp. Cremoris, Lb. fermentum, Lb. perolens , Lb. casei, P. pentosaceus, Lb. plantarum*), among which Lb. perolens showed the highest buttery aroma which is related to the high concentrations of diacetyl. Harlé et al. (2020) reported in fermentation of soy juice with different LAB, only some strains of *S. thermophilus, Lb. plantarum, Lactobacillus pentosus, Lb. lactis* and *Lactobacillus coryniformis*, presented high level of buttery, creamy and dairy which is related to the presence of butane-2,3-dione. They also stated that the acid and cheese-like odor associated with some of these strains is related to the sucrose limitation which induces the heterolactic fermentation in lactobacilli strains.

The proteolytic activity of LAB can lead to the formation of various volatile compounds such as alcohols, aldehydes, acids, esters, sulfur compounds and so on. The amino acids released

during proteolysis are not only an important source of energy for the growth of lactic acid bacteria but also the precursors of flavor compounds via oxidative deamination and/or transamination reactions. These are called aminotransferase and/or carboxylase reactions, during which a number of volatile compounds identified in fermented products, including 3-methylbutanal and benzaldehyde, can be recognized as products of amino acid catabolism. The conversion of leucine, isoleucine, and valine takes place via transamination of the amino acids to the corresponding α-keto acids and, subsequently, via a chemical or enzymatic decarboxylation step, to 3-methylbutanal, 2-methylbutanal, and 2-methylpropanal, respectively (Ayad et al., 2001; Yvon et al., 1998). Aguirre et al. (2008) observed the generation of hydrophilic peptides during the fermentation of soy protein with 12 different lactic acid bacteria strains from *Lb. paracasei, Lb. fermentum, Lc. lactis, Lb. plantarum, Lb. helveticus, Lb. reuteri*, and *P. pentosaceus.* Hydrophilic peptides are normally correlated with desirable fermented soy flavors (Smit et al., 2005). Ben-Harb et al. (2019) reported that the presence of roasted and grilled notes in lactic fermented pea protein isolate could be related to the proteolysis of pea vicilin by LAB. It seems the hydrophobic free amino acids and hydrophilic peptides produced during the hydrolysis of vicilin by microorganisms are responsible for these specific aroma compounds (Anal et al., 2020; Crafack et al., 2013).

Lipolysis is one of metabolic pathways of LAB during which triglycerides degrade to free fatty acids, di- and mono-glycerides and glycerol. Lipase and esterase are the responsible enzymes for lipolysis. LAB are a source of lipases and esterase (Chen et al., 2017). The activity of these two enzymes can be distinguished since esterase hydrolize short chain fatty acid (2-8 carbon atoms) and water soluble substrates while lipases are active on water insoluble substrate and hydrolyze longer chain fatty acids (10 or more). The activity of these two enzymes, doesn't have any nutritional role for the bacterial growth. However, their presence lead to the release of fatty acids which are the precursors of different volatile compounds such as methylketones, secondary alcohols, esters and lactones with positive attributes such as cheese-like, creamy, floral and fruity (Dellali et al., 2020; Smit et al., 2005). The majority of LAB have intracellular esterase and they need cell lysis to have access to the food lipids. Hence their contribution to lipolysis is limited. However, esterase of LAB is able to catalyse the direct synthesis of esters through the reaction between an alcohol and organic acids (Liu et al., 1998). Yi et al. (2021) reported the reduction of the main beany flavour aldehydes in mung beans fermented with *Lb. plantarum,* is related to the conversion of aldehydes such as hexanal to hexanoic acid and further to esters through the enzymatic activity of bacteria. Liang et al. (2022) reported the

formation of higher quantity of esters in fermented mung bean flour with *L. lactis* and *Lb. casei* compare to unfermented samples. X. Zhang et al. (2022) reported the formation of esters such as heptyl formate with fruity note in fermented chickpea with *Lb. rhamnosus* could be through the enzymatic activity of the bacteria. Lactones are reported to be formed from β-oxydation of free fatty acids to 4/5-hydroxyacids and further to γ- and δ- lactones (Chen et al., 2017). Masiá et al. (2021) reported the presence of δ-decalactone, γ-octalactone for fermented soy milk with *Lb. rhamnosus*. Clearly, changes in aromatic profile of fermented samples depends highly on the type of fermenting microorganism. Schlegel et al. (2019) showed fermentation with eight different microorganisms; the use of *Lb. reuteri, Lactobacillus amylolyticus, Lb. helveticus, Lb. brevis,* and *Lb. delbruekii* noticeably decreased the "green notes" of a lupine protein isolate. Depending on the bacteria, new pleasant aroma notes were perceived. Harlé et al. (2020) reported in fermentation of soy juice with different *S. thermophilus* strains, only some of these strains were able to reduce hexanal. They also observed an increase in the level of hexanal in soy juice for some other *S. thermophilus* strains. Hence, selection of the right LAB is important to obtain the desirable properties in legume protein.

II.Objective and strategy

The principal objective of this work was to explore an alternative extraction method based on alkaline solubilization/isoelectric precipitation, in which the decrease of pH was achieved by the activity of LAB during fermentation, instead of mineral acid addition. There were three main questions to be answered in this study:

(i) Is it possible to achieve protein isolate by application of fermentation in acid precipitation step?

As it was already mentioned in the previous chapter, LAB consume a part of the substrate as a nutrient to grow and decrease the pH. Hence, the first objective here was to see whether it is possible to obtain the albumin and the globulin fractions by alkaline solubilization/isoelectric precipitation technique assisted by fermentation. The strategy was to use three different commercial starters (i.e. *S. thermophilus* (S), *S. thermophilus* and *Lb. acidophilus* (SL), *S. thermophilus*, *Lb. acidophilus* and *B. lactis* (SLB)) directly added to the solubilized protein from a commercial pea flour, and incubate them at optimal temperature until the pH reach 4.8 which is close to the isoelectric point of the globulin fraction. The effect of fermentation on protein profile and denaturation was then assessed. Total and non-protein nitrogen content of pea flour, pea protein extracts and protein isolates (albumin and globulin) were measured by Kjeldahl method. The hydrolysis of protein isolated during fermentation was characterized by quantification of free amino groups and the presence or absence of protein fractions were characterized through the electrophoresis by SDS-PAGE. Finally, the state of denaturation of protein during fermentation was measured by differential scanning calorimetry (DSC) analysis. The results obtained for the fermented samples were compared with the samples obtained by controls (addition of HCl or lactic acid).

(ii) What would be the impact of fermentation on the protein, nutritional and antinutritional factor composition of the residual pea albumin fractions?

Following the results obtained from the first question, which indicated an increase in protein content of albumin fraction, the second question was raised. Indeed, alkaline solubilization/isoelectric precipitation is a recognized method in industry for production of isolate with high yield (~60-70%). While, albumin fraction is usually discarded in industry due to inconvenient profile such as the presence of antinutritional components and lower content of protein compared to globulin fraction (Malley et al., 1975; Sell et al., 2005; Yang et al., 2022). However, this fraction could offer interesting properties, and high content of sulfur

containing amino acids compared to globulin fraction (Croy et al., 1984; Davies, 1976; Lu et al., 2000). Hence, the question here was to assess the effect of fermentation on protein profile, and nutritional and antinutritional properties of fermented albumin fraction. Pea cultivars with contrasted polypeptide composition were used to observe the effect of fermentation in possible enrichment of certain protein fractions. Fermentation here was performed by application of different LAB species either alone or in co-culture in order to observe the effect of different strains on pea protein composition. Four LAB strains were selected including *Lb. plantarum, S. thermophilus, Lb. helveticus* and *Lb. rhamnosus*. The selection of bacteria was based on their acidification capacity, their proteolytic activity, and their contribution to organoleptic properties on legume-based foods.

One strain of *Lb. plantarum* was selected because it is well adapted to pea proteins (Ben-Harb et al., 2019; Djeghri-Hocine et al., 2007) and it has been widely used in pea fermentation (Arteaga et al., 2021; Cabuk et al., 2018a; Cabuk et al., 2018b; Czarnecka et al., 1998; Li et al., 2021). Its proteolytic activity has also been reported (Byanju et al., 2021; Diana et al., 2014). Furthermore, many studies reported the improvement of organoleptic properties and reduction of beany off-flavor of pea protein fermented by *Lb. plantarum* (Schindler et al., 2012; Shi et al., 2021; Xiang et al., 2022). *S. thermophilus*, is widely used in dairy industry for its high acidification rate and its contribution to organoleptic properties (Galia et al., 2009; Iyer et al., 2010). There are also some evidences of high growth and acidification of this strain in pea substrate (Narala et al., 2022; Yousseef et al., 2014) and the positive organoleptic attributes in fermented pea protein such as creamy, dairy and sweet (Yousseef et al., 2016). *Lb. helveticus*, is an important industrial starter for having an efficient proteolytic system (Griffiths & Tellez, 2013). High proteolytic activity of *Lb. helveticus* in legume protein has been reported previously (Meinlschmidt et al., 2016; Vermeirssen et al., 2003). It also contributes to the reduction of the negative attributes like beany, bitter and astringent (Li et al., 2021; Schlegel et al., 2019). *Lb. rhamnosus*, exhibited a high proteolytic activity in pea protein isolate (Stanisavljevic et al., 2015) and pea flour (Pei et al., 2022). It is famous for its positive contribution to aromatic profile of the legume proteins (Masiá et al., 2021; Pei et al., 2022).

After preliminary tests, among four strains only *S. thermophilus* (ST) and *Lb. plantarum* (LP) were selected for fermentation and further analysis. The co-culture of these two strains (STLP) was also used to investigate the possible synergies on protein profile and properties. Three pea cultivars (Cartouche (CAR), Ascension (AC), Assas (AS)) were chosen for their differences in

polypeptide profile and protein composition. Cartouche was rich in vicilin, Ascension was rich in convicilin and Assas was rich in albumin. Total and non-protein nitrogen of three pea cultivars flours, their alkaline protein extracts and the resulting protein isolates (albumin and globulin fractions) of all the samples obtained without or with fermentation were measured by Kjeldahl method. The proteolytic activity of bacteria on protein isolates was assessed by analysis of free amino groups content of all the samples. The polypeptide profile by SDS-PAGE of protein isolates was characterized to observe the purity and presence of protein fractions. The presence of peptides (<10kDa) was measured by size exclusion chromatography (HPLC-SEC). DPPH scavenging activity and Trolox equivalent antioxidant capacity (TEAC) assay were performed to measure the anti-oxidant activity of the produced peptides by fermentation. Antinutritional compounds (trypsin inhibitors, α-glactosides and phytates) were measured in all the samples. The content of antinutritional compounds in protein isolates was then compared with pea flour.

(iii) Would the extraction method change the aromatic profile of albumin and globulin fractions?

The enzymatic activity of LAB during the metabolism of different nutrients produce precursors of volatile compounds which can positively improve the aromatic profile of legume proteins either by reducing or producing new ones. Hence, the objective here was to characterize the effect of extraction assisted by fermentation on volatile compounds of albumin and globulin fractions. Only one pea cultivar was used to obtain albumin and globulin fractions without (conventional acidification or control) and with fermentation by using different LAB strains (either alone or in co-culture). The volatile compounds were extracted by SAFE technique since it allows maximum isolation of volatile compounds, and they were identified and quantified by GC-MS. To have a complete characterization of aromatic profile of the samples, odor active compounds were characterized by GC-O.

The overall strategy and the analytical methods applied to the different samples are summarized in Fig. II-1.

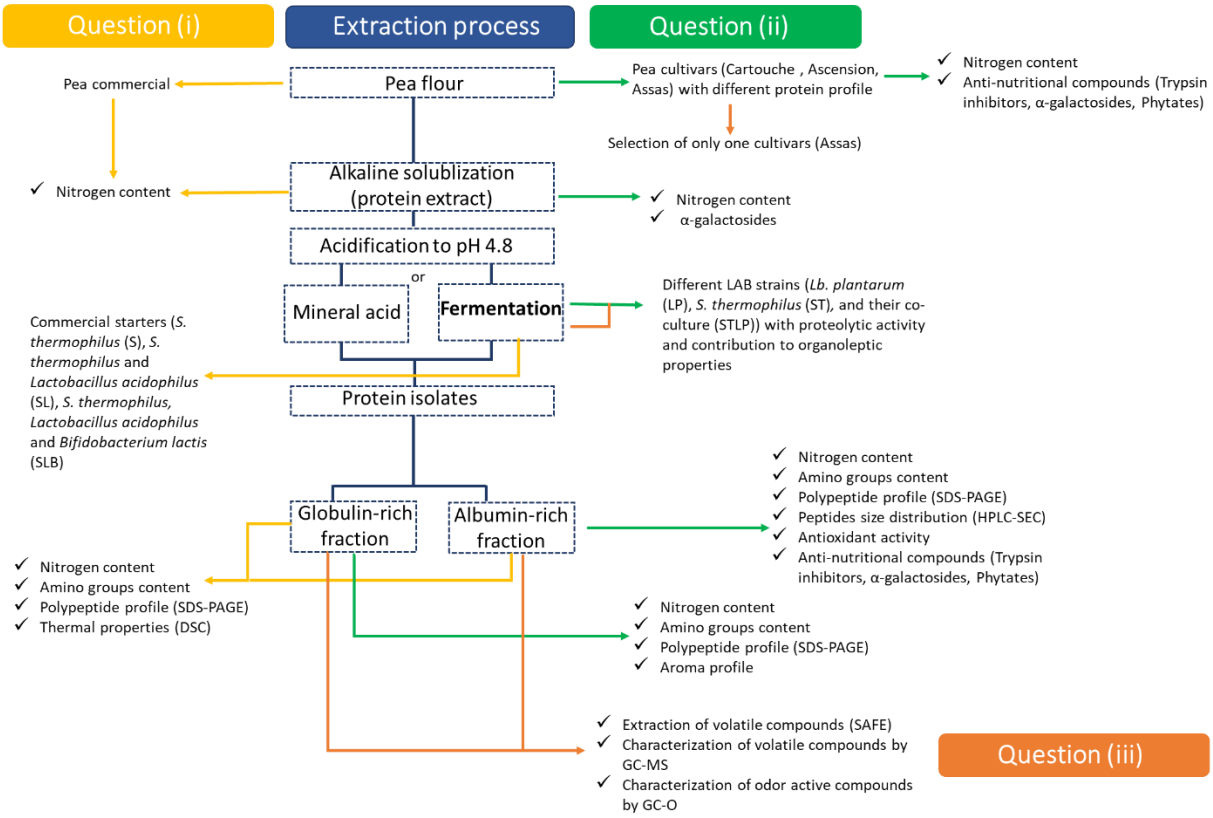


Figure II-1. General overview of research objectives and strategy

III.Materials and methods

III.1. Materials

All the materials were of analytical grade and supplied by Honeywell FlukaTM (Gillman, SA, Australia) or ThermoFisher (Dardilly, France), unless the contrary was indicated. Two different sources of pea (*Pisum sativum* L.) flour were used in this study. The primary studies were carried out on smooth yellow pea flour supplied by COSUCRA (Warcoing, Belgium. The complimentary analyses were performed on three different pea cultivars (Cartouche, Ascension, Assas) with different grain characteristics and polypeptide profiles (Appendix. Table IX-1). The cultivars were provided by INRAE (UMR Agroécologie, Dijon, France), as part of Peavalue project ANR-19-CE21-0008-03.

Commercial freeze-dried starters supplied by DuPont Danisco (Paris, France) consisted of *Streptococcus thermophilus* (S) (ChoozitTM TA 52 LYO 25 DCU), thermophilic multiple-species culture containing *Streptococcus thermophilus* and *Lactobacillus acidophilus* (SL) (YO-MIX® 101 LYO 100 DCU) and multiple species containing *Streptococcus thermophilus, Lactobacillus acidophilus* and *Bifidobacterium lactis* (SLB) (YO-MIX 202 FRO 500 DCU). Four other freeze-dried lactic acid bacteria including, *Streptococcus thermophilus* (102303T) (ST) and *Lactiplantibacillus plantarum* (CNRZ211) (LP), *Lactobacillus helveticus* (DSM20075) (LH) and *Lacticaseibacillus rhamnosus* (CNRZ212) (LR) were purchased from the International Center for Microbial Resources-food Associated Bacteria (CIRM-BIA, Rennes, France).

III.2. Sample preparation

III.2.1. Rehydration of freeze-dried LAB and preparation of bacterial stock

LAB were received freeze-dried. Rehydration was performed by adding two hundred microliters of the optimal broth medium (MRS for LP, LH, LR and M17 for ST) to the powder. Then the whole content was transferred into another tube containing 10 mL of optimal broth medium. The tube was incubated for 24 h at the optimal growth temperature which was 37 °C for LP, 30 °C for LR and 43 °C for LH and ST.

The bacteria were then plated on optimal agar medium using an inoculation loop to isolate single colonies and were incubated for 24 hours at their optimal growth temperature. The isolated colonies could be used later for experiments. The plates were stored at 5 °C up to 4 weeks. They then constituted a short-term stock of bacteria.

Bacterial cultures were prepared in liquid medium (MRS for LP, LH and LR and M17 for ST). First, a pre-culture was grown in the same medium as the main culture for physiological adaptation of the bacteria to the liquid medium and to reduce the lag time. The pre-culture was prepared by inoculating 10 mL of a fresh optimal broth medium with a single bacterial colony. It was then incubated for 24 h at the optimal growth temperature. The final culture was prepared by adding 1 mL of pre-culture to 10 mL of the same fresh broth medium. Figure III-1 represented the schematic of rehydration of freeze-dried LAB and preparation of bacteria.

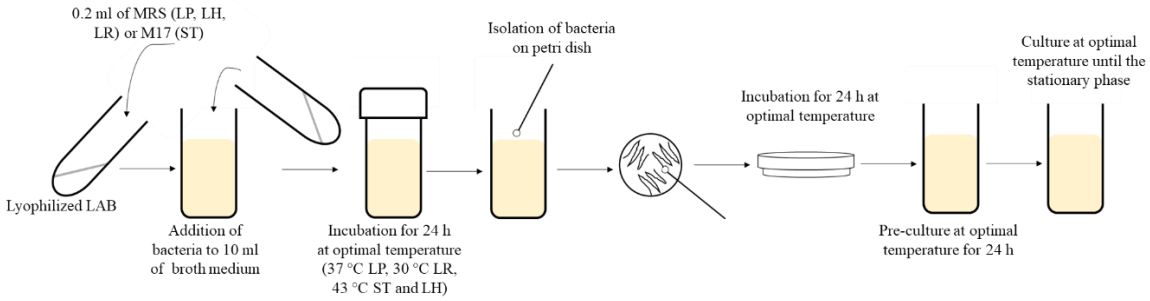


Figure III-1. Schematic of rehydration of freeze-dried LAB and bacterial preparation

To store the bacteria, a culture in liquid medium was prepared from a pre-culture. The culture was then stopped in the beginning of the stationary phase of the bacterial growth (see section III.3.1). The content was centrifuged (4,000×g, 20 °C, 5 min) and the supernatant was discarded. The pellet was resuspended in 1 mL of a fresh optimal broth medium. Then 1 mL of glycerol 30% was added to this suspension. The content was transferred into cryotubes of 1 mL and it was stored at -80 °C. These cryotubes constituted a long-term stock of bacteria since bacterial cells can be maintained at -80°C for several years. For each strain, 20 cryotubes were prepared from the first culture obtained after rehydration of the freeze-dried bacteria and plating on agar medium for isolation.

III.2.2. Bacterial culture

For the protein extraction experiments, large bacterial cultures were required and cultures obtained from a single colony of the short-term stock were not concentrated enough. Cultures were therefore prepared directly from frozen bacteria, which also had the advantage of always using the original bacteria. The contents of a cryotube were transferred to a sterile Eppendorf tube. Glycerol was removed from the bacteria by centrifugation (4,000×g, 5 min, 4 °C) and

replaced with 1 mL of fresh broth medium. The pellet was washed three times. Then the entire contents were added to 10 mL of fresh broth medium and incubated for 24 h at the optimal growth temperature of the bacteria. 1 mL of pre-culture was added to 10 mL of fresh medium and incubated until the beginning of the stationary phase. Bacteria were harvested at a cell density of 10^7 CFU/mL for mono-culture. While for co-culture, ST and LP had the ratio of 1:1 and they were added at concentration of $0.5*10^7$ CFU/mL. The bacteria were centrifuged (4,000×g, 10 min, 4°C) and they were transferred to phosphate-buffered saline (PBS) before adding to the protein solution. LH and LR were also cultivated in anaerobic condition in which the pre-culture, culture and Petri dishes were sealed in an anaerobic jar (anaerocult®A, MilliporeSigma, MA, US) and gas generation sachets (Thermo Scientific™ Oxoid AnaeroGen 3.5 L Sachet, ThermoFisher, Dardilly, France).

The commercial starters were added directly to the protein solution (see section III.2.4) in quantity at usual recommended concentrations indicated by DCU values, and conditions indicated by suppliers (see section III-1). The values, in 1.4 liter of protein suspension, were 0.119, 0.3 and 0.3 g of S, SL and SLB respectively, and at 37 °C. These commercial starters were only used in chapter IV.

III.2.3. Pea flour preparation

The seeds from Cartouche (CAR), Ascension (AC) and Assas (AS) cultivars were cracked in a Rotor Beater Mill SK300 (RETSCH GmbH, Haan, Germany) in order to coarsely break grains and separate hull. Hull was removed by blowing air. Dehulled cracked grains were milled in the same Rotor Beater Mill SK300 (RETSCH GmbH, Haan, Germany) up to particle size < 1 mm. Flours from the different varieties were then sieved (<800 µm). This method permitted a flour yield around 70% (related to initial grain weight). Hull meant 6-10% from the initial grain weight, and other losses due to grinding and handling meant 20-22% (initial weight) (data not shown). Table III-1. represents the characteristics of pea cultivars flour.

Table III-1. Dry matter, ashes and protein contents of pea flour from three cultivars (CAR, AC, AS)

Cultivars	Dry matter	Ashes	Protein content (% db)[2]
CAR	90.46±0.03 a[1]	12.53±0.46 a	19.3±0.6 a
AC	91.99±0.01 c	12.53±0.46 a	23.60±0.5 b
AS	91.48±0.06 b	13.00±0.47 a	23.99±0.8 b

[1]Different letters represent significant differences the cultivars

[2] Measured by Kjeldhal method with a nitrogen conversion factor of 5.4 (Mosse, 1990))

III.2.4. Pea protein extraction

The schema of extraction is shown in Figure III-2. Pea proteins were extracted by a modification of the traditional AEIEP method, using fermentation to decrease pH up to the isoelectric point of globulins. Hence, pea flour was mixed with water (10% w/w) and the pH of the solution was adjusted to 7.5 with NaOH (0.5 M). The suspension was then stirred overnight at 4 °C, and pH was readjusted to 7.5. Insoluble material was removed by centrifugation (10,000xg, 30 min, 20 °C) and the supernatant was collected. Acid precipitation was used to separate pea globulin and albumin fractions. The acidification was achieved by fermentation. The selected lactic acid bacteria were added to the protein solution at pH 7.5 as either mono- or co-culture (S, SL, SLB, ST, LP, STLP). The samples were then placed into an incubator (Sanyo incubator MIR-153 w, Osaka, Japan) at optimal temperature (37 °C for starters, 37 °C for LP, 43 °C for ST and 40 °C for STLP) under moderate stirring. Each fermentation was prepared in triplicate. The incubation was also performed under anaerobic condition for LH and LR in which the protein extracts containing the bacteria were placed in an anaerobic jar. The pH of the incubated pea protein was measured continuously and automatically recorded at 5-min intervals (pH meter 3310, WTW GmbH, Weilheim, Germany). The acidification was stopped at pH 4.8 which is close to the isoelectric point of the globulins. The obtained suspensions were then centrifuged (10,000×g, 30 min, 4°C) and the soluble part which corresponded to the albumin fraction was separated from the non-soluble part as pellet. Pellet contained mainly globulins together with biomass. In order to recover the globulin fraction, pellet was suspended in water (5% w/v) and the pH was adjusted to 7.5 by NaOH 0.5

M to solubilize globulins. The sample was stirred overnight and the pH readjusted to 7.5. The suspension was again centrifuged (10,000×g, 20 min, 20°C) and the supernatant was collected as the globulin fraction, free from biomass that decanted. Pea protein fractions were also obtained with conventional acidification by using hydrochloric acid (HCl) 0.5 M or lactic acid (LA) 0.5 M following the same other steps. The obtained fractions with conventional acidification were then compared with the ones obtained by fermentation. The samples were stored in a freezer at -18 °C until utilization. A part of the samples was freeze-dried (Heto PowerDry PL6000, Thermo Scientific, Waltham, MA, USA) and stored at -5 °C (maximum four weeks) for analysis such as Differential Scanning Calorimetry and Size Exclusion Chromatography. The freeze-drying conditions were the same as the one indicated by Oliete et al. (2019).

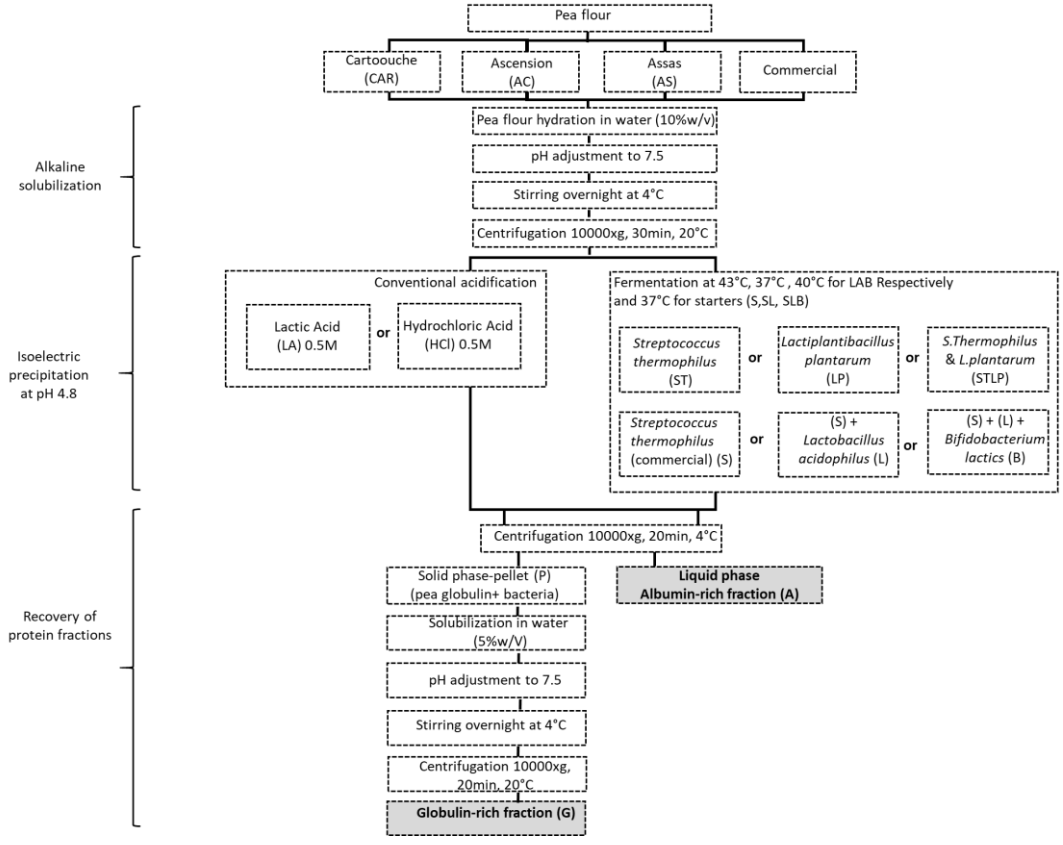


Figure III-2. Extraction strategy of albumin and globulin fractions of pea protein by using fermentation with commercial starters (*S. thermophilus* (S), *S. thermophilus* and *Lb. acidophilus* (SL), *S. thermophilus*, *Lb. acidophilus* and *B. lactis* (SLB)) and LAB strains (*S. thermophilus* (ST), *Lb. plantarum* (LP), *S. thermophilus* and *Lb. plantarum* (STLP)), or with conventional acidification with hydrochloric acid (HCL), lactic acid (LA).

III.3. Microbial analysis

For the protein extraction experiments, the bacteria added to the protein solution always had to be in the same physiological state. It was therefore necessary to obtain the growth curve of the different bacteria. In addition, the cell concentration of the bacterial suspension added to the pea proteins had to be constant. This was measured by optical density, which required matching the cell concentration, determined by counting colony forming units (CFU) on the plates, to the optical density for each strain.

III.3.1. Bacterial growth and viability

The growth curve of the four strains (ST, LH, LR, LP) was obtained by automatic optical density measurements using a plate reader (Paradigm Detection Platform, Beckman Coulter, Harbor, Oregon, USA) (Fig III-3). A fresh culture was prepared as previously described. Immediately after adding 1 mL of pre-culture to 10 mL of fresh culture medium into a sterile tube, 3 × 200 µL of the cell suspension was transferred in 3 wells of a 96-well plate, for replicates, and 3 × 200 µL of culture medium was poured into 3 other wells as blanks. The bacteria were incubated at their optimal growth temperature in the plate reader. The optical density was automatically measured every 30 minutes during 24 h.

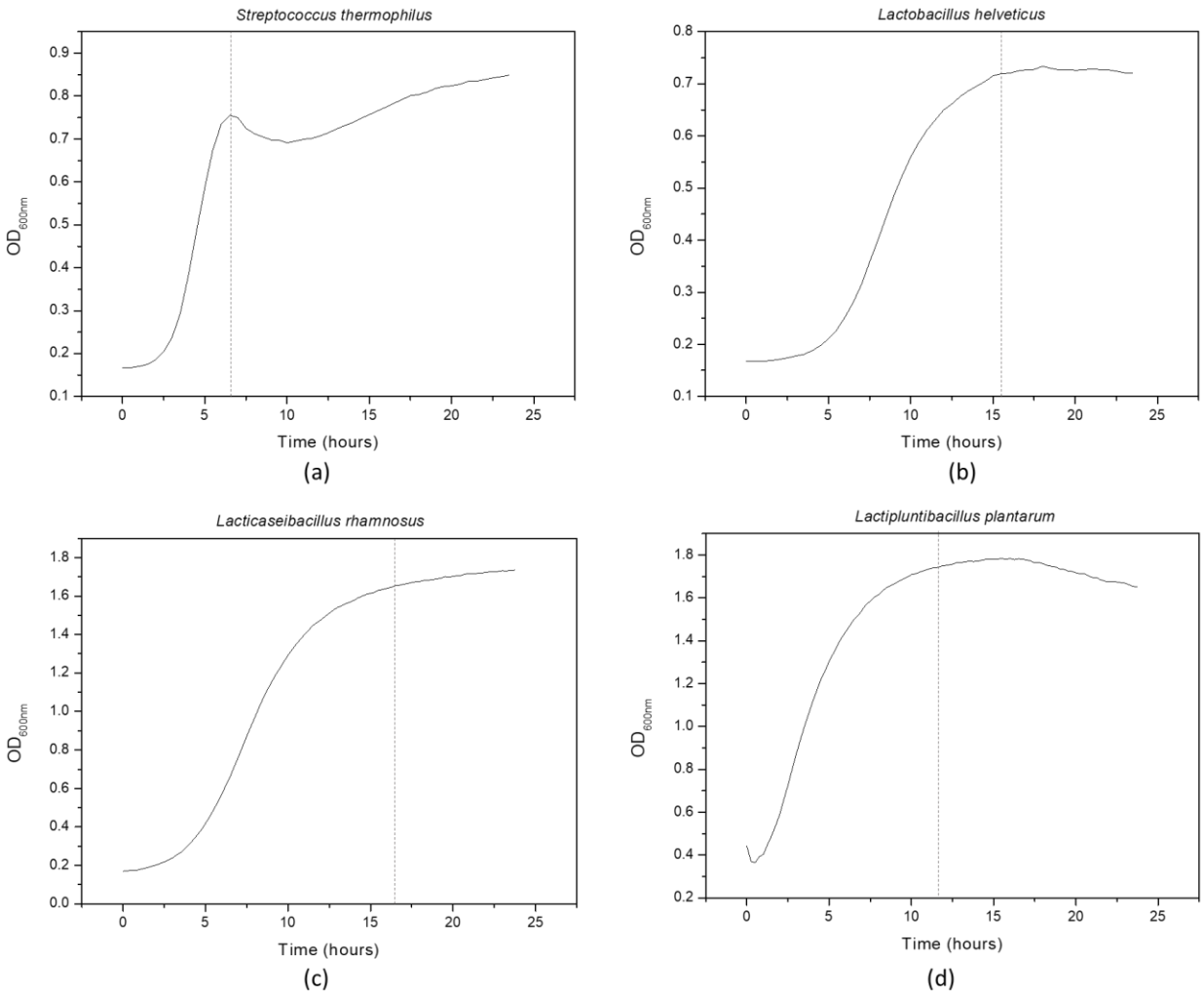


Figure III-3. The growth curves of LAB, *S. thermophilus* (ST) (a), *Lb. helveticus* (LH) (b), *Lb. rhamnosus* (LR) (c) and *Lb. plantarum* (LP) (d).

The beginning of the stationary phase was chosen for the collection of the bacterial suspension for the protein extraction experiments since it is the stage when growth ceases but cells remain metabolically active. The growth curves give information on the required growth time to collect the bacteria (approximately 7, 15, 16 and 12 h for ST, LH, LR and LP, respectively).

Meanwhile, the bacteria in the Erlenmeyer flask were incubated at their optimal growth temperature. Their growth was monitored by measuring the optical density every hour for 24 hours using a UV/VIS spectrophotometer (Biochrom LTD, Cambridge, England). This allowed to confirm the shape of the growth curve for each bacterial strain. In addition, for 2 samples, both measurement of optical density and counting of CFU were performed to match the optical density measured with this spectrophotometer to cell concentration. The equation giving the

cell concentration as a function of the optical density was obtained for each strain (results not shown).

For pea protein extraction, a sample of cell suspension was taken from a culture at the beginning of the stationary phase, according to the growth curve of the bacteria. The optical density of the cell suspension was measured using the spectrophotometer to determine the cell concentration and adjust it if necessary.

III.3.2. Acidification kinetic parameters

The acidification kinetic parameters were characterized according to Spinnler and Corrieu (1989). The evolution of pH was measured automatically at 5 min interval and carried out in triplicates. The time variation of pH (dpH/dt) was then calculated and the maximum rate of acidification (V_{max}) was expressed as pH units/h. The other kinetic parameters including; time (t_{vmax})and pH (pH_{Vmax}) at which the maximum acidification rate was observed, final pH (pH_f) at which the pH was stable, the time (t_{pHf}) and ($t_{Ph4.8}$) required to reach to pH_f and $pH_{4.8}$, respectively, were also measured.

III.4. Proximate analysis

III.4.1. Dry matter

The dry matter was determined according to the 935.29 AOAC method (AOAC, 2000). The samples (either lyophilized or liquid) were weighted and placed in the oven at 105 °C until constant weight (approximately 72 h). They were then placed in a desiccator for at least 1 h before being weighted. The dry matter was then calculated following equation III-1*:*

$$\%Dry\ matter = (mf/mi) \times 100\% \qquad \text{Equation III-1}$$

with m_i (g) being the initial weight of the sample and m_f (g) the final weight of the sample after desiccation.

III.4.2. Ashes content

The content of ashes according to the 942.05 AOAC method (Windham, 1995), with some small modifications. The samples were pre-calcinated on the laboratory hot plate. They were then placed in a Nabertherm Muffle Furnace (Lt 15/11/B180 L-150H1CN, Lilienthal,

Allemagne) at 550 °C for 5 h. After being cooled in desiccator, the residues were taken and the content of ashes were calculated following equation III-2:

$$\%Ashes = (mAshes/minitial) \times 100 \quad \text{Equation III-2}$$

In which m_{Ashes} was the mass of the residue after calcination and $m_{initial}$ was the mass of the sample before calcination.

III.4.3. Nitrogen content

The total nitrogen content of pea flour and obtained protein fractions was determined by Kjeldahl method according to the AOAC International method 920.87 (Helrich, 1990). Approximately 10-200 mg of protein samples were hydrolyzed with 10 mL of concentrated sulfuric acid (96%) and one copper catalyst tablet (Kjeltab copper mini, BÜCHI AG, Switzerland) and glass balls, in a digestion unit (SpeedDigester K-435, BÜCHI AG, Switzerland) at 400 °C for 2 h. After cooling, 25 mL of distilled water plus 1 droplet of phenolphthalein was added to the hydrolysates before neutralization and titration. The distillation and boric acid titration were performed with the BÜCHI distillation unit K-350 / K-355. Released ammonia was absorbed in solutions of 2% boric acid and nitrogen contents were determined by titration with 0.1 M HCl. The amount of total nitrogen was then calculated from the equation 3 in which V (in L) was the volume of hydrochloric acid used during the titration of samples, N was the normality of HCl and m was the weight of the samples (in grams).

$$Total\ nitrogen\ (\%) = (N \times V(L) \times 14)/m \times 100 \quad \text{Equation III-3}$$

The content of non-protein nitrogen was obtained following the method of Awolumate (1983) with a small variation. 10-200 mg of protein samples were mixed with trichloroacetic acid (TCA) 12% (1:5 ratio). The samples were kept under stirring for 15 min and then the suspensions were filtered. The content of nitrogen in the filtrate was measured as explained for total nitrogen. Finally, the amount of protein nitrogen was obtained by subtracting the non-protein nitrogen from the total nitrogen. The nitrogen conversion factor of 5.4 was used here (Mosse, 1990).

III.5. Polypeptide analysis

III.5.1. Protein composition by SDS PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

The polypeptide composition of albumin and globulin fractions was characterized by SDS-PAGE for all the extraction conditions. NovexTM electrophoresis gels at 10% to 20% Tris-Glycine were used. Samples were diluted at least half in sample buffer: 187.5 mM Tris-HCl, pH 8.9, 10% (w/v) glycerol, 2% (w/v) SDS, and 0.05% (w/v) bromophenol blue, in the presence (reducing conditions) or absence (non-reducing conditions) of 2% (w/v) dithiothreitol (DTT). The samples under reducing conditions were heated in a water bath for 10 min at 95 °C. All the samples were prepared and then deposited in the wells of the gel to have 10 µg of protein per well. Mw protein markers from Sigma–AldrichR (SigmaMarkerTM S8445, wide range, Mw 6.5 to 200 kDa) were used for all samples except the samples obtained by STLP which was from Thermofisher (Thermo Scientific™, PageRuler™ unstained broad range protein ladder, Mw 5 to 250). The migration was carried out at 35 mA per gel, with the following migration buffer: 0.3% (w/v) trizma base, 1.45% (w/v) glycine, and 0.1% (w/v) SDS, in a ScientificR Mini Gel Tank of Migration (Thermo Fisher Scientific Inc). The gels were then rinsed with distilled water, and the fixation was performed in four successive distilled water baths heated for 1 min in a microwave at 550 W. The staining of the gels was performed with Coomassie blue, Thermo ScientificTM PageBlueTM Protein Staining Solution, overnight. The discoloring was then achieved in several baths of distilled water, until the desired color. The gels were then scanned using the Odyssey infrared imaging system (LI-COR Biosciences, https://www.licor.com). Protein band detection and quantification were performed using CLIQS (TotalLab, http://www. totallab.com). In each well, the normalized volume of each band was calculated as the ratio of each band volume to the total band volume after 'rolling ball' background reduction (radius300). Each sample was analysed twice. The quantitative data (i.e. relative protein abundance) were subjected to the statistical analysis.

III.5.2. Free amino group

The content of free amino groups was measured with o-phthaldialdehyde (OPA) following the method of Church et al. (1985). The following compounds were diluted with water to 100 mL: 80 mg OPA (dissolved in 2 mL 95% ethanol); 50 mL 0.1 M sodium tetraborate buffer solution with pH 9.5; 5 mL 20% SDS; 0.2 mL of 2-mercaptoethanol. The OPA reagent was prepared immediately before use. Globulin and albumin fractions with a concentration of 10 mg

protein/mL (measured by Kjeldhal method with a nitrogen conversion factor of 5.4 (Mosse, 1990)) were prepared in phosphate buffer 0.1 M pH 7.0. An amount of 0.05 mL of the sample was added to 2 mL of OPA reagent. This solution was briefly stirred, and the absorbance at 340 nm was measured after a 2-min incubation period at room temperature. A standard curve was obtained by using leucine as a reference compound. Reference samples with a concentration ranging from 1 to 3 mM were prepared in phosphate buffer pH 7.0, and the leucine determination was performed as described above.

The content of free amino group was also measured by trinitrobenzene Sulfonic acid TNBS following the method of Adler-Nissen (1979). Briefly, globulin and albumin fractions with a concentration of 50 mg protein/mL (measured by Kjeldhal method with a nitrogen conversion factor of 5.4) were prepared in phosphate buffer 0.1 M pH 8.2, 2% SDS w/v. An amount of 250 µL of the sample was added to 2 mL of TNBS reagent (0.5 g/L) and 1.75 mL phosphate buffer 0.2 M pH 8. The TNBS reagent was prepared immediately before use. This solution was incubated for 60 min at 50 °C, and the absorbance at 340 nm was measured after addition of 4 ml HCl 0.1 M to stop the reaction. A standard curve was obtained by using L-leucine (at concentration 0-3 mM) as the control.

III.5.3. Size distribution of peptides by Size Exclusion Chromatography (SEC-HPLC)

Size distribution of the peptides in the albumin fraction produced during the lactic fermentation was determined by high pressure liquid chromatography (HPLC Shimadzu Corporation, Kyoto, Japon), equipped with an isocratic pomp (Shimadzu LC-20AT) and a UV-visible detector (Shimadzu SPD-20AV), adapted with a size exclusion column of Protein-Pak SEC Column, 60Å, 10 µm (7.8 mm X 300 mm, 500 - 20K) (Waters, Milford, MA). The column was equilibrated at 25 °C with a mobile phase including a phosphate tampon (Na_2HPO_4) 100Mm, pH 7 containing 0.3 M NaCl filtered through a 0.22 µm (Durapore® Membrane Filter, filter hydrophilic PVDF, 47 mm membrane) (Sigma Alrich, Merk SA, Darmstadt, Germany). The column was pre-calibrated by the protein standard including Insulin B chain, Leucine, Myoglobin and Cytochrome C from bovine heart, supplied by Sigma Aldrich (Merk SA, Darmstadt, Germany) to determine the elution volume. Solution of standard and freeze-dried protein samples were dissolved in filtered phosphate buffer (Na_2HPO_4) 100 mM, pH 7 containing 0.3 M NaCl with a concentration of about 1 mg/mL. The Mw fraction less than 10 kDa was separated from the protein solution by centrifugal filters (Amicon Ultra-15 Centrifugal Filter Unit, Merk SA, Darmstadt, Germany), and then they were filtered through a

syringe filter (0.45 µm, 13 mm, Restek France, Lisses, France). The standard and protein solutions were then injected (20 µl) at a flow rate of 0.1 mL.min^{-1} for 120 min. The absorbance was then measured at different wavelength 214, 280 and 254 nm. The wavelength at 254 was to assess the interference with phenolic compounds. The best results were achieved at 214 nm due to the better absorption coefficient of protein. Tests were performed in triplicate. A calibration curve was obtained over a range of 0.3 to 16.7 kDa. The calibration curve equation and the correlation coefficients were y=-0.18x+1.503, R^2=0.986. Chromatograms were recorded and processed by LabSolution LC (HPLC Shimadzu Corporation, Kyoto, Japon).

III.6. Nutritional and antinutritional compounds analysis

III.6.1 Determination of antioxidant activity

Trolox equivalent antioxidant capacity (TEAC) was analyzed in albumin fractions by total antioxidant capacity assay kit (MAK187, Sigma-Aldrich, Merk SA, Darmstadt, Germany) according to the manufacturer's instructions. The method is based on the reduction of Cu2+ to Cu+ by antioxidant molecules which give an absorbance at 570 nm. Briefly, 100 µL of albumin fractions were added to 96 well plate with clear flat bottom. Different dilutions of samples were also prepared and the volume was adjusted to 100 µL by distilled water in order to ensure that the readings are within the standard value range. 100 µL of Cu2+ reagent was added to the samples and incubated in darkness at room temperature for 90 min, and the absorbance was measured at 570 nm using a microplate reader (Paradigm Detection Platform, Beckman Coulter, Harbor, Oregon, USA). Trolox solutions ranging from 0 to 20 nmol per well were used to prepare a calibration curve. The antioxidant activity was expressed as nmol Trolox equivalents per µL sample (nmol Trolox/µL).

2,2-diphenyl-picryl hydrazyl (DPPH) free radical scavenging ability was performed according to the method proposed by Brand-Williams et al. (1995). In this assay, antioxidant compounds present in the sample reduced the DPPH• radicals, which had an absorption maximum at 517 nm. The DPPH• radical solution was prepared by dissolving 10 mg of DPPH in 25 mL of 80% methanol. First, the extinction of the disposable cuvette with 250 µL of the methanolic DPPH• solution and 2.1 mL 80% methanol was measured as blank. 100 µL of sample was then added to 250 µL of the methanolic DPPH• solution and 2 mL of 80% methanol. The mixture was shaken and allowed to stay at room temperature in the dark for 20 min. The decrease in absorbance of the resulting solution was monitored at 517 nm for 20 min using a

spectrophotometer (UV/Visible Jenway 6305, Barlo world scientific, Dunmov, UK) The results were expressed as percentage of reduction of the initial DPPH adsorption.

III.6.2. Trypsin inhibitor analysis

Trypsin inhibitory activity of pea flour and albumin fraction was determined following the method of Smith et al. (1980) and some modifications. Briefly, 10 mg of finely ground pea flour and freeze-dried albumin fraction were mixed with 5 mL of NaOH 10 mM (pH adjusted to ~9 by NaOH 1M), for 3 h at room temperature. The solution (called extract shown by V) was centrifuged (10,000×g, 30 min, 20°C) and the supernatant was separated. Trypsin (20 µg/mL) (Trypsin from bovine pancreas, Merck/MilliporeSigma, Burlington, MA, United States) solution was dissolved in Tris-HCL buffer (20 mM pH 7.5). An amount of 200 µL of prepared sample were mixed with 200 µL of trypsin and incubated for 10 min at 37 °C. The reaction started by addition of 500 µL of 1 mM Nα-benzoyl-DL-arginine-ρ-nitroanilide (BApNA) (Merck/MilliporeSigma, Burlington, MA, United States) prepared in 1% (v/v) of dimethyl sulfoxide and Tris-HCL buffer (20 mM pH 7.5). The BApNA reagent was prepared immediately before use. The assay tubes were then incubated for 10 min at 37 °C. The reaction was stopped by adding 100 µL of acetic acid 30% (v/v). It was then centrifuged at 2,000×g for 10 min. Absorbance of samples was measured at 410 nm and symbolized as As. The absorbance was compared to a trypsin standard which was determined by using the same procedure except for replacing the extracted trypsin inhibitors with water. The corresponding absorbance was symbolized as Ac. A trypsin inhibitor unit (TIU) was defined as an increase of 0.02 absorbance at 410 nm. With this definition, trypsin inhibitor activity (TIA) is defined as TIU per mg sample and it was calculated as follow:

$$TIA = TIU/mg\ sample = \{[(Ac - As/Ac)/0.02 \times V\ (mL)\}/m\ (mg) \qquad \text{Equation III-4}$$

where As was the absorbance of the sample, Ac was the absorbance of standard, V was the mL of extract (5 mL), m was the mg of sample. Trypsin inhibitor activity assay was performed in triplicate.

III.6.3. α-galactoside quantification

Oligosaccharides of the raffinose family (RFOs), consisting of raffinose, stachyose and verbascose, as well as the sucrose and D-glucose contents were measured in protein solution after alkaline solubilization, and in the albumin fractions by an enzyme-based assay kit

(Megazyme Raffinose/d-Glucose Assay Kit, Megazyme International, Ireland). The kit consisted of α-galactosidase (from *A. niger*), invertase (from yeast) and glucose determination reagent i.e. glucose oxidase/peroxidase (GOPOD) for colorimetric estimation of sucrose and RFOs content. The kit is based upon the principle to stepwise hydrolysis of complex soluble carbohydrates to glucose followed by its colorimetric measurement. Soluble sugars such as sucrose and RFOs were hydrolyzed with α-galactosidase and invertase into D-glucose, D-galactose and D-fructose. D-glucose concentration was determined using GOPOD reagent. The concentration of raffinose, stachyose, verbascose and other higher homologues of the RFOs in samples were measured as a group, because α-galactosidase hydrolyses all members of the RFO family. Since 1 mol of each of the RFO contains 1 mol of D-glucose, the RFO concentrations were presented on a molar basis (mmol/100 g sample).

Briefly, 0.5 g of each sample was treated with 95% ethanol (to digest the endogenous enzymes completely) at 85 °C for 20 min, and the final volume was made up to 50 mL using sodium acetate buffer (50 mM, pH 4.5). The obtained digested mixture was incubated at room temperature for 20 min and vortexed to obtain uniform slurry. Subsequently, 2 mL chloroform was added to 5 mL slurry obtained, and vortexed for 15 s followed by centrifugation at 1,000×g for 10 min. A volume of 0.2 mL from the aqueous phase of the supernatant was taken in three tubes (namely, A, B, and C). A volume of 0.2 mL sodium acetate buffer (50 mM, pH 4.5), 0.2 mL of invertase (8.3 U/mL) and a mixture of invertase + α-galactosidase (invertase 8 U/mL and α-Galactosidase 40 U/mL) was added into tubes A, B, and C, respectively. All three tubes were incubated at 50 °C for 20 min. Reagent blank (0.4 mL sodium acetate buffer) and glucose control (0.1 mL standard glucose solution, which contained 0.556 μmol of glucose + 0.3 mL sodium acetate buffer) were also taken simultaneously. Subsequently, 3 mL of GOPOD reagent was added in all of the tubes and incubated again at 50 °C for 20 min. The glucose concentration for tubes A, B, and C and glucose control was determined by measuring the change in absorbance at 510 nm against the reagent blank using a spectrophotometer (UV/Visible Jenway 6305, Barlo world scientific, Dunmov, UK). Glucose, sucrose and RFOs concentrations were shown in mmol/100 g sample. The concentrations of glucose, sucrose and RFOs were calculated as follows:

$$Glucose\ (mmol/100\ g) = \Delta A \times F \times 250 \times 200 \times 1/1000 \qquad \text{Equation III-5}$$

$$Sucrose\ (mmol/100\ g) = (\Delta B - \Delta A) \times F \times 250 \times 200 \times 1/1000 \qquad \text{Equation III-6}$$

$$RFOs\ (mmol/100\ g)\ =\ (\Delta C - \Delta B)\ \times\ F\ \times\ 50\ \times\ 250\ \times\ 200 \times\ 1/1000$$ Equation III-*7*

where ΔA, ΔB and ΔC were the absorbance of sample plus sodium acetate buffer, sample plus invertase and sample plus invertase and α-Galactosidase enzyme solution, respectively

F = Factor to convert from absorbance to μmol of glucose= 0.556 (μmol of glucose) / GOPOD absorbance for 0.556 μmol of glucose

250 = conversion to 50 mL of extract

200 =conversion from 0.5 to 100 g of sample

and 1/1000 = conversion from μmol to mmol.

All enzymatic assays were performed in three technical replicates (n = 3) for each sample. The consumption patterns of D-glucose, sucrose and RFOs in albumin fraction of samples obtained without fermentation (control) and with added fermentation by ST, LP and STLP were shown as a ratio calculated from the content of these sugars in protein extract.

III.6.4. Phytic acid content

The determination of phytate content of pea flour and albumin fractions was done according to the method developed by Davies and Reid (1979) with some modifications. Briefly, 0.5 g of pea flour and freeze-dried albumin samples were mixed with 20 mL of HNO_3 0.5 M and the suspensions were put under continuous stirring for 3 h. Each sample was then filtered by Whatman No.1 filter paper to obtain the extract. The stock solution of ferric ammonium sulphate (FAS) (2.16 mg/mL) was prepared freshly. The working solution was prepared by diluting one volume of stock solution to 24 volumes of distilled water. 0.2 mL of the previous extract was mixed with 0.2 mL of the working solution of FAS in a test tube. Then the test tube was kept in a boiling water bath for 20 min. After the tube cooled to the room temperature, 1 mL isoamyl alcohol was added to the tube followed by 0.02 mL of ammonium thiocyanate (5 g/50 mL). The tube was centrifuged (3,000×g, 10 min). Finally, the intensity of the color in the isoamyl alcohol layer was determined at 465 nm using a spectrophotometer against an isoamyl alcohol 'blank', exactly 15 min after addition of the HN_4CNS. Since the principle of this method is based on an indirect measurement of phytic acid. Hence, the idea is to precipitate the ferric ion complex with phytate at acidic pH. The excess of ferric ion will later make characteristic pink complex with thiocyanate ion. The extinction at 465 nm in the amyl layer

is inversely related to the phytate anion concentration. The phytate content can be obtained by reference to a calibration curve prepared with the same quantities of iron, thiocyanate and acid, and a standard phytate preparation. The standard stock solution was prepared by dissolving 50 mg sodium phytate in 20 mL distilled water and making the final volume to 100 mL with distilled water. The working solution was of 0.5 mg/mL concentration. Under these conditions an inverse linear relationship was found over a range of 0 to 500 µg of phytate.

III.7. Aromatic profile

The aromatic profiles of albumin and globulin fractions obtained for fermented and control samples were characterized by Gas Chromatography-Mass Spectroscopy (GC-MS) and Gas Chromatography (GC)-Olfactometry. Only one pea cultivars (AS) was used for the aromatic profile study.

III.7.1. Extraction of the volatile compounds

The volatile compounds of albumin and globulin were extracted by solvent assisted flavor evaporation (SAFE) technique as previously described by Murat et al. (2013), Karolkowski et al. (2023). Sample preparation was started with mixing 70mL of ultra-pure water (MilliQ system, Millipore, Bedford, MA, USA) with 200 µL of an internal standard (hexanal D12 in water, 109.8 ng/µL) (CAS 66-25-1; 96% pure; CDN Isotopes, Cluzeau Info Lab, Sainte Foy La Grande, France) for 30 min on ice. Then 50 g of sample were added and stirred for 10 min. The mixture was then transferred to the dropping funnel of SAFE apparatus (described by Engel et al. (1999)) where the entire sample was allowed to flow into the flask over 40 min at 8×10^{-3} mbar, and vacuum distillation (5×10^{-2} mbar) was performed for 45-75 min at 30 °C under stirring. The resulting frozen hydro-distillate was reached to the room temperature. Then after, the liquid extraction was performed with dichloromethane (CH_2Cl_2) as the solvent (Carlo Erba, Val de Reuil, France; purity > 99.9%, distilled just before use). Three successive 15-min extraction steps were carried out under stirring using 3 × 15 mL of CH_2Cl_2 in a water-ice bath and the recovered organic extracts were pooled and dried over anhydrous Na_2SO_4 (4 g). The extract was then filtered through glass wool before being concentrated to 300 µL (the adjusted volume obtained using CH_2Cl_2) by using two successive Kuderna-Danish apparatuses (Merck, Darmstadt, Germany) of decreasing size equipped with a Snyder column. The extracts were obtained in triplicate for each fraction sample and stored at -20 °C before analysis.

III.7.2. Separation and identification of volatile compounds by GC-MS

A 1-µL volume of each SAFE extract was analysed by liquid injection into an Agilent 7890A GC (Hewlett-Packard, Palo Alto, Santa Clara, USA) equipped with a fused-silica capillary DB-Wax column (30 m x 0.25 mm id., 0.5-µm film thickness) (Agilent J & W, Santa Clara, USA) with a constant helium flow of 1.2 mL/min. The oven temperature was held at 40 °C and then programmed to increase to 240 °C at a rate of 4 °C/min and held at this temperature for 10 min. The volatile compounds were identified using a 5975C mass selective detector (MSD) (Agilent J & W, Santa Clara, USA). Mass spectra were taken at 70 eV over a scan range between 29 and 350 amu. Data were acquired using ChemStation software (ver. A.03.00, Agilent J & W, Santa Clara, USA). The volatile compounds were identified by comparing their mass spectra with mass spectra libraries (the Wiley 138, NIST, and INRAE databases achieved using standard compounds) and comparing the liner retention index (LRI) calculated using a series of alkanes (C10 to C30) with the LRI from published data (NIST, 2022). Moreover, when standards were available in our collection of aroma compounds, their mass spectra and LRIs obtained on an equivalent DB-Wax column were used to confirm the identification of the volatile compounds. Semi-quantitative data for each compound was obtained by automatic or manual integration of the total ion count (TIC) peak area (arbitrary units) and expressed as a concentration (µg/mL of sample) based on the concentration of a hexanal D12 standard of 109.8 ng/µL.

III.7.3. Identification of odor-active compounds by GC-Olfactometry

For each fraction, the 3 SAFE extracts were mixed to obtain a homogenous sample. Two microlitres of the final extracts were injected into an Agilent 6890A GC (Hewlett-Packard, Palo Alto, Santa Clara, USA) coupled to a FID and an olfactometric detection port (ODP) that constituted the GC-O system. The same column (fused-silica capillary DB-Wax) and chromatographic conditions as GC-MS analysis, was used for GC-O. The column was equipped with a Y-splitter to divide the effluent between the FID and ODP (in a 1:1 ratio). Humidified air (25 mL/min) was added to the transfer line to prevent nasal mucosa dehydration. Linear retention indices (LRIs) were calculated by a weekly injection of a reference solution of n-alkanes (C10 to C30; Sigma-Aldrich) according to Van Den Dool and Kratz (1963).

This study was performed following the relevant institutional and national regulations and legislation (Inserm (French National Institute for Health and Medical Research) Ethic

Evaluation Committee, N°21-773, approved the 09/02/2021). Participants were requested to sign an informed consent form and were not informed of the aims of the experiment. They were compensated €10 per hour. Nine assessors who were both sensitive to odor and experienced in the use of GC-O described the odor of the detected area. During a 40-min analysis, the assessors were asked to describe the perceived odor in their own words. Data were acquired using Openlab software (6850/6890 GC system, V2.3, Agilent Technologies) for the chromatography study and an ODP recorder (Gerstel, Mülheim an der Ruhr, Germany) for the descriptive study (a button and a microphone were used to record the description of odor events). The data were processed based on the detection frequency (DF) method following Pollien et al. (1997). Odor (OAs) areas were characterized in terms of (i) the number of assessors that simultaneously detected an odor-active compound at the ODP (corresponding to the DF); (ii) the odor described by the entire panel in accordance with the literature (INRASnif (internal database), VCF online (www.vcf-online.nl), The Good Scents Company (www.thegoodscentscompany.com) and Fenaroli's Handbook of Flavor Ingredients (Burdock, 2016) and (iii) the LRI for the column (NIST, 2022). An OA was determined to be odor-active if the DF was higher than 44% ($\geq$ 4/9 assessors) (Deuscher et al., 2020; Paravisini et al., 2012). The descriptors were assorted to 9 odor classes of lactic, fruity, floral, chemical, mushroom, grilled, green, potato and metallic, based on literature (Chigwedere et al., 2022; Karolkowski et al., 2023; Roland et al., 2017)

III.8. Thermal properties

Thermal properties of the albumin and globulin fractions were studied by differential scanning calorimetry (DSC). Onset temperature (T_{onset}), temperature of denaturation (T_d) and enthalpy of denaturation (ΔH_d) were determined using a MicroSC-4c microcalorimeter (SETARAM instrumentation, Caluire, France). Obtained protein solutions at 5% (w/w) (protein concentration measured by Kjeldhal method with nitrogen conversion factor of 5.4) were weighed in an aluminum pan, hermetically sealed and heated from 20 to 110 °C at 5 °C/min rate. Another pan filled with water served as reference. The experiments were repeated at least 3 times.

III.9. Statistical Analysis

All the statistical data treatments were performed using the software packages XLSTAT (Addinsoft, Paris, France) and/or Statistica (ver. 13.3, TIBCO Software Inc., Tulsa, OK, USA).

Comparison between the samples obtained by commercial starters and conventional method (chapter IV) and the effects of fermenting bacteria and pea cultivar (chapter V) were studied by one-way analysis of variance (ANOVA) using Statistica software. The significant differences in the volatile compounds content between the samples obtained without or with fermentation was evaluated by by one-way analysis of variance (ANOVA) using XLSTAT software (chapter VI). Tukey's post hoc least significant differences method was used to describe means with 95% confidence intervals.

Principal component analysis (PCA – Pearson correlation) was performed on the volatile compounds quantified by GC–MS as the variable and their chemical classes as supplementary variable. Any volatile compound that corresponded to at least one co-elution was excluded from the samples. Moreover, only volatile compounds with significantly different concentrations ($p < 0.05$; Tukey's test) were considered in the construction of the first PCA. Another PCA (Pearson correlation) was performed on the 9 odor classes to highlight differences in odor attributes between the samples obtained without or with fermentation. For each odor class, the DF of volatile compounds higher than 44% ($\geq 4/9$ assessors) (a percentage that defined a compound as odor-active) was summed. Agglomerative hierarchical clustering (AHC) was performed to group the samples based on their dissimilarities in volatile compounds and odor active compounds. The dissimilarity matrix was performed on the basis of the Euclidean distance with a Ward's method of aggregation. PCA and AHC were both performed by using XLSTAT

IV. Pea protein extraction assisted by lactic fermentation: Impact on protein profile and thermal properties

IV.1. Introduction

The popularity of legumes is growing as consumers increasingly demand healthier and novel sources of protein, which can totally or partially replace those from animals (El Youssef et al., 2020). Although animal-based foods might be considered as optimal in essential amino acid composition, their consumption is associated with health problems and environmental issues. Therefore, it is urgent to find alternative sources of protein that are available, cost-effective and more promising for both human health and environment. Legumes are a possible solution that contribute toward sustainable and healthier diet (Pihlanto et al., 2017). Among various legume proteins, pea protein is of great interest for its worldwide production, high-quality amino acids and low allergenicity (Lam et al., 2018). However, the consumption of pea protein is still limited by its imperfect techno-functional properties, low solubility and low accepted sensory profile (Barać et al., 2015).

In fact, pea is a great source of protein, approximately 20–30% of total dry seed (Koyoro & Powers, 1987). It can be consumed either as a part of grain components (e.g., flour milled from grains) or as enriched protein ingredients such as protein concentrate (50–55%) and protein isolate (80–90%) (Barać et al., 2015; Boye et al., 2010). The majority of pea proteins consists of albumins (15–20%) and globulins (60–70%) (Crévieu et al., 1996). These two fractions can be purified, since they have different solubility. Albumins are water soluble, while globulins are salt soluble. The two main proteins of the albumin fraction are PA1 and PA2 belonging to the 2S group (Lu et al., 2000). PA1 molecules have a Mw of approximately 4–6 kDa (Gressent et al., 2011). PA2 proteins have a Mw of about 24–26 kDa and are thought to associate non-covalently to form homodimers of 48–53 kDa (Gruen et al., 1987). Both 2S pea albumins have a high sulfur amino acid content compared to globulins (Higgins et al., 1986). The three abundant pea globulins are legumin, vicilin and convicilin with an isoelectric point (pI) close to pH 4.8. Legumins belong to the 11S group and have a multimeric structure with a high Mw of 360–400 kDa. A mature legumin consists of six subunit pairs (~60 kDa) that interact non-covalently. Each of these subunit pairs consists, in turn, of an acidic subunit of ~40 kDa and a basic subunit of ~20 kDa, linked by a single disulfide bridge. Vicilin is a trimer of 150–200 kDa. It lacks cysteine, so it cannot form a disulfide bond. Each monomer (~50 kDa) has two cleavage sites, which make possible the post-translational formation of various polypeptide fragments of about 20 kDa (α), 13 kDa (β) and 12–16 kDa (γ), 30–36 kDa (α + β) and 25–30 kDa (β + γ). Finally, convicilin subunits (~70 kDa) are thought to associate non-covalently in

a multimer of 210–280 kDa (Liang & Tang, 2013; O'Kane et al., 2004). The variety in structures and conformations of pea proteins affects both their functional and nutritional properties. For instance, vicilin has less compact structure, thus, it is more readily extractable and offers better functional properties than legumin (Dagorn-Scaviner et al., 1986). However, the low content of sulfur amino acids in vicilin make it less interesting from a nutritional point of view (Tzitzikas et al., 2006).

Nutritional, sensory and technological properties of pea proteins can be influenced by the extraction process (Gao et al., 2020). That is why food researchers and the industry are constantly trying to develop new cost-effective and safe extraction methods with optimal extractability leading to adequate techno-functional and sensory properties (Stone et al., 2015b). Indeed, numerous methods have been proposed for the extraction of protein from pea flour including alkaline extraction-isoelectric precipitation (AEIEP), salt extraction-dialysis, micellar precipitation and aqueous extraction (pH > 7) (Lam et al., 2018). However, each method might be selected for different protein type, which in turn, influences the final composition and functionality of the protein isolate (Stone et al., 2015b). AEIEP is a common technique with high yield for producing pea protein isolates in the food industry. The method aims at separate albumins and globulins thanks to differential solubility. The reason is the high solubility of both legumin and vicilin at alkaline pH, but minimal solubility at their pI while the albumin part remains soluble (Gao et al., 2020). This process starts with the solubilization of protein by suspending pea flour in water at alkaline pH (~8). It then follows by the isoelectric precipitation step in which the pH of the protein-rich extract is lowered up to ~4.5–4.8 by the addition of mineral acid in order to reach pI of the globulins. Then, the precipitated globulins can be neutralized and dried to produce protein isolate as powder.

Food processing including physical, chemical and biological (i.e., fermentation, germination) process is also determinant in controlling protein composition and properties. Fermentation is a traditional technique that serves as a practical method for food preservation (Holzapfel, 2002; Matejčeková et al., 2019). It has been used to enhance the bioaccessibility and bioavailability of nutrients (Cabuk et al., 2018a; Rui, 2019; Stanisavljevic et al., 2015; Xing et al., 2020) or to improve the organoleptic properties (Meinlschmidt et al., 2016; Schindler et al., 2011; Schlegel et al., 2019; Yousseef et al., 2016) and shelf life of various legume proteins. Fermentation consists of modifying food by microorganisms (bacteria, molds and yeasts) that grow and consume part of the substrates and enrich it with the products of their metabolism (Fernandez-

Orozco et al., 2007). However, selection of the right microorganism is necessary, since some microorganisms including yeasts and molds might concern food safety (Nkhata et al., 2018). Lactic acid bacteria (LAB) with the generally recognized as safe (GRAS) status are of great interest in food fermentation (Klupsaite et al., 2017). They are known for contributing to the improvement of desired sensory properties and improvement of food's aroma (Coda et al., 2015). LAB have been increasingly used for legume fermentation in the last decade. However, its effect is highly related to the legume type, LAB strain and fermentation conditions (Bartkiene et al., 2015; Rui et al., 2017). Lactic acid fermentation can affect the structure and content of legume protein. This can be attributed to the proteolytic activity of bacteria mechanism during fermentation (Meinlschmidt et al., 2016), by which the polypeptide chain is broken down, and new polypeptides with a lower Mw are formed (Lampart-Szczapa et al., 2006; Schlegel et al., 2019). The changes in protein conformation and structure alter the functionality and nutritional properties of the final products (Masiá et al., 2021; Sozer et al., 2019). The LAB species such as *S. thermophilus*, *Lb. bulgaricus, Lb. acidophilus*, Lb. helveticus and Lb. plantarum have been frequently reported for their positive effects on the organoleptic properties of legume protein (Yousseef et al., 2016). The development of LAB during pea protein fermentation helps the improvement of aroma and flavor by either reducing the occurrence of compounds responsible for off-flavor or masking undesirable green notes (Ben-Harb et al., 2019). LAB fermentation is also an effective way for partial or complete degradation of antinutritional factors and improvement of protein bioavailability and digestibility (Cabuk et al., 2018b; Coda et al., 2015; Czarnecka et al., 1998; Xing et al., 2020).

Taking into account the positive effects of LAB fermentation on the legume properties and the drop in pH due to lactic acid formation, the aim of the present study was to explore an alternative extraction method of pea proteins based on alkaline solubilization/isoelectric precipitation, where the decrease in pH was achieved by lactic fermentation instead of mineral acid addition. Three different commercial LAB strain or starters were selected for their aptitude for acidification and/or their recognized positive effect on legume protein properties: *S. thermophilus* (S), *S. thermophilus* + *Lb. acidophilus* (SL) and *S. thermophilus* + *Lb. acidophilus* + *B. lactis* (SLB). The fermentation-assisted extraction was expected to modify the protein profile of the globulin and the albumin fractions isolated with this process. To evaluate this effect, extraction yield and polypeptide profile of the albumin and globulin isolates were evaluated by comparison to unfermented systems produced by common AEIEP. Free amino

group content and thermal properties of the samples were further analyzed to check the denaturation of proteins.

IV.2. Results and discussion

IV.2.1. Acidification kinetics

Figure IV-1. shows the typical acidification kinetics of the cultures used (S, SL, SLB). Pea protein extract was a good substrate for the strains, as they were all able to reduce the pH up to 4.8. As pea seeds contain a part of fermentable sugars (a-galactosides and sucrose) (Wang & Daun, 2004), it is likely that these sugars were also solubilized in the extracted protein suspension in a non-limiting amount for bacteria growth. It is well known that many LAB strains possess enzymes to metabolize these sugars into lactic acid (Gänzle & Follador, 2012). Otherwise, the time required to reach the target pH was shorter for the mixed cultures. S showed the largest lag phase compared to the mixed cultures. It changed from lag phase to log phase in 5 h and then reached the stationary phase after 4 h. SLB had the highest growth rate in pea protein substrate. It took about 3 h for it to reach log phase and 2.5 h to reach the plateau. SL showed the same behavior as SLB, although it reached the stationary phase after approximately 3 h growth. Indeed, the acidification rate in lactic fermentation highly depends on the strains and the bacteria mixture (Boulay et al., 2020; Yousseef et al., 2016). For instance, fermentation of soymilk with different strains of *Lb. acidophilus* and *B. lactis* revealed a sharp difference in the rate of acid production (Blagden & Gilliland, 2005; Murti et al., 1993). Furthermore, the synergistic effect of combined bacteria strains could cause an accelerated and efficient organic acid production during fermentation process itself, as already indicated by Matejčeková et al. (2019). The importance of such synergy on acidification activity of lactic acid bacteria has been studied extensively. Wang et al. (2002) showed that lower pH and higher titrable acidity were obtained with mixed culture of *Streptococcus* and *B. lactis* compared to *S. thermophilus* alone. This was confirmed by de Souza Oliveira et al. (2012), who reported that the mixed cultures of *S. thermophilus* and *B. lactis* had better acidification profile than their mono-cultures. Helland et al. (2004) also showed that *B. lactis* had better growth in co-culture fermentation with *Lb. acidophilus*. Moon and Reinbold (1976) also compared the developed acidity of mixed and pure cultures. The authors reported that the growth of *S. thermophilus* increased in mixed culture with *Lb. acidophilus*. Garcia et al. (2020) showed the incorporation of *S. thermophilus* with *Lactobacilli* strains led to higher acidification and significant reduction in the pH. Besides,

S. thermophilus is always the dominant species in the mixed cultures, with the greatest growth regardless of the substrate (Božanić et al., 2008).

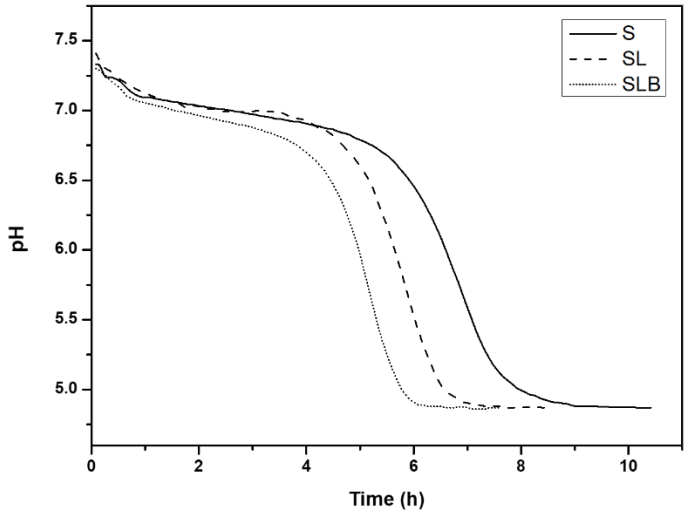


Figure IV-1. Acidification kinetics of bacteria (*S. thermophilus* (S), *S. thermophilus* + *Lb. acidophilus* (SL), *S. thermophilus*+ *Lb. acidophilus* + *B. lactis* (SLB)) during the acid precipitation step of pea protein extraction

IV.2.2. Nitrogen content in albumin and globulin fractions

Total nitrogen (protein + non-protein nitrogen) content of globulin and albumin fractions obtained by both lactic fermentations and controls is shown in Figure IV-2. As illustrated, globulin fractions obtained by controls (GHCl and GLA) showed higher total (~16%) and protein nitrogen content (~12%) than the fermented samples (GS, GSL, GSLB) (~14.5% and 9.5%, respectively). The majority of nitrogen was measured as protein nitrogen (Figure IV-2 a). The non-protein nitrogen content for globulin fractions (~4%) did not differ significantly in all the samples, representing ~25–30% of the total nitrogen as already reported (Gueguen & Barbot, 1988). In the case of albumin fractions, the trends observed for total nitrogen or protein nitrogen between controls and fermented samples were reversed (Figure IV-2 b). Lactic fermented samples had higher values for both total (~11%) and protein nitrogen content (~7%), as well as non-protein nitrogen (~4%) compared to controls (~9%, 6% and 2%, respectively). However, this difference was significant only for total and non-protein nitrogen. Changes in nitrogen content of globulin and albumin factions when fermentation was used might be attributed to a combined effect of enzymatic proteolysis and acid-induced hydrolysis, implied by bacteria metabolism (Meinlschmidt et al., 2016). Indeed, during lactic fermentation, bacteria consumed part of the present nutrients for biomass production and eventually break the

polypeptide chains to smaller polypeptides and amino acids (Aguirre et al., 2008). During this metabolism, bacteria also produced organic acid (i.e., lactic acid) as a side product, which will interrupt the ionic interaction between side chains (Lampart-Szczapa et al., 2006). Changes in conformation and size of globulin would favor their solubility at pH 4.8, and thus, they would be released in the albumin fraction. Furthermore, extensive hydrolysis of protein would produce amino acids that will increase the non-nitrogen content of the albumin fraction. This could explain the increase in the total and non-protein nitrogen contents in albumin fractions for fermented samples compared to the controls and the decrease in total and protein nitrogen contents in globulin fractions, respectively. Increase in legume protein content by lactic fermentation was reported previously. However, previous authors did not study the changes in each fraction (Lampart-Szczapa et al., 2006; Rui et al., 2017).

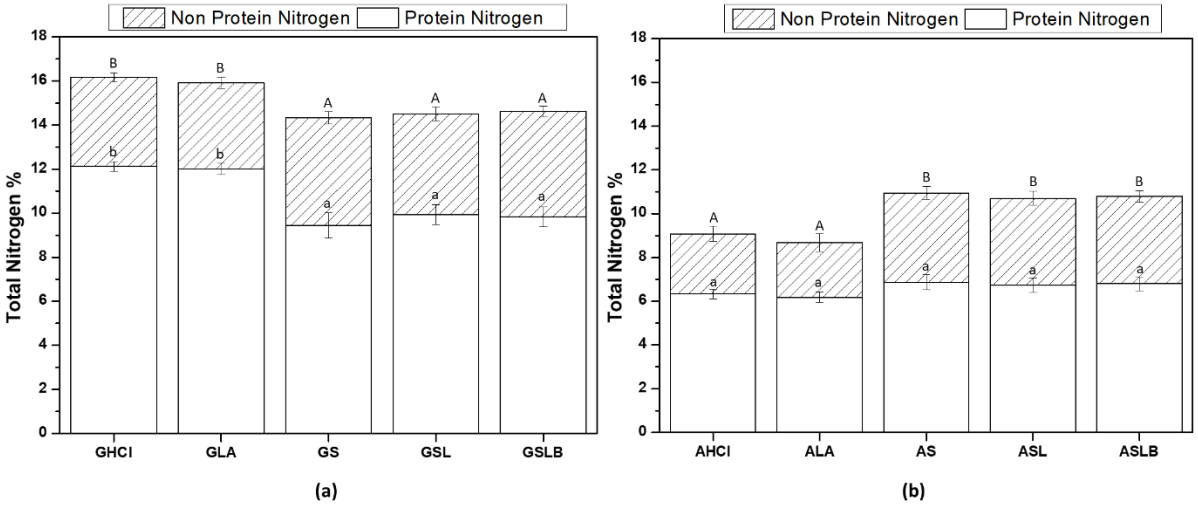


Figure IV-2. Total nitrogen (non-protein and protein nitrogen) content of globulin (G) (a) and albumin (A) (b) fractions obtained by controls (HCl, LA) and fermentations with *S. thermop*hilus (S), *S. thermophilus* + *Lb. acidop*hilus (SL), *S. thermophilus*+ *Lb. acidophilus* + *B. lactis* (SLB). Different capital letters represent significant differences in total nitrogen content and small case letters represent significant differences in protein nitrogen among different samples (Tukey's post hoc test).

IV.2.3. Nitrogen extraction yield

The nitrogen extraction yields of all fractions was shown in Figure IV-3. Lactic fermented samples showed higher yields for albumin fraction and quite smaller for globulin one compared to the controls. These data confirmed the same trends as observed for nitrogen content in albumin and globulin fractions. Moreover, the results indicated no significant difference in

extraction yield neither between controls (HCl and LA), nor between fermented samples. The sum of globulin and albumin fraction was inferior to 100% in all the samples showing a reduction in yield of approximately 15%. It was probably the result of removing insoluble globulins and/or biomass after the final centrifugation applied to obtain the globulin suspension. For fermented samples, it could be hypothesized that biomass incorporated a significant amount of nitrogen or more likely that part of globulins was adsorbed at the surface of bacteria cells and were de-canted simultaneously.

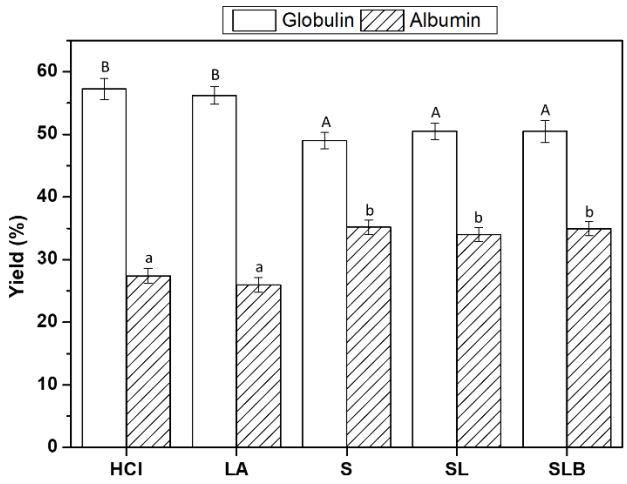


Figure IV-3. Nitrogen extraction yield of globulin and albumin fractions obtained by controls (HCl, LA) and fermentations with *S. thermophilus* (S), *S. thermophilus* + *Lb. acidophilus* (SL), *S. thermophilus*+ *Lb. acidophilus* + *B. lactis* (SLB). Different capital letters represent significant differences in nitrogen extraction yield between globulin fractions and small case letters represent significant differences between albumin fractions (Tukey's post hoc test).

IV.2.4. Free amino groups content

The content of the free amino groups (-NH_2) was determined by the standard method with OPA (Figure IV-4). All the fermented samples (for both albumin and globulin fractions) revealed higher amino groups content compared to the controls. An increase in the content of the free amino groups for fermented samples could be explained by the proteolytic activity of bacteria during fermentation probably releasing smaller polypeptides (<10 kDa), peptides and free amino acids (Montemurro et al., 2019; Rui et al., 2019; Vermeirssen et al., 2003; Verni et al., 2017; Xu et al., 2019). Although the proteolytic activity and acid production capacity of the bacteria are known to be strain-dependent (Rui et al., 2019), no significant differences were observed between fermented samples in the present work. The value of the free amino group

in albumin fraction (Figure IV-4 b) was about tenfold higher for fermented samples compared to the globulin fractions, indicating that the smaller molecules produced were primarily present in albumin fraction as a soluble part. Free amino groups in the globulin fraction would correspond to those retained by insoluble protein and/or biomass when decreasing pH up to 4.8. The results obtained for the free amino group were consistent with the data measured for nitrogen content and extraction yield. An increase in the nitrogen content of albumin fractions in fermented samples could be explained by the enrichment of the extracts by soluble polypeptides and peptides originating from proteolysis. On the other hand, a decrease in nitrogen content in fermented globulin fractions could be attributed more likely to the elimination of some globulin polypeptides with the biomass.

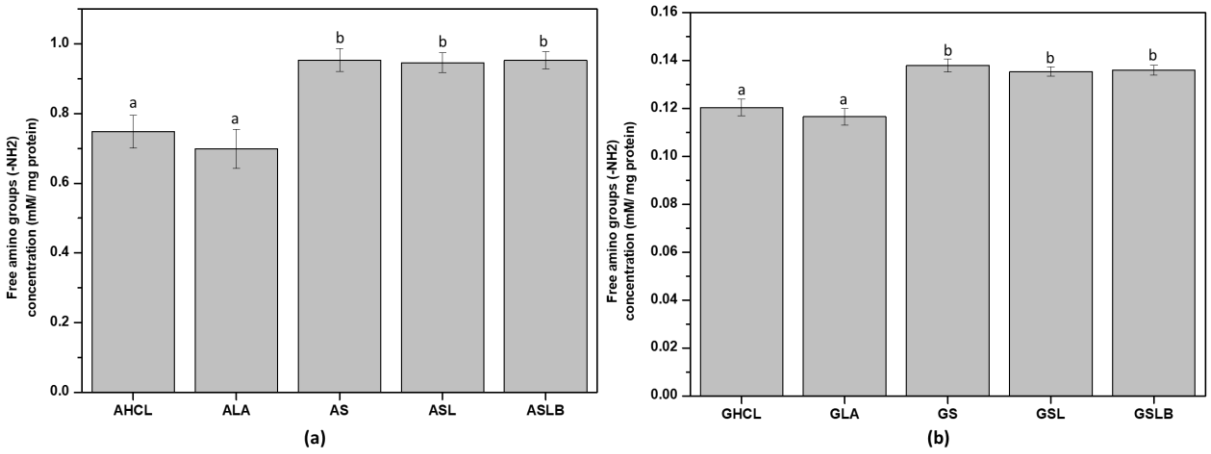


Figure IV-4. Free amino groups content (mM/mg protein) of globulin (G) (a) and albumin (A) (b) fractions obtained by controls (HCl, LA) and fermentations with *S. thermophilus* (S), *S. thermophilus* + *Lb. acidophilus* (SL), *S. thermophilus*+ *Lb. acidophilus* + *B. lactis* (SLB). Different letter represents significant differences among different samples (Turkey's post hoc test).

IV.2.5. Polypeptide profile

SDS-PAGE was performed in non-reducing and reducing conditions to determine the effect of extraction method on polypeptide composition of the recovered globulin (Figure IV-5) and albumin (Figure IV-6) fractions. In non-reducing conditions (NRC), the globulin samples' profiles (Figure IV-5) revealed the presence of bands ranging from 10 to 99 kDa, characteristic of pea proteins (Dziuba et al., 2014). The majority of these polypeptide bands represented various subunits of vicilin including the monomer (Vαβγ, ~50 kDa) and the cleavage resulting polypeptides (Vαβ, ~30–36 kDa; Vβγ, ~25–30 kDa; Vα, ~20kDa; Vβ, ~13kDa; Vγ, ~12–16

kDa) (Dziuba et al., 2014; Gatehouse et al., 1982). There is also the presence of legumin monomer (Lαβ, ~60kDa), which separated to acid (Lα, ~40 kDa) and basic (Lβ, ~21–23 kDa) subunits in reducing condition (Liang & Tang, 2013). The higher-molecular-weight bands corresponded to lipoxygenase (LOX, ~94 kDa) and convicilin (CV, ~71 kDa) (Shand et al., 2007). The results indicated no significant difference in polypeptide profile of the globulin fractions obtained by either chemical acidification or lactic fermentation. The final polypeptide composition of globulin isolate was apparently not affected by the extraction method, although a global loss of globulins was observed, as indicated before. The presence of smaller polypeptides than 6 kDa, not observable on the electrophoretic profile because they migrated beyond the limit of the gel, cannot be excluded in the case of fermented samples, since free amino group content increased, as observed above.

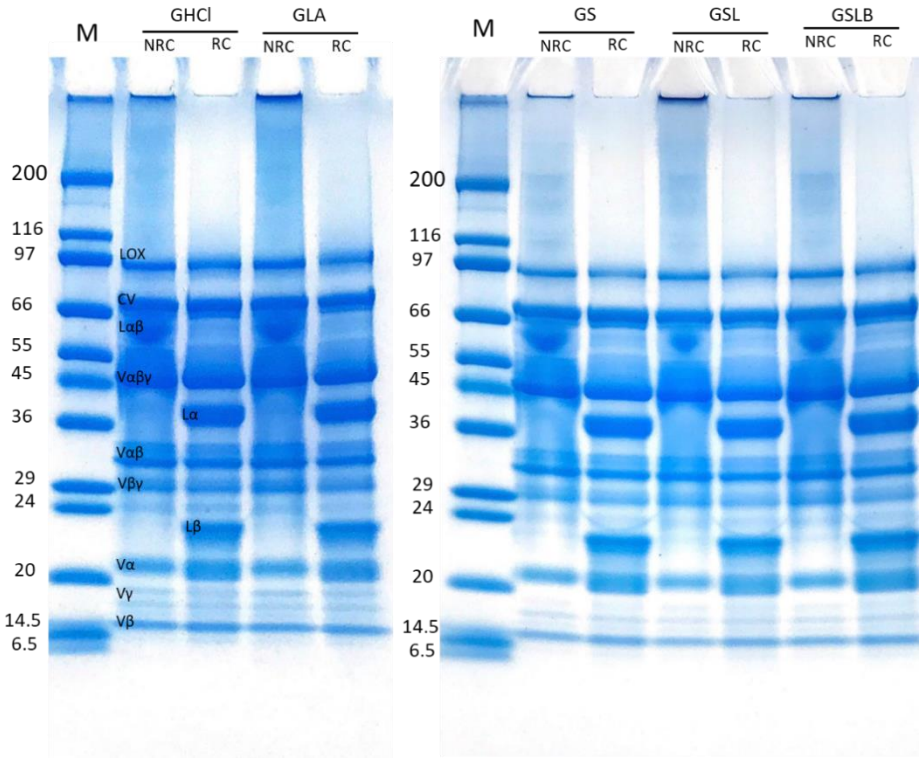


Figure IV-5. Electrophoretic profiles of globulin fractions obtained by controls (HCl, LA) and fermentations with *S. thermophi*lus (S), *S. thermophilus* + *Lb. acidophilus* (SL), *S. thermophilus*+ *Lb. acidophilus* + *B. lactis* (SLB) in reducing (RC) and non-reducing (NRC) conditions.

Clearly absent in the previous patterns, the main 2S albumin subunits (PA2, ~26kDa; PA1, ~6kDa) (Dziuba et al., 2014; Gruen et al., 1987) were observed in the electrophoretic profiles of albumin samples, which also showed clear bands of LOX, lectine (Lect, ~17 kDa) (Crévieu

et al., 1996) and some contaminations by globulin polypeptides, mainly Vαβ (Figure IV-6). The main polypeptides did not seem to be affected by the extraction method. Nevertheless, a few differences were observable for other contamination bands. The band of convicilin (~70 kDa) seemed to disappear completely in fermented samples. It might also be noticed that some bands (between 40–80 kDa) disappeared in fermented samples compared to the controls. Barkholt et al. (1998) also claimed the disappearance of some high-molecular-weight bands in the protein profile of lactic-fermented pea flour compared to the non-fermented.

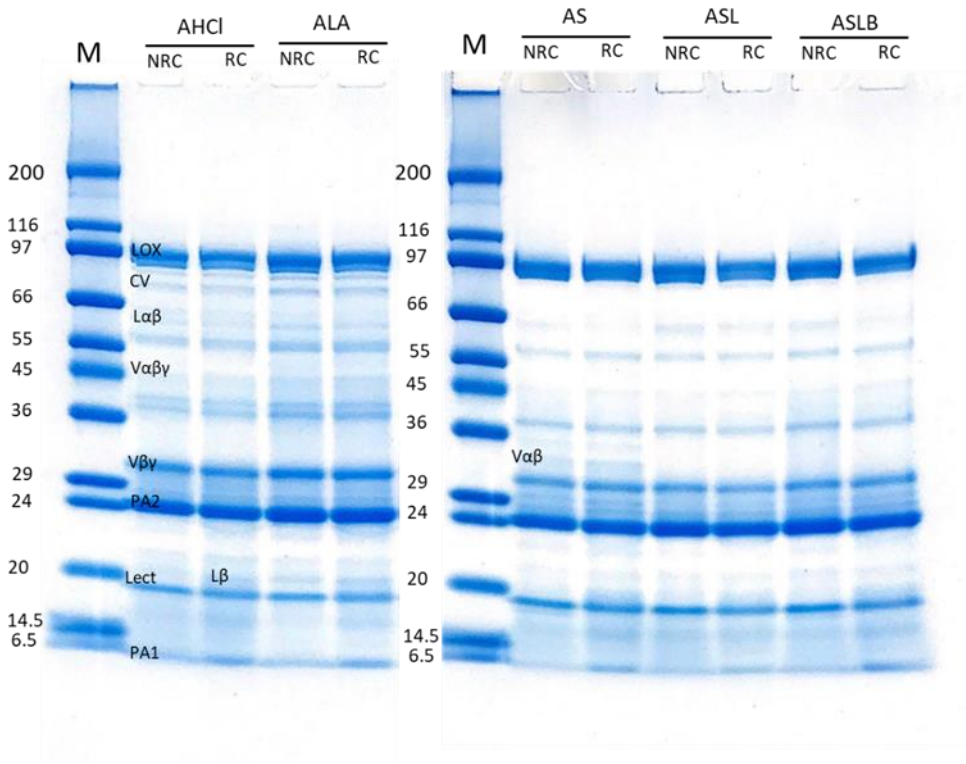


Figure IV-6. Electrophoretic profiles of albumin fractions obtained by controls (HCl, LA) and fermentations with *S. thermophilus* (S), *S. thermophilus* + *Lb. acidoph*ilus (SL), *S. thermophilus*+ *Lb. acidophilus* + *B. lactis* (SLB) in reducing (RC) and non-reducing (NRC) conditions.

IV.2.6. Differential scanning calorimetry (DSC)

Thermal properties of native globulin fractions were studied by differential scanning calorimetry (DSC). Onset temperature (T_{onset}), temperature of denaturation (T_d) and enthalpy of denaturation (ΔH_d) of globulin fractions are presented on Table IV-1. Thermograms (data not shown) displayed one broad peak with a small shoulder, which according to literature, corresponded to the denaturation of 7S and 11S pea globulins, respectively (Mession et al.,

2013). T_{onset} for all the samples was approximately 61 °C. The first denaturation peak, with major surface, (T_{d1}) had the highest value for GS (72.9 °C), followed by GLA (72.4 °C) and GSL (72.1 °C) samples. Td_1 value for GS was significantly different from those evaluated for GHCl (71.7 °C) and GSLB (71.9 °C). The small differences observed between samples could be attributed to small variations in protein composition (Ricci et al., 2018) and/or reflect the effect of ions added for acidification (chloride, lactate) or the metabolites produced during fermentation (Liu et al., 2011; Sun & Arntfield, 2010, 2012). The minor denaturation peaks (T_{d2}) corresponding to legumin did not vary significantly (~82–83 °C) between samples.

Table IV-1. Thermal parameters of globulin fractions obtained by controls (HCl, LA) and fermentations with *S. thermophilus* (S), *S. thermophilus* + *Lb. acidophilus* (SL), *S. thermophilus*+ *Lb. acidophilus* + *B. lactis* (SLB)

Sample	T_{onset} (°C)	T_{d1} (°C)	T_{d2} (°C)	ΔH_d total (J/g protein)	Peak 1/Peak2 area ratio
GHCl	61.58 ± 0.76 a[1]	71.69± 0.3 a	83.29 ± 0.69 a	12.57 ± 0.24 a	3.5 ± 0.4 b
GLA	61.83 ± 0.18 a	72.43 ± 0.14 ab	83.05 ± 0.34 a	13.05 ± 0.26 a	3.5 ± 0.2 b
GS	61.93 ± 0.36 a	72.90 ± 0.04 b	83.31 ± 0.31 a	15.11 ± 0.40 b	2.2 ± 0.2 a
GSL	61.79 ± 0.06 a	72.15 ± 0.65 ab	81.97 ± 0.44 a	14.66 ± 0.33 b	2.6 ± 0.3 a
GSLB	61.84 ± 0.46 a	71.91 ± 0.45 a	82.23 ± 0.78 a	14.76 ± 0.17 b	2.4 ± 0.3 a

[1]Different letters in the same column and type of sample represent significant differences among samples (Tukey's post hoc test).

The most noticeable differences were observed in ΔH_d, which was calculated from the total area of denaturation peaks. The enthalpy change reflects the extent of ordered structure of the globulins as the transition from native to denatured state took place (Ricci et al., 2018). ΔH_d of GS (15.11 J/g), GSL (14.66 J/g) and GSLB (14.76 J/g) were significantly higher compared to GHCl (12.57 J/g) and GLA (13.05 J/g). This value for GHCl was similar to alkaline-extracted pea globulin reported by Mession et al. (2012). Higher ΔH_d values in fermented samples could be representative of a higher-ordered structure of the protein in the globulin fractions obtained after fermentation. To explain these values, the vicilin/legumin area ratio was calculated from peak curve deconvolution (Table IV-1). The vicilin/legumin area ratio was lower in the fermented samples compared to the controls, indicating that more legumin was present in the case of fermented samples. As legumin has a more complex structure than vicilin, the extent of legumin denaturation energy was higher (Mession et al., 2012) leading to higher value for ΔH_d. These results also indicated a relative depletion of vicilin and convicilin in the globulin isolate meaning fermentation released more 7S globulins in the albumin fraction or some

polypeptides from 7S globulins were consumed by bacteria, which was consistent with SDS-PAGE results.

Thermal properties of the albumin fractions obtained by both controls and lactic fermentations are shown on Table IV-2. The thermograms of albumin samples showed two peaks (data not shown) at around 63 °C and 77 °C, respectively. Although there were no available data in literature regarding thermal denaturation temperature of pea albumins, this observation was in accordance with a previous study in our team (Djoullah, 2015). The first peak considered as albumin denaturation was followed by a broad small peak, which was representative of the contamination by globulin polypeptides, corresponding to only around 1% of the total surface of both peaks. T_{onset} was close to 55 °C for all the samples. T_{d1} for controls (~62 °C) was slightly lower than fermented samples (~63 °C), which might reflect small differences in protein and co-solute composition. A similar trend was observed for ΔH_d among albumin fraction samples. ΔH_d values were significantly higher for fermented samples (~9.5 J/g protein) compared to controls (~8 J/g protein). This might be related to the improved protein purity in fermented samples as observed above. It could be also hypothesized that changes in albumins as a result of fermentation (chemical composition, conformation) exerted a better protective effect on protein structure upon thermal denaturation.

Table IV-2. Thermal parameters of albumin fractions obtained by controls (HCl, LA) and fermentations with *S. thermophilus* (S), *S. thermophilus* + *Lb. acidophilus* (SL), *S. thermophilus*+ *Lb. acidophilus* + *B. lactis* (SLB)

Sample	T_{onset} (°C)	T_{d1} (°C)	T_{d2} (°C)	ΔH_d total (J/g protein)
AHCl	54.16 ± 0.24 a[1]	62.14 ± 0.29 a	76.64 ± 0.18 a	7.87 ± 0.5 a
ALA	55.17 ± 0.89 a	62.21 ± 0.25 a	76.69 ± 0.04 a	8.36 ± 0.55 a
AS	54.11 ± 0.71 a	63.88 ± 0.13 b	78.56 ± 0.72 a	9.42± 0.49 b
ASL	55.70 ± 0.35 a	63.95 ± 0.16 b	78.15 ± 0.77 a	9.55 ± 0.54 b
ASLB	55.20 ± 0.47 a	63.71 ± 0.03 b	77.81 ± 0.38 a	9.43 ± 0.45 b

[1]Different superscript in the same column and type of sample represent significant differences among different samples (Tukey's post hoc test).

IV.3. Conclusion

The pea protein extraction assisted by lactic fermentation successfully led to the production of globulin-and albumin fractions. Compared to the common alkaline extraction isoelectric precipitation method, fermentation led to an increase in total nitrogen content and protein yield

of the albumin fraction. The increased protein solubility in the albumin fraction was attributed to proteolytic activity of lactic acid bacteria, affecting probably some vicilin/convicilin polypeptides. As a consequence of fermentation effects, the recovery of protein in particular 7S globulins in the globulin extract was decreased. Slight differences in thermal properties associated to the main pea proteins were observed, which could be explained by the changes in the composition of the protein fractions by fermentation. These findings are decisive for pea protein extraction in the food industry. Further studies need to be undertaken to better understand the impact of extraction assisted by different LAB strains on the functional, sensorial and nutritional properties of the isolated pea proteins. In particular, the identification of bioactive peptides released in the albumin fraction during fermentation would be useful for the valorization of enriched protein extract.

V. Impact of fermentation on the protein composition and nutritional aspects of pea protein fractions obtained by a modified isoelectric precipitation method

V.1. Introduction

Over the past decade, there has been a significant interest toward the legume-based proteins as a promising alternative to the animal derived ones. Legumes are sustainable, available and low cost. They comprise oil-seed legumes such as soybean, and pulses such as lentils, chickpeas, peas, lupines and beans (Semba et al., 2021). Among which peas are of great interest for their high protein content, good quality of amino acids and low allergenicity. However, the application of pea protein in food industry is still challenging for its low solubility and imperfect sensorial properties (Emkani et al., 2021). Pea proteins obtained in industry are globulins. Other fractions such as albumins are discarded as by-products.

Precisely, pea consists of approximately 20–30% protein of its total dry seed. It can be used either as grain components (e.g., flour milled from grains) or as enriched protein ingredients such as protein concentrate (50–55%) and protein isolate (80–90%) (Barać et al., 2015; Boye et al., 2010). The majority of pea proteins are globulins (60–70%) and albumins (15–20%). Globulins consist of legumin, vicilin and convicilin with an isoelectric point (pI) close to pH 4.8 (Derbyshire et al., 1976). Legumin belongs to the 11S group with a high Mw of 360–400 kDa. It consists of six subunit pairs (~60 kDa) and each of which consists, of an acidic subunit of ~40 kDa and a basic subunit of ~20 kDa, linked by a single disulfide bridge(Liang & Tang, 2013). Vicilin is a trimer of 150–200 kDa. Each monomer (~50 kDa) has two cleavage sites, for the post-translational formation of various polypeptide fragments of about 20 kDa (α), 13 kDa (β), 12–16 kDa (γ), 30–36 kDa (α+ β) and 25–30 kDa (β+γ) (Dziuba et al., 2014; Gatehouse et al., 1982). Finally, convicilin subunits (~70 kDa) are considered to associate non-covalently in a multimer of 210–280 kDa (Shand et al., 2007). The two principal protein of albumins are PA1 and PA2 which belong to the 2S group (Lu et al., 2000). PA1 contributes about 50% of the seed's sulfur amino acids (Gressent et al., 2011). In the mature grain, PA1 consists of two components, PA1a (~6 kDa) and PA1b (~4 kDa) (Lu et al., 2000). The PA1 polypeptide has an isoelectric point of ~7.2 (Bérot et al., 2007). PA2 has a Mw of approximately 24–26 kDa and it consists of two subunits of PA2a and PA2b which combined by non-covalent bonds form homodimers of Mw 48–53 kDa (Gruen et al., 1987; Lu et al., 2000). The isoelectric point of PA2 is between 5 to 5.6 (Crévieu et al., 1996). Both pea 2S albumins have a high sulfur amino acid content compared to globulins (Higgins et al., 1986).

Albumin and globulin fractions can be purified, since they have different solubility. Globulins are salt soluble, while albumins are water soluble. Other compounds such as trypsin inhibitors,

lipoxygenase, phytate, lectine and α-galactosides, are also soluble in water and are recovered in the soluble albumin fraction after separation from globulin (Le Gall et al., 2007; Szymanowska et al., 2009). These compounds are considered as non-nutritive compounds since they interfere with nutrient availabilities or cause host digestive discomfort or health problems. For instance, trypsin inhibitors are low Mw proteins capable to bind to digestive enzyme (i.e. trypsin) and inactivate it. The presence of these proteins in human and animal diets could lead to pancreatic enlargement, reduced digestibility of protein, reduced absorption of amino acids and reduced availability of minerals (Bacon et al., 1995a). In general, peas have 5-20 times lower trypsin inhibitor compared to soybean. Still this value could be significantly dependent on the cultivars (Gwiazda et al., 1979). The α-galactosides of sucrose, also known as the raffinose family of oligosaccharides (RFOs) are the second most abundant soluble carbohydrate in legumes (Jones et al., 1999). α-galactosides are soluble low-molecular weight oligosaccharides such as raffinose (trisaccharide), stachyose (tetrasaccharide), and verbascose (pentasaccharide) (Tester & Karkalas, 2003). α-galactosides are responsible for digestive discomfort and flatulence due to their fermentation by gut bacteria in the large intestine. However, moderate doses of α-galactosides favour the metabolism of beneficial intestinal microorganisms such as Bifidobacteria (Teixeira et al., 2012). Phytic acid (myo-inositol hexaphosphoric acid (IP6)) consists of a cyclic ring ($C_6H_6O_6$) where each oxygen is connected to a phosphate group ($P(OH)_3$) (Amat et al., 2022). The particular structure of phytic acid allows binding with different molecules (e.g. proteins) through different types of interactions (bridge formation, complex formation and electrostatics). Phytic acid can interact with protein through ionic interaction (below the isoelectric point of protein) and it decrease the solubility of protein (Urbano et al., 2000). The complex phytate-protein is resistant to proteolysis so it interferes with protein digestion. Phytate can also bind with enzymes such as proteases and amylases and decrease the protein digestibility (Samtiya et al., 2020; Tiwari & Singh, 2012). Additionally, phytic acid forms complexes with certain minerals, such as calcium, copper, magnesium, iron, manganese, zinc, and amino group derivatives in protein moieties, and thus decreases their absorption in the gastrointestinal tract. For people with high daily pulse consumption, this can result in anemia due to iron deficiency (Tiwari & Singh, 2012).

The composition of pea protein can be modified by different parameters, such as the cultivar, the extraction method or the use of biological process such as fermentation. First and foremost, the genetic and phenotypic variation of pea cultivars which can affect the ratio of globulins (i.e. 11S/7S) and globulin to albumin fraction (Schroeder, 1982). The variation of protein fractions

can modify the properties of protein. For instance, the less compact structure of vicilin offers better functional properties compared to legumin (Tzitzikas et al., 2006). However, the low content of sulfur amino acids in vicilin make it less interesting from a nutritional point of view (Wang & Daun, 2004).

Another important parameter controlling pea protein composition is the extraction method. Different methods have been proposed for the extraction of protein from pea flour including alkaline extraction-isoelectric precipitation (AEIEP), salt extraction-dialysis, micellar precipitation and aqueous extraction (pH > 7) (Lam et al., 2018; Owusu-Ansah & McCurdy, 1991). AEIEP is a common technique with high yield for producing pea protein isolates in the food industry. The extraction process starts by solubilization of protein at alkaline pH (~8). It then follows an isoelectric precipitation of globulin fraction at pH ~4.5-4.8 by a mineral acid (Tanger et al., 2020). The method separates albumins and globulins since both legumin and vicilin have high solubility at alkaline pH, and minimal solubility at their pI, while albumin remains soluble in a large pH range (Djemaoune et al., 2019).

Biological process such as lactic acid fermentation could be as important as the two other parameters (cultivar and extraction method) in modifying the composition of protein. Indeed, lactic acid bacteria (LAB) obtain the required energy for growth by metabolism of different compounds in the substrate such as proteins and this way it can modify the protein composition (Emkani et al., 2022). The polypeptides can break down to smaller peptides and amino acids through the enzymatic pathway by microbial proteases or the disruption of the ionic interactions between protein side chains during organic acid production (Klupsaite et al., 2017; Meinlschmidt et al., 2016). The small peptides released by LAB during fermentation can possess bioactive properties such as antioxidant activities (Stanisavljevic et al., 2015).

A recent study in our group have proposed an alternative extraction method based on AEIEP (Emkani et al., 2021). In this method, lactic acid fermentation by using LAB commercial starters was applied during acid precipitation step. This way the reduction of pH was obtained thanks to the production of lactic acid by specific starters during fermentation which leads to the precipitation of non-soluble fractions (globulins) and its separation from the soluble ones (albumins). This study showed that the albumin fraction obtained during fermentation had higher protein content than the one obtained by traditional AEIEP method. Albumin fraction is generally discarded in industry due to the presence of non-protein soluble compounds and antinutritional components, the possible allergenic activity of this fraction, and most

importantly the lower content of protein in albumin compare to globulin fraction (Malley et al., 1975; Moreno & Clemente, 2008; Sell et al., 2005; Yang et al., 2022). While this fraction can be interesting from the nutritional point of view, since it considers to have higher content of sulfur containing amino acids (Croy et al., 1984; Davies, 1976). Pea albumin may play a bioactive role or it can be precursor of biologically active peptides (e.g. Bowman-Birk inhibitors (BBI)) with various physiological functions (Utrilla et al., 2015). These bioactive compounds are resistant to gastric acid or proteolytic enzymes, thus reaching the large intestine in active form and offer significant health benefits including anti-inflammatory, anti-carcinogenic, or anti-obesity (Duranti, 2006; Liu et al., 2022; Utrilla et al., 2015; W. Zhang et al., 2022). Pea albumin (PA1 b) is also known for its outstanding insecticidal activity against certain insects which make it a promising alternative to the chemical pesticides reducing the concern on their toxicity and persistence in the environment (Da Silva et al., 2010; Eyraud et al., 2013; Rahioui et al., 2014). Moreover, previous studies on pea albumin fraction reported a good emulsion and foam stabilizing properties (Lu et al., 2000). Especially interface and foam stabilizing properties of this fraction seem to be promising compared to globulin owing to the smaller Mw, lower protein charge and the specific distribution in hydrophobicity of albumin fraction (Kornet et al., 2022; Yang et al., 2022).

Hence the objective of this work was to evaluate more precisely the effects of fermentation with specific LAB strains on the under-valorised pea albumin fraction obtained with the previous proposed extraction method assisted by fermentation. Protein and peptide content and other nutritional aspects (α-galactoside and phytic acid contents, and trypsin inhibitor and antioxidant activities) were further discussed in this work. Here, we aimed to use different pea cultivars with contrasted initial protein composition in 7S, 11S and 2S to observe whether the method could preferentially lead to the enrichment of certain protein fractions. Two LAB strains (*S. thermophilus*, *Lb. plantarum*) were applied either alone or in co-culture for their promising impact on legume protein properties. *Lb plantarum* is a facultative heterofermentative bacteria (Mandha et al., 2022). It is famous for its versatility and its adaptability in different substrates and conditions (Smetanková et al., 2012). It has been used numerously in pea ingredient fermentation (Arteaga et al., 2021; Cabuk et al., 2018a; Cabuk et al., 2018b; Czarnecka et al., 1998; Li et al., 2021). *S. thermophilus* is homofermentative aerotolerant and is widely used in dairy industry for its high acidification rate and its contribution to organoleptic properties (Galia et al., 2009; Iyer et al., 2010). There are some

evidences of high growth and acidification of this strain in pea substrate either alone or in co-culture (Ben-Harb et al., 2019; Emkani et al., 2021; Narala et al., 2022; Yousseef et al., 2014).

V.2. Results and discussion

V.2.1. Monitoring the pH reduction and bacterial selection

The reduction of pH during fermentation of pea protein extract by four strains (ST, LH, LR, LP), was monitored (Fig V-1). The first attempts were carried out on protein extract from commercial pea flour. The result showed that LP had the fastest pH reduction and it was followed by ST. It took around 7 h for LP to reach pH 4.8 (the isoelectric point of the globulin). While for ST, it took around 10 h to reach this pH. On the contrary to the two previous bacteria, LH and LR could not be selected for fermentation in the tested condition since it took a long time (around 25 h) for LR to reach pH 4.8 or it was impossible to reach this pH with LH.

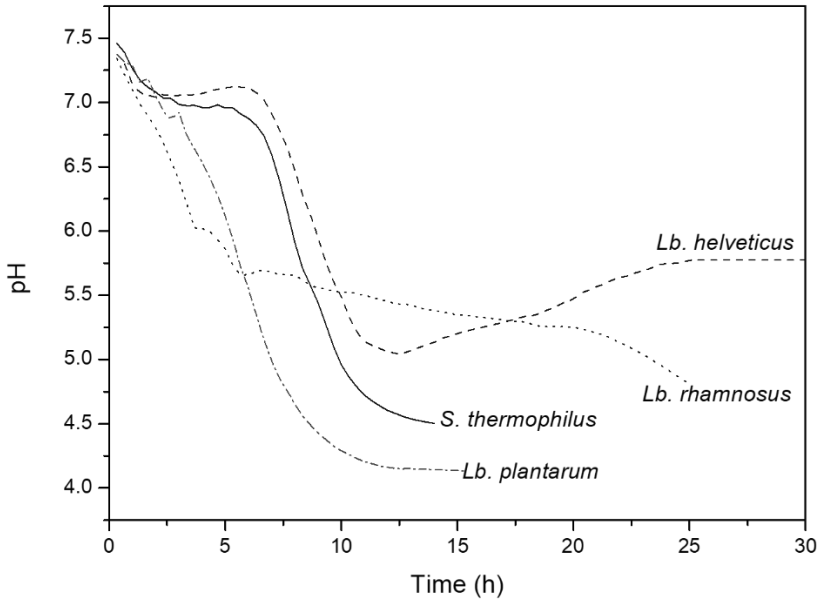


Figure V-1. Acidification kinetics of lactic acid bacteria cultures (*S. thermophilus* (ST) (solid line), *Lb. helveticus* (LH) (dash line), *Lb. rhamnosus* (LR) (dot line), *Lb. plantarum* (LP) (dash dot line)) in protein extract obtained from commercial pea flour.

Furthermore, the distribution and appearance of LH, LR and ST in broth medium under aerobic condition showed (Fig. V-2) that for LH and LR, a dense layer of bacteria at the bottom of the broth medium tube was observed. Indeed, the bacteria developed in the bottom of the tube, as far as possible from the oxygen due to the obligate anaerobic nature of these two strains, despite

the information provided by the supplier. While ST, had a uniform fine turbidity because of its aerotolerant nature.

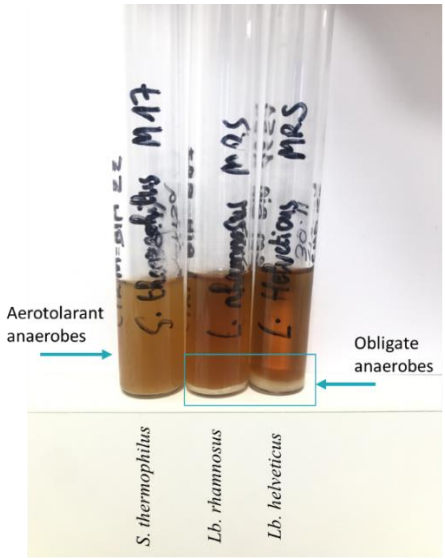


Figure V-2. Appearance of selected LAB (*S. thermophilus* (ST), *Lb. rhamnosus* (LR) and *Lb. helveticus* (LH)) growth in broth medium under aerobic condition.

Considering the anaerobic character of these two strains, the fermentation was performed under anaerobic condition. Fig V-3 compared the pH evolution of LH and LR in both aerobic and anaerobic condition. The diagrams showed that in anaerobic condition, it took around 9 h for LH (Fig V-3. a) and 6 h for LR (Fig V-3. b) to reach pH 4.8 compared to aerobic condition in which it was not possible to reduce the pH enough.

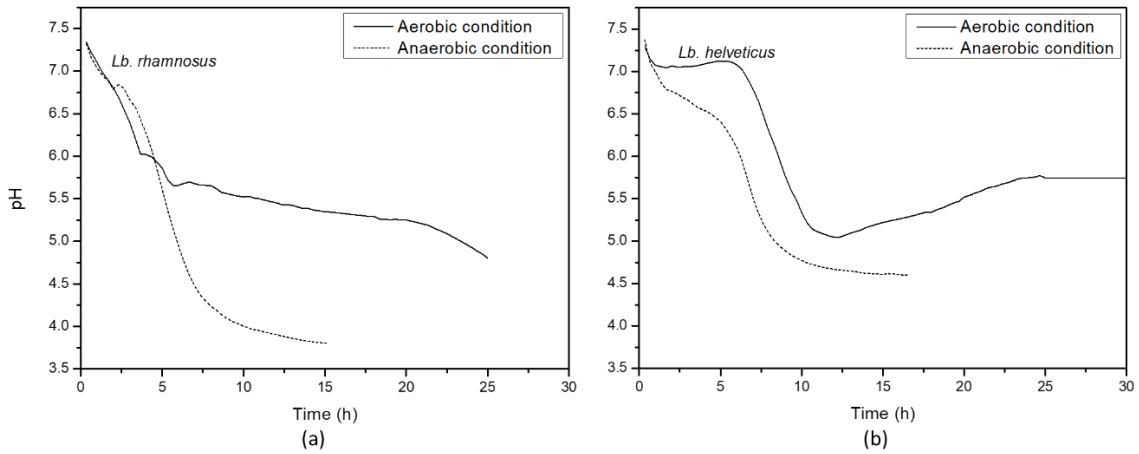


Figure V-3. Comparing the acidification kinetics of *Lb. helveticus* (LH) (a) and *Lb. rhamnosus* (LR) (b) in protein extract obtained from commercial pea flour, in aerobic (solid line) and anaerobic (short dash line) condition.

All these considered it could be possible to performed the experiments for ST and LP in aerobic and for LH and LR in anaerobic condition. However, LAB cultivation under aerobic and anaerobic conditions could affect bacterial growth, metabolic end products and consequently the properties of the final fermented sample. Murphy and Condon (1984) reported that the main end product of aerobic and anaerobic cultivation of LP were acetic acid and lactic acid, respectively. Fu and Mathews (1999) found that LP had higher cell yield under aerobic conditions, while lactic acid production was higher under anaerobic condition. The differences in growth rate and metabolites concentration is related to the differences in metabolic pathways under aerobic and anaerobic condition (Goffin et al., 2004). When grown in presence of oxygen, some LAB have pyruvate oxidase which convert pyruvate to acetylphosphate and result in increased acetate production (Wagner et al., 2005). These findings demonstrate the importance of aeration condition in fermentation process. Thus, it was preferred in this study to cultivate all the bacteria in the same aeration condition. Therefore, only ST and LP were selected for the further analysis.

V.2.2. Acidification kinetics

Figure V-4 shows the pH evolution during fermentation of soluble pea protein extracts obtained from pea protein flours of three different varieties (CAR, AC, AS), by ST, LP and their co-culture. The diagrams showed that pea protein suspensions were a suitable substrate since all the bacteria were able to grow and reduce the pH. However, the time required to reduce the pH, was quite different between the strains.

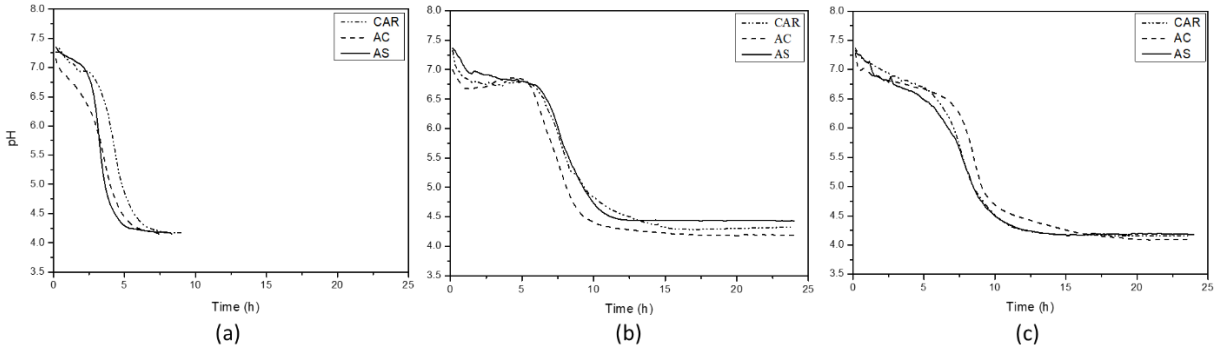


Figure V-4. Acidification kinetics of LAB strains (*S. thermophilus*+ *Lb. plantarum* (STLP) (a). *S. thermophilus* (ST) (b) and *Lb. plantarum* (LP) (c) in protein extracts obtained from different pea cultivars (CAR, AC, AS).

Table V-1 shows the acidification kinetic parameters of pea cultivars fermented with mono- or co-culture. The highest acidification rate (V_{max}~1.2-2.5 pH units/h) and lowest t_{Vmax} (~3.6 h) values were related to the co-culture compared to the mono-cultures (V_{max} ~0.8-0.9 pH units/h; t_{Vmax}=7.5-8.5 h), with significant differences. The highest V_{max} value (2.5 pH units/h) in co-culture was related to the variety AS. While there was no significant difference between the varieties in mono-cultures. The pH_{Vmax} represents the pH at maximum acidification rate, corresponding to the maximum acid producing ability of the strains. There was no significant difference in $pH_{Vmax,}$(~5-6), between any variety fermented either with mono- or co-cultures. The pH_f represents the final reducing pH after which the pH was stable. The value of pH_f was higher in ST (~4.2-4.4) compared to LP and co-culture (~4.1) indicating lower acidification. The time required to reach this pH (t_{pHf}) was higher in mono-cultures (~14-16 h) compared to co-culture (~7-7.5 h). $t_{pH4.8}$, indicating the time required to reach pH 4.8 (isoelectric point of globulins), had higher value in ST (~9.5-10.1 h) and it followed by LP (~9-9.5 h). Co-culture (~3.9-4.7 h) showed the lowest $t_{pH4.8}$ compared to the mono-cultures.

Higher value of pH_f and $t_{pH4.8}$ in ST could be explained by the lower acidification capacity of this strain compared to LP. Indeed, the acidification capacity is a strain-dependent metabolic feature that could be influenced by many factors such as metabolism of sugar and proteolytic system (Iyer et al., 2010; Mital & Steinkraus, 1979). Metabolism of sugar by LAB lead to the production of organic acids and reduction of pH. LP is considered as heterofermentative facultative which make it possible for this strain to consume pentoses in limiting hexoses (Mandha et al., 2022). While ST is homofermentative and is not able to ferment pentoses (Pastink et al., 2009). The highest V_{max}, the lowest t_{Vmax}, pH_f, t_{pHf} and $t_{pH4.8}$ observed in the co-culture could be explained by the synergetic effect of the combined bacteria culture which resulted an accelerated acid production as pointed out by many authors. Mishra and Mishra (2013) observed that both *S. thermophilus* and *Lb. plantarum,* once in combination, resulted in increased rate of fermentation and reduced fermentation time. Emkani et al. (2021) showed the mixed culture of ST, with *Lb. acidophilus* and *B. lactis* had better acidification profile in pea protein compared to the mono-cultures. Li et al. (2020) reported the combination of *S. thermophilus* and *Lb. plantarum* in milk fermentation had higher pH reduction compared to the co-culture of *S. thermophilus* and *B. lactis*.

Table V-1. Acidification kinetic parameters for fermented pea protein suspensions obtained from flours of different varieties (CAR, AC, AS) with mono-cultures and co-culture of *S. thermophilus* (ST) and *Lb. plantarum* (LP).

Strain	Pea cultivars	V_{max} (pH units/h)	t_{Vmax} (h)	pH $_{Vmax}$	pH f	t_{pHf} (h)	$t_{pH\ 4.8}$ (h)
STLP	CAR	1.53± 0.15c	3.75± 0.95a	6.1± 0.5a	4.16± 0.096a	7.5± 0.2a	4.7±0.6a
	AC	1.2± 0.2bc	3.5± 1.05a	5.5± 0.27a	4.17± 0.12a	7.03± 0.25a	4.1±0.72a
	AS	2.5± 0.2d	3.6± 1.1a	5.9± 0.3a	4.17±0.09a	6.8± 0.57a	3.9±0.64a
ST	CAR	0.89±0.05ab	8.1± 0.9cd	5.6± 0.4a	4.29±0.10b	15.36± 0.9bc	10.1±0.19c
	AC	0.82±0.05a	7.5± 1.5b	5.7± 0.5a	4.2±0.12ab	15.5± 0.47bc	9.5±0.32bc
	AS	0.84±0.07a	7.5± 1.1b	6.02± 0.7a	4.43±0.13c	14.3± 0.8b	10±0.24c
LP	CAR	0.83±0.12a	8.33± 1.4d	5.5± 0.31a	4.16±0.15a	14.6± 0.48b	9±0.21b
	AC	0.91±0.10ab	8.5± 1.3d	5.5± 0.5a	4.13±0.14a	16.16± 1.22c	9.5±0.38bc
	AS	0.85±0.07a	8.1± 0.87cd	5.4± 0.3a	4.16±0.16a	14.1± 0.59b	9±0.26b

[1] The values were presented as average ± standard deviation, n (n=3). Different letters in the same column represent significant differences among different samples (Tukey's post hoc test, P value < 0.05).

V.2.3. Total and non-protein nitrogen contents

The isoelectric precipitation extraction method was applied to recover albumin and globulin fractions from pea flour of three different cultivars (CAR, AC, AS) without (control) or with fermentation using mono-cultures of ST and LP and their co-culture. Total nitrogen (TN) content of albumin and globulin fractions was shown in Fig V-5. The results indicated that in albumin fractions (Fig V-5. a), TN content was significantly higher for fermented samples (~4-5%) compared to control (~3%). Also, co-culture (~5%) had higher values compared to mono-cultures (~3.7-4.5%), while there was no significant difference between the mono-cultures of ST and LP. Comparing pea varieties, it seemed AS (~4.5%) fermented with both ST or LP had the highest content of TN in albumin fraction. This is not surprising since the variety AS was initially richer in PA2. Regarding the globulin fractions (Fig V-5. b), the TN content was higher in control (~14.3-14.7%) compared to the fermented samples with co-culture (~12%) having the lowest value. This result was coherent with what was observed in albumin fractions. The decrease in the TN content of globulin fraction was supposed to result from the hydrolysis of some pea proteins to smaller polypeptide chains which probably solubilized during the extraction process and were recovered in the albumin fraction.

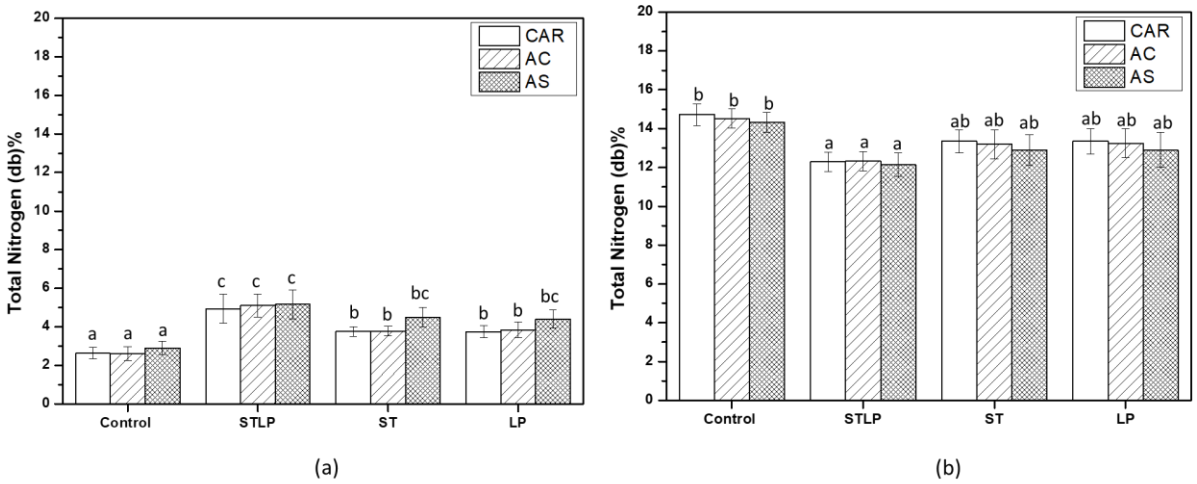


Figure V-5. Total nitrogen content of albumin (a) and globulin (b) fractions obtained without (control) or with added fermentation by *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP). Comparison between different pea cultivars: CAR, AC and AS. Different letters represent significant differences in total nitrogen content among different samples (Tukey's post hoc test, P value < 0.05).

Non-protein nitrogen (NPN) content of albumin (Fig V-6. a) and globulin (Fig V-6. b) fractions of pea cultivars obtained by both control and fermentation was measured. The results showed that fermented samples had the highest content of NPN compared to the control (albumin: ~0.8% and globulin: ~0.3%) in both fractions. Also, co-culture (albumin: ~2.6% and globulin: ~0.7%) sample had the highest values in both protein fractions compared to the mono-culture ones. NPN in fermented samples, represented around 5% and 50% of TN in globulin and albumin fractions, respectively, which was approximately two-fold higher than control. An increase in the content of NPN could be explained by hydrolysis of protein during fermentation (Ibrahim et al., 2005; Soni & Sandhu, 1989; Yousif & El Tinay, 2001). In fact, non-protein nitrogen is defined as a complex fraction of nitrogen containing compounds having the Mw of less than 10 kDa. This fraction mainly consists of amino acids and peptides as well as alkaloids ammonium salts and the nitrogenous glucosides and lipids (Holt & Sosulski, 1981). Higher content of NPN in fermented samples compared to control could also explain the higher content of total nitrogen content (Niveditha & Sridhar, 2015). Generally, the content of NPN depends on cultivars (Periago et al., 1996). However, in this study there was no significant differences between the varieties.

It is worth saying that despite the application of fermentation in isoelectric precipitation step, the total protein content in globulin fractions was ~72% in fermented samples compared to

~77% in control, considering the N to protein conversion factor of 5.4. By applying a N to protein conversion factor of 6.25 classically used for commercial pea protein isolates, these contents were ~83% and ~88% respectively in the range of values usually observed by applying the AEIEP method (Lam et al., 2018).

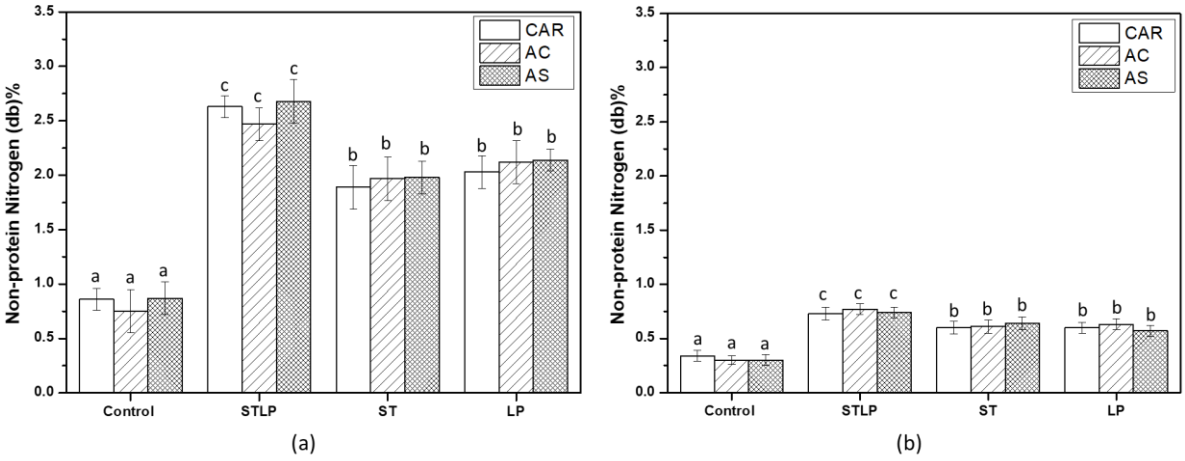


Figure V-6. Non-protein nitrogen content of albumin (a) and globulin (b) fractions obtained without (control) or with added fermentation by *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP). Comparison between different pea cultivars: CAR, AC and AS. Different letters represent significant differences in non-protein nitrogen content among different samples (Tukey's post hoc test, P value < 0.05).

V.2.4. Free amino groups

Free amino groups were measured to better understand the proteolysis effect occurring in fermented samples. The content of free amino groups in albumin and globulin fractions obtained without (control) or with fermentation with ST, LP and their co-culture from different pea cultivars (CAR, AC, AS), was shown in Figure V-7 a and b, respectively. Free amino group content was significantly lower in control albumin (~0.4 mM/mg protein) and globulin (~0.01 mM/mg protein) fractions compared to the fermented samples in agreement with the previous NPN data. Also, the samples fermented with co-culture seemed to have higher content of free amino groups in albumin (~1.3 mM/mg protein) and globulin (~0.03 mM/mg protein) fractions compared to the mono-culture samples (albumin: ~1 mM/Mg protein and globulin: ~0.02 mM/mg protein). Higher content of free amino groups in fermented samples could be explained by the proteolytic activity of the bacterial strains leading to the release of small peptides and amino acids during fermentation. Indeed, the proteolytic system of LAB is a complex system

that depends on the function of cell-envelope proteinase (CEP), peptide transport systems and intra-cellular peptidases (Kunji et al., 1996). A strong proteolytic activity was reported in some previous studies for LP. Rui et al. (2019) reported the high proteolytic activity of LP in fermentation of soy protein causing an increase in the content of small peptides. Oyedoh et al. (2020) also observed high proteolysis and high concentration of peptides when cowpea was fermented with LP. ST is known for its high proteolytic activity in milk (Galia et al., 2009). Moreover, there are some evidence of its proteolytic activity in legumes. For instance, Hati et al. (2018) reported a maximum proteolysis and peptide generation for ST in fermentation of both bovine milk and soy milk. Boulay et al. (2020) studied the role of CEP in the growth of ST in soy protein and they observed a high proteolytic activity of ST resulted to the generation of more peptides and consequently a better growth of this strain. The increase in proteolytic activity of these LAB once in a co-culture has been reported previously. For instance, Madjirebaye et al. (2022) observed an increased in the content of small peptides for co-culture of ST and LP compared to their mono-culture in fermentation of soy milk. Li et al. (2019) also reported a higher proteolytic activity of STLP compared to the mono-culture of ST or its co-culture with *B. animalis*, which lead to the production of more free amino groups in fermented milk. Although the proteolytic activity and acid production capacity of the bacteria are known to be strain dependent (Lim et al., 2019), there was no difference between the samples fermented with ST and LP. Despite the initial differences in protein profile of the pea cultivars, there was no significant difference in their free amino groups content. In albumin fraction, AS fermented with ST and LP had lightly higher content of free amino groups content compared to the two other cultivars, but differences did not reach to be significant.

Also the content of free amino groups was about 40-60 times lower in globulin fraction compared to the albumin ones as the released peptides during fermentation are more soluble and are mostly recovered in the latter fraction.

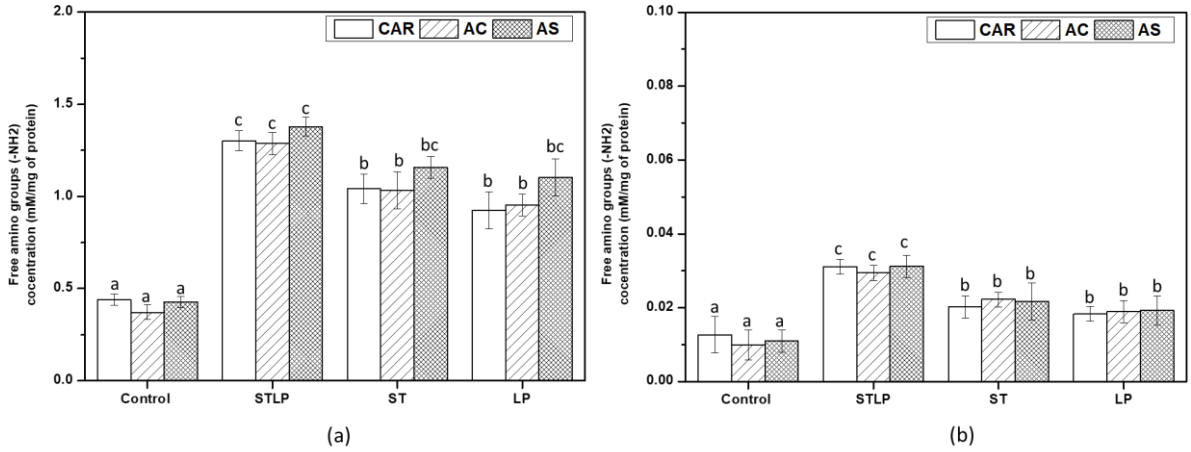


Figure V-7. Free amino groups content of albumin (a) and globulin (b) fraction obtained without (control) or with added fermentation by *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP). Comparison between different pea cultivars: CAR, AC and AS. Different letters represent significant differences in free amino groups content among different samples (Tukey's post hoc test, P value < 0.05).

V.2.5. Protein profiles

V.2.5.1. SDS PolyAcrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed in non-reducing and reducing conditions to determine the effect of extraction method on polypeptide composition of the recovered albumin and globulin fractions of different pea cultivars (CAR, AC, AS). In non-reducing conditions (NR), the polypeptide profile of albumin (Fig. V-8) showed the presence of bands ranging from ~6 to ~99 kDa. In control, the bands corresponding to lipoxygenase (Lox, ~94 kDa) (Shand et al., 2007), Convicilin (CV, ~71 kDa) (Shand et al., 2007), vicilin monomer (Vαβγ, ~50 kDa) and the cleavage resulting polypeptides (Vαβ ,~30–36 kDa; Vα, ~20 kDa; Vβ, ~13kDa; Vγ, ~12–16 kDa) (Dziuba et al., 2014; Gatehouse et al., 1982), lectine (Lect, ~17 kDa) (Crévieu et al., 1996), and the main 2S albumin subunits (PA2, ~26kDa; PA1, ~6kDa) (Dziuba et al., 2014; Gruen et al., 1987), were observed. While in fermented samples, CV and Vαβγ were absent. Other globulin contamination could be observed depending on LAB strains and cultivars. For instance, Vβγ (~25–30 kDa) was mainly observed in cultivars CAR and AC fermented with ST and LP. These two cultivars had initially higher content of vicilin compared to cultivar AS. Different vicilin subunits (Vα, ~20 kDa; Vβ, ~13kDa; Vγ, ~12–16 kDa) were mainly observed for cultivars fermented with co-culture. Legumin acidic subunit (Lα, ~40 kDa) (Liang & Tang, 2013) which was separated from legumin monomer (Lαβ ~60kDa) under reducing condition,

was observed for cultivars fermented with co-culture, and cultivars AS and AC fermented with both ST or LP. The high intensity of bands smaller than 20 kDa in the electrophoretic profile of co-culture could indicate the presence of more peptides in this region. Production of small protein fractions and the disappearance of high Mw proteins was related to the proteolytic activity of LAB as revealed before by amino group quantification. The disappearance of high Mw protein in electrophoretic profile after lactic acid fermentation has been previously shown in pea flour (Barkholt et al., 1998) and pea protein isolate (Emkani et al., 2021). Boulay et al. (2020) explained the proteolytic activity of ST in soy protein by the disappearance of high Mw protein and presence of low Mw compounds in polypeptide profile.

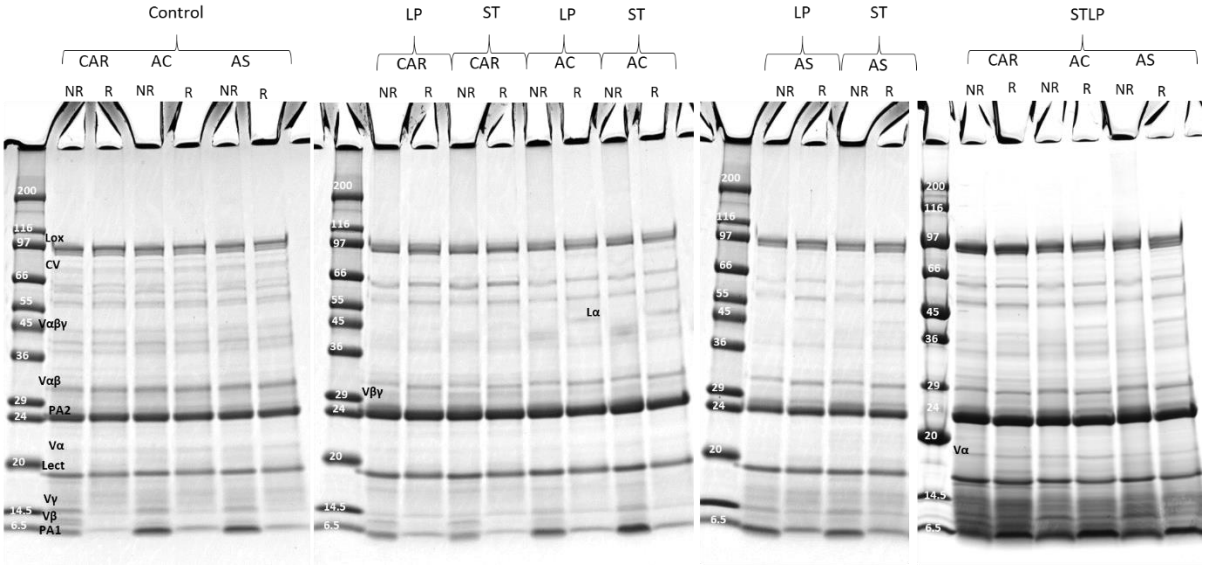


Figure V-8. Electrophoresis profile of albumin fractions obtained without (control) or with added fermentation by *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP). Comparison between different pea cultivars: CAR, AC and AS in non-reducing (NR) and reducing (R) conditions.

The polypeptide profile of globulin fraction (Fig V-9) in non-reducing condition revealed the presence of bands ranging from ~10 to ~99 kDa. In globulin fraction, the 2S albumin subunits (PA2, ~26kDa; PA1, ~6kDa) (Dziuba et al., 2014; Gruen et al., 1987) were clearly absent for all the samples. While, different group of Vαβγ (~50 kDa) and derived subunits, Lαβ (~60 kDa) and its acidic (Lα, ~40 kDa) and basic (Lβ, ~20 kDa) subunits, CV, LOX were observed for all the samples. The band corresponding to Vγ (~12–16 kDa) was absent in cultivar AC obtained without fermentation (control) and with co-culture. While this fraction was present for AC obtained by ST and LP. This is probably related to the hydrolysis of vicilin monomer by ST

and LP. The bands corresponding to vicilin subunits (<24 kDa) in globulin fraction of co-culture seemed to be narrow which might be also representing the proteolysis of vicilin. In the same time, this could explain why different groups of vicilin subunits were observed in albumin fractions related to co-culture samples.

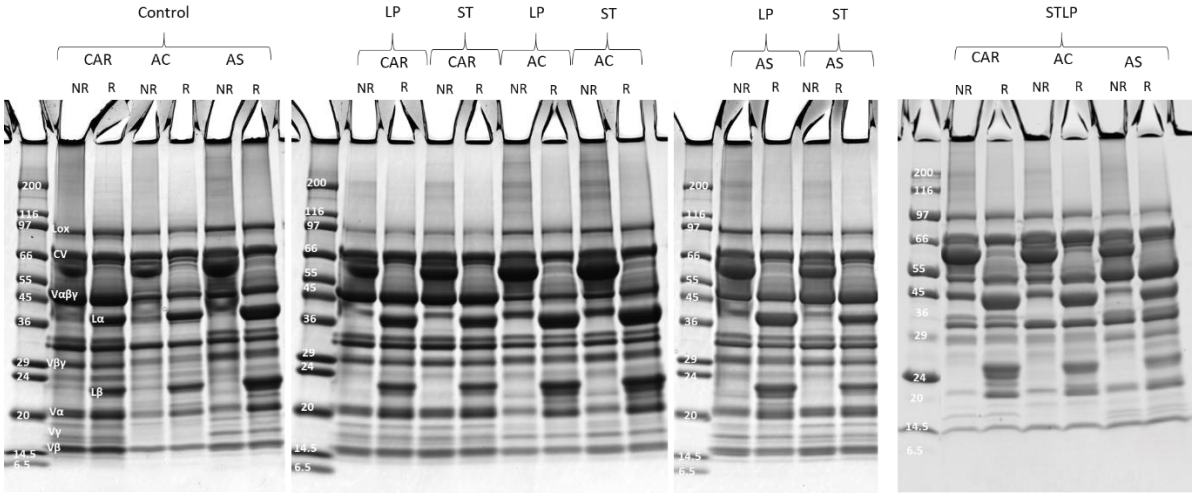


Figure V-9. Electrophoresis profile of globulin fractions obtained without (control) or with added fermentation with *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP). Comparison between different pea cultivars: CAR, AC and AS in non-reducing (NR) and reducing (R) conditions.

V.2.5.2. Size distribution of peptides

SEC-HPLC was performed in order to study in detail the Mw distribution of peptides (<20 kDa) in the albumin fraction. Fig.V-10 showed the representative chromatograms for albumin fractions obtained without (control) and with fermentation with STLP, ST and LP, by comparing the three different pea varieties. Seven different classes of Mw were distinguished in term of their elution volume. The area of the peaks corresponding to each class were integrated to define the proportions corresponding to the different fractions, considering their sum equal to 100% (Table V-2).

The elution profiles of pea albumin fractions corresponding to control samples (Fig V-10 a), showed 3 major peaks (from ~4 to ~10 mL). Two minor peaks at around 4 and 6 mL elution volume which were related to the highest Mw protein components (>2 kDa) of the samples. These two peaks could be assigned to the PA1 protein which were also observed in electrophoresis pattern of all the samples at Mw around 4-6 kDa. These calculated values

matched with the ones from literature (Eyraud et al., 2013; Gressent et al., 2011). The percentage of integrated area of these two peaks was significantly lower for control (~0.5%) compared to the fermented samples (~13%) (Table V-2) which meant fermented samples contained higher content of smaller peptides.

The third peak was the sharp one which was observed for all the samples at around 8 mL elution volume representing peptides with Mw of around 0.8 kDa. This peak had higher integrated area for the samples fermented with STLP and ST (~12%) compared to LP (~7.2-9.4%). The lowest value was obtained for the control samples. The four other peaks at higher elution volumes (~9, ~10, ~10.5, ~11.5 mL) representing lower Mw peptides and amino acids (<0.8 kDa) were only present for fermented samples. The proportion of these peaks was significantly different between the strains and cultivars (Table V-2). Fermentation with co-culture led to higher proportion of these peaks compared to the mono-culture. A peak at around 10 mL elution volume was only observed for samples fermented with STLP and cultivar AS fermented with ST and LP. In the last case (i.e. for AS), the proportion of this peak seemed to be higher in co-culture compared to ST and LP mono-cultures. The next two peaks at around 10.5 and 11 mL elution volume were detected for all the samples. These peaks had higher integrated area for the samples fermented with STLP. To resume, fermentation, especially with the co-culture, released higher number of small oligopeptides/peptides (with Mw ranging from 10 to 0.2 kDa) and in higher proportion compare to the controls. The majority of these peptides were in the range of 0.8 to 0.2 kDa. An increase in the content of small peptides during fermentation is associated with many beneficial health activities such as angiotensin l-converting enzyme (ACE)-inhibitory activity, opioid, antioxidant, antidiabetic, immunomodulatory, and antimicrobial activities (Emkani et al., 2022).

Regarding cultivars, the most significant differences were observed at elution volume ~4 and ~6 mLwhich belonged to the polypeptides higher than 2 kDa. At elution volume ~4, the highest value was related to AC fermented with ST and it followed by CAR fermented with LP. While cultivar AS seemed to have richer profile in lower Mw peptides than the other two cultivars. This result agreed with NPN and free amino groups content data.

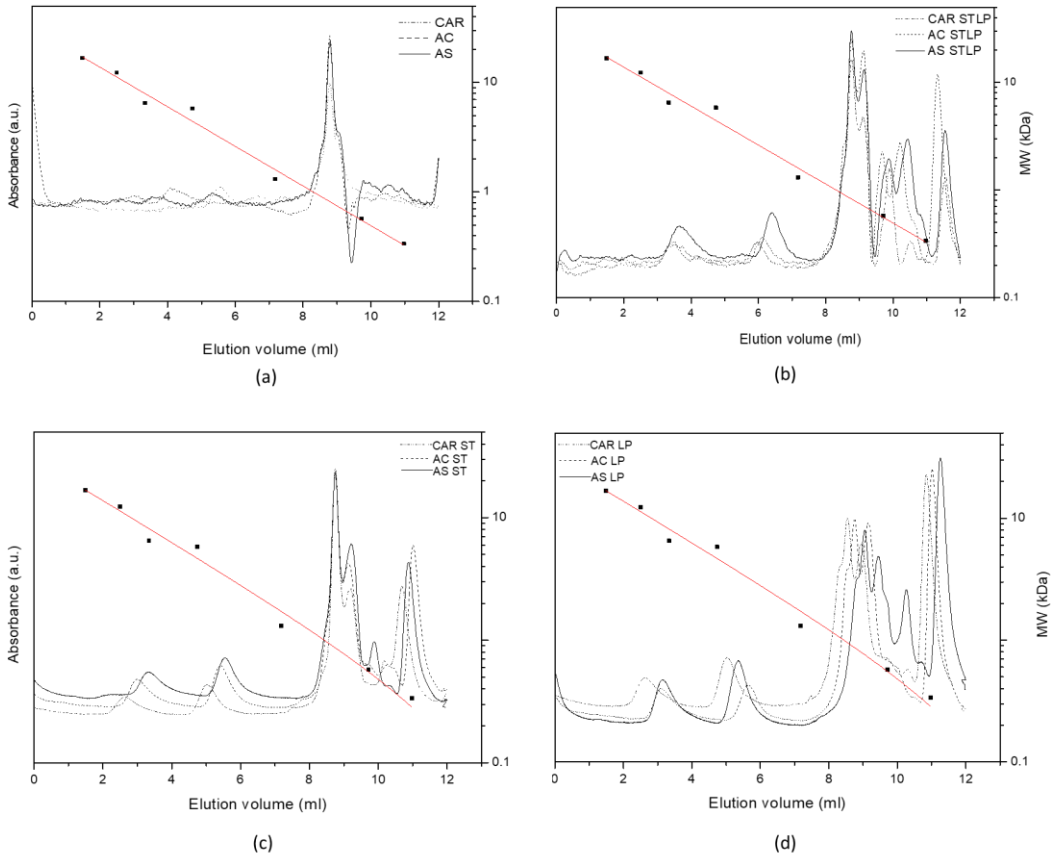


Figure V-10. SEC-HPLC chromatograms of albumin fractions obtained without (control) (a) or with added fermentation by *S. thermophilus* (ST) (c), *Lb. plantarum* (LP) (d) or their mixture (STLP) (b). Comparison between different pea cultivars: CAR, AC and AS. The calibration curve was represented as red line. Standards name with their corresponding Mw (■) was given in (c). The calibration curve equation and the correlation coefficients were y=-0.18x+1.503, R2=0.986.

Table V-2. Percentage of integrated area of SEC-HPLC chromatograms of albumin fractions obtained without (control) or with added fermentation by *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP). Comparison between different pea cultivars: CAR, AC and AS.

Elution volume (mL)	**~4**	**~6**	**~8**	**~9**	**~10**	**~10.5**	**~11.5**
Mw (kDa)	**~9-5.5**	**~1.8-4**	**~0.8-0.7**	**~0.7-0.6**	**~0.5-0.45**	**~0.4-0.35**	**~0.35-0.2**
CAR control	Trace	0.6± 0.02a[1]	0.8± 0.02a	ND	ND	ND	ND
AC control	0.5± 0.03a	0.6± 0.02a	0.8± 0.02a	ND	ND	ND	ND
AS control	0.5 ± 0.02a	0.4 ± 0.04a	0.9± 0.01a	ND	ND	ND	ND
CAR STLP	1.8± 0.03b	14.7± 0.2f	11.9± 0.6d	16.7± 0.9d	31.6± 0.48b	23.2± 0.9c	13.2± 0.8de
AC STLP	1.5± 0.04ab	13.5± 0.3e	11.6± 0.5d	18.9± 0.7e	31.3± 0.5b	23.6± 0.6c	14.8± 0.7e
AS STLP	1.3± 0.03ab	20.9± 0.5g	12.4± 0.6d	14.4± 0.6c	30.8± 0.5b	31.5± 0.9d	14.8± 0.6e
CAR ST	9.0± 0.2c	3.3± 0.02b	12.5± 0.6d	11.3± 0.3b	ND	9.8± 0.5a	9.8± 0.9b
AC ST	15.7± 0.5f	11.6± 0.1d	11.8± 0.7d	7.7± 0.5a	ND	11.4± 0.6b	8.8± 0.8ab
AS ST	13.2± 0.4de	8 ± 0.1c	11.5± 0.7d	11.0± 0.4b	3± 0.2a	Trace	10.3± 0.7bc
CAR LP	13.5± 0.6e	13± 0.1e	9.4 ± 0.4c	6.7± 0.3a	ND	Trace	9.8± 0.8b
AC LP	8.2± 0.1c	3.5 ± 0.03b	7.2± 0.6b	6.5± 0.5a	ND	Trace	7.9± 0.7a
AS LP	12.32± 0.5d	7.21 ± 0.1c	8.9± 0.4bc	6.3± 0.4a	3± 0.3a	Trace	11.7± 0.7cd

[1] Proportion of integrated area was presented as average ± standard deviation, n (n=3). Different letters in the same column represent significant differences among different samples (Tukey's post hoc test, P value < 0.05). ND: not detected by SEC-HPLC.

V.2.6. Effects of added fermentation on antioxidant activity and antinutritional factors

V.2.6.1. Antioxidant activity

The antioxidant activity (AO) of albumin samples obtained without (control) and with fermentation with ST, LP and their co-culture (STLP), was evaluated. As shown in FigV-11, DPPH scavenging activity (Fig V-11. a) and TEAC (Fig V-11. b) in fermented samples was approximately 2-3 times higher than control. This indicated a correlation between the two methods used for measuring antioxidant activity. The value of DPPH scavenging was around 23% in control. While this value was around 45% for mono-cultures and around 56% in co-cultures. TEAC was around 0.1 (nmol Trolox equivalent/µL sample) for control. In fermented samples this value was ~0.5 (nmol Trolox equivalent/µL sample) for mono-cultures and ~0.65 (nmol Trolox equivalent/µL sample) for co-culture. The improved antioxidant activity for fermented samples could be associated to the production of peptides during fermentation. Release of small peptides with antioxidant activity from legumes has been reported numerously. For instance, Leksono et al. (2022) reported an increase in antioxidant activity of black soybean milk fermented with *S. thermophilus* and *Lb. plantarum* mono-cultures. They also observed similar pattern in increasing the antioxidant activity between the strains. Sáez et al. (2021) observed that chickpea fermented with *Lb. plantarum* had 40% increase in antioxidant activity compared to control. Torino et al. (2013) also reported an increase in

antioxidant capacity of lentil fermented with LP. Naprasrt et al. (2019) observed that the antioxidant activity of fermented red bean milk was 2 times higher than unfermented and the highest value among different LAB was related to LP. On the contrary to the results of this study, Lee et al. (2015) reported that in fermentation of black soymilk with *S. thermophilus* and its co-culture with *Lb. plantarum*, the highest antioxidant activity belonged to mono-culture compared to co-culture.

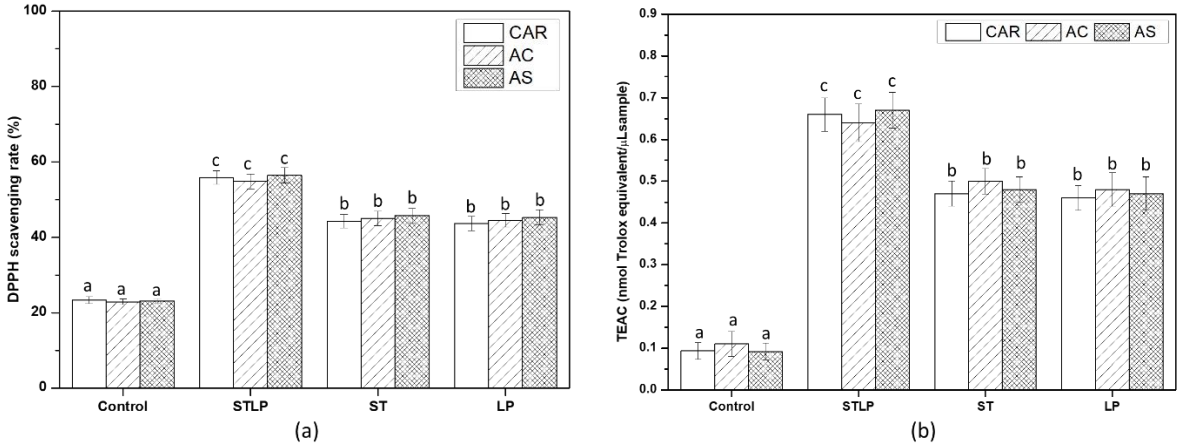


Figure V-11. Radical scavenging capacity on DPPH (a) and Trolox equivalent antioxidant capacity (TEAC) (b) of albumin fractions obtained without (control) or with added fermentation by *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP). Comparison between different pea cultivars: CAR, AC and AS. Different letters represent significant differences among different samples (Tukey's post hoc test, P value < 0.05).

V.2.6.2. Consumption of sucrose, glucose and α-galactosides

Fig. V-12 showed the ratio of glucose (a), sucrose (b) and RFOs (c), in albumin fractions obtained without (control) and with fermentation with ST, LP and their co-culture (STLP) calculated from protein extract of different pea cultivars (CAR, AC, AS). In control, the ratio of these carbohydrate was close to 1. Indeed, these carbohydrates are all water soluble and their majority supposed to be found in albumin fraction after the acidification step. The content of glucose in albumin fractions obtained by control was around ~0.038 M (6.8 g/L sample), sucrose around ~0.016-0.02 M sample (5.4 g/L sample) and RFOs was around ~0.03-0.045 M sample. No significant differences were observed in the content of glucose between the cultivars obtained by control. However, cultivar AC had higher content of sucrose and RFOs in control.

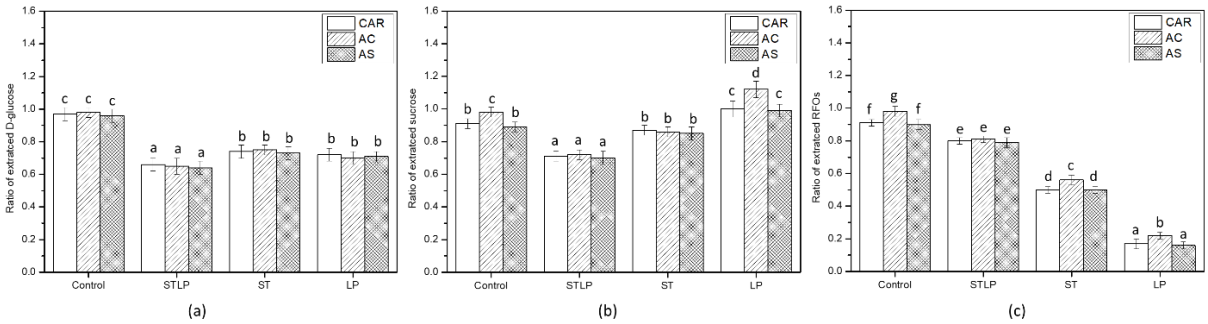


Figure V-12. The ratio of extracted glucose (a), sucrose (b), RFOs (c) of albumin fractions obtained without (control) or with added fermentation by *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP), calculated from protein extract. Comparison between different pea cultivars: CAR, AC and AS. Different letters represent significant differences among different samples (Tukey's post hoc test, P value < 0.05).

While in fermented samples, LAB were able to reduce the content of these sugars by consuming them as a source of energy for growth. The content of glucose was reduced by all the strains (Fig V-12. a). The lowest value of glucose and sucrose was related to STLP. The ability of ST and LP to consume glucose was reported previously (Cui et al., 2021; Galia et al., 2009). Both ST and LP had α-galactosidase activities since they were able to reduce the content of RFOs (Fig V-12. c). The α-galactosidase activity of ST (Donkor et al., 2007; Simović et al., 2016) and LP (Delgado-Fernandez et al., 2020; Silvestroni et al., 2002) have been shown previously. However, the activity of this enzyme seemed to be strain dependent. The highest consumption of α-galactosides was associated to the fermentation with LP. The reduction of RFOs was around 62% in LP and around 40% in ST regardless of pea cultivar. In samples obtained by ST and LP, cultivar AC had higher content of RFOs compared to the two other cultivars. The lowest α-galactoside consumption was observed for the co-culture (~20%). In general, the pH optimum pH for bacterial α-galactosidases is in the range of 6.0 to 7.5 (Anisha, 2022). It could be then suggested that the rapid reduction of pH in co-culture limited the action of this enzyme. Simultaneously to the α-galactoside content reduction, an increase (~10%) in sucrose content was measured for LP, especially with cultivar AC (Fig V-12. b). This result could be related to the combination of two effects: (i) the noticeable reduction in RFO content increased sucrose release due to the α-galactosidase activity and (ii) LP is not able to catabolize sucrose efficiently as already indicated by Wang et al. (2022). Also, no significant change in sucrose content was observed for ST compared to control. It is known that ST is able to consume sucrose (Galia et al., 2009). The consumption of sucrose by ST would be contracted by the reduction of RFOs.

The increase in the content of sucrose during fermentation has been previously reported for the fermentation of soymilk by *Lb. fermentum* (Garro et al., 1998).

V.2.6.3. Trypsin inhibitor activity

Trypsin inhibitor activity (TIA) was measured for pea flours of three cultivars (CAR, AC, AS) and the corresponding albumin fraction obtained without (control) or with fermentation with ST, LP or their co-culture (STLP) (Fig.V-13). The amount of TIA in pea flours was around ~14.8 TIU/mg sample with no significant differences between the cultivars. In general, pea is known for having lower amount of trypsin inhibitor compared to other legume (Bacon et al., 1995a). However, the amount of TIA can be significant in some cultivars and it can vary from 1 to 15 TIU/mg sample depending on the cultivars (Bacon et al., 1995b; Griffiths, 1984; Wang et al., 1998). The analysis also showed that the value of TIA in control (~15 TIU/mg sample) of albumin fraction samples did not change significantly from pea flours. This result confirmed the fact that trypsin inhibitor was recovered in the albumin fraction during extraction (Park et al., 2010; Reinkensmeier et al., 2015). However, the TIA decreased to around ~8 TIU/mg sample for the fermented albumin fraction samples with again no significant difference within pea varieties. This might be related to the proteolytic activity of LAB that could affect the native protein structure of trypsin inhibitor. The decreased level of TIA after fermentation with LAB in pea have been reported previously. For instance, Ma et al. (2018) reported a reduction of approximately ~50% in TIA of pea seeds fermented with a mixed LAB culture (containing *S. thermophilus*, *L. bulgaricus*, and *L. acidophilus*). Cabuk et al. (2018a), observed the content of TIA in pea protein isolate dropped around 18 to 50% (as the fermentation time increased) after fermentation with *Lb. plantarum.* Byanju et al. (2021) reported the fermentation of green pea with *Lb. plantarum* reduced TIA by ~47%. In the present study, no significant difference was observed between the mono-culture samples. However, the value of TIA was lower for the samples fermented with co-culture (~6.8 TIU/mg sample) compare to the mono-culture samples, pointing out again higher proteolytic activity of STLP compared to ST or LP alone. As it was previously observed in soy milk fermentation with co-culture of *S. thermophilus,* and *Lb. plantarum* by Madjirebaye et al. (2022).

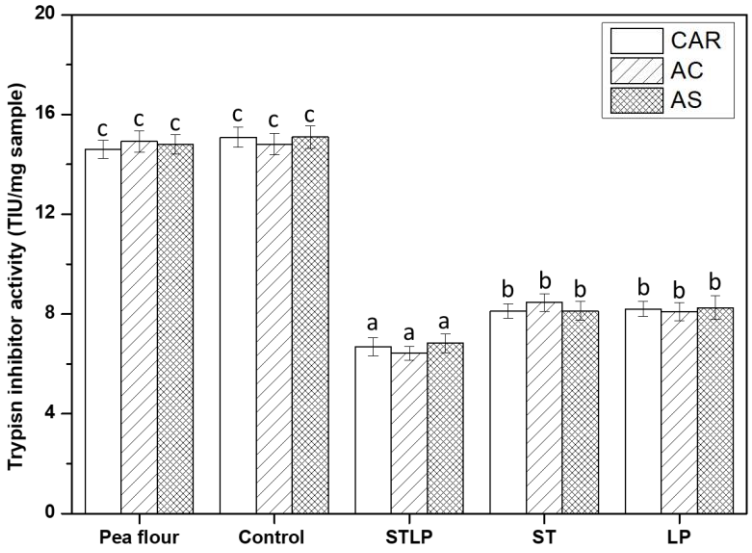


Figure V-13. Trypsin inhibitor activity (trypsin inhibitor unit/mg sample) of albumin fractions obtained without (control) or with added fermentation by *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP). Comparison between different pea cultivars: CAR, AC and AS. Different letters represent significant differences among different samples (Tukey's post hoc test, P value < 0.05).

V.2.6.4. Phytic acid

Phytic acid (PA) content was measured for pea flour of the three cultivars (CAR, AC, AS) and the corresponding albumin fractions obtained without (control) or with fermentation with ST, LP or their mixture (STLP) (Fig V-14). The content of PA in pea cultivars AS and AC was around 0.9 mg/g of flour sample in dry basis. While, this value was significantly lower in CAR (around 0.8 mg/g flour). Also, there was a significant reduction in the amount of PA for albumin fractions in control (~0.2 mg/g freez-dried albumin sample in dry basis). It could be possible that during the extraction of pea protein, PA initially present in protein bodies was not totally solubilized or that insoluble complexes were formed with other compounds such as globulins and divalent cations (Amat et al., 2022). The reduction of PA in albumin fractions can also be explained by endogenous phytase activity of legume, as phytase is primarily localised in the protein bodies (Scott, 1991). The content of PA in fermented (~0.06-0.09 mg/g freez-dried albumin sample in dry basis) samples was 2-3 times lower than control. The degradation of PA in fermented samples could be mainly related to the microbial phytase activity of both ST and LP (Noureddini & Dang, 2009). LP and ST are both able to produce phytase. Sumengen et al. (2013) reported a high intra- and extra-cellular phytase activity for *Lb. plantarum.* A high phytase activity also was reported for *S. thermophilus* by Priyodip and

Balaji (2018, 2020). Although, in the present study, no significant differences were observed between the strains which could suggest the similar activity of phytase among ST and LP. Several works have proved the PA reduction in other fermented legume samples by these bacteria species. For instance, Xing et al. (2020) showed the degradation of PA in chickpea protein concentrate after fermentation with different LAB species. Fritsch et al. (2015) also observed a reduction of PA content by more than half in lupin protein isolate fermented with *Lb. plantarum*. Rui et al. (2017) reported a reduction of 50% in the content of phytic acid (~3mg/g sample) for soy seeds fermented with *Lb. plantarum*. Mefleh et al. (2022) reported ~70% reduction in PA content for chickpea protein isolate fermented with *S. thermophilus* mono-culture and its co-culture with *Lb. plantarum*.

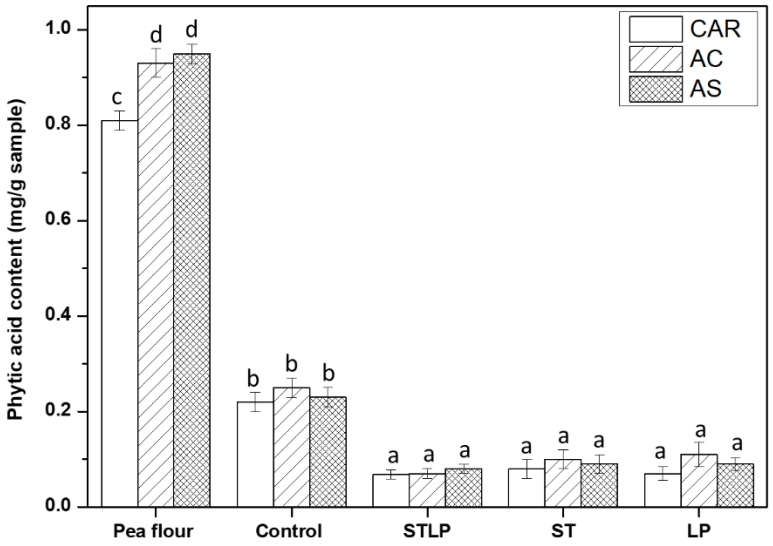


Figure V-14. Phytic acid content of albumin fractions obtained without (control) or with added fermentation by *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP). Comparison between different pea cultivars: CAR, AC and AS. Different letters represent significant differences among different samples (Tukey's post hoc test, P value < 0.05).

V.3. Conclusion

Results from the study support the hypothesis that AEIEP extraction assisted by LAB fermentation improves the nutritional quality of albumin fraction from peas compared to the traditional AEIEP method without impairing the protein profile of the globulin fraction which is usually recovered to produce pea protein isolate. Using LP, ST and their co-culture allowed modifications in protein composition by increasing nitrogen and small peptides content, the increase in antioxidant activity and the reduction of antinutritional compounds such as trypsin

inhibitor, α-galactosides and phytic acid. These effects were mainly associated to the proteolytic activity of the selected LAB strains during fermentation. However, these changes depended on the microorganism used. The albumin fractions obtained by LP fermentation significantly reduced the RFO, and that obtained by the co-culture showed the highest level of proteolysis and the highest production of small peptides which was related to the synergetic effect of the bacteria. These results indicate the possibility of tailoring properties of the albumin fraction depending on its application. To industrially optimize the use of pea albumin fraction, it would be necessary to study the effect of this method on the organoleptic quality of the albumin fraction. The physicochemical and technological properties of this fraction should be also considered.

VI. Identification of volatile compounds and odor-active compounds in pea protein fractions

VI.1. Introduction

Despite its popularity, pea protein is associated with off-flavors which can hinder their application in food products. The off-flavors in peas are mainly described as green and beany. These off-flavors can be inherent to pea or developed during various steps of pea protein production such as harvest, storage, physical treatment, chemical and biological process (Roland et al., 2017). The main reason in development of off-flavors is the oxidation of fatty acids during the harvest or storage which can be enzymatic (lipoxygenase) or non-enzymatic (autoxidation). The off-flavors can also be formed during the biodegradation of amino acids or the effect of heat on sugar and amino acids (Maillard reaction), oxidative and thermal degradation of carotenoids as well as the contaminations during the extraction process (Murray et al., 1976; Roland et al., 2017).

Several molecules are responsible for beany off-flavors in peas among which 3-methylbutan-1-ol, pentan-1-ol, oct-1-en-3-ol, (2*E*,4*E*)-hepta-2,4-dienal, acetophenone, oct-1-en-3-one and 3-isopropyl-2methoxypyrazine have been reported numerously (Fischer et al., 2022b; Trindler et al., 2021). However, these compounds can develop the beany note only at certain concentration of these compounds (Vara-Ubol et al., 2004). Moreover, the perception of an off-flavors is for the presence of a combination of volatile compounds rather than one molecule. For instance, hexanal has no beany odor by itself, but it can increase the intensity of beany note in combination with another volatile compound (Bott & Chambers IV, 2006).

The presence of off-flavors in pea protein also depends on the nature of the volatile compounds and protein (Guichard, 2002). For instance, non-polar volatile compounds are likely to retain in protein fraction with more hydrophobic site. While polar volatile compounds are soluble and retained in the soluble fraction (Heng et al., 2004). The purity of the protein also affects the retention and subsequently the perception of off-flavors. For instance, Heng (2005) indicated the presence of more volatile compounds in pea flour compare to pea protein.

Hence, controlling the conditions of raw material, extraction and food processing could alter the aromatic profile of pea protein. There are some evidences that lactic acid fermentation can improve the aromatic profile of legume proteins by either reducing these off-flavours during enzymatic activity and/or by masking them by formation of new volatile compounds through the metabolic pathways (Ben-Harb et al., 2019; Pei et al., 2022; Schindler et al., 2011; X. Zhang et al., 2022). Moreover, there has been a growing interest on application of LAB in food

processing and in particular in legume-based dairy products partly for their contribution on nutritional value, health promoting and techno-functional properties of the products (Knez et al., 2023; Leksono et al., 2022; Sáez et al., 2021; Singh et al., 2020; Verni et al., 2019).

A recent study in our team represented an alternative extraction method to alkaline solublization/ isoelectric precipitation in which the reduction of pH achieved during the fermentation thanks to the metabolism of bacteria, instead of mineral acid addition (Emkani et al., 2021). It was also shown in previous chapter that, depending on the strains, LAB had different enzymatic activity (proteolytic, glycolytic) which can lead to the production of different volatile compounds. For instance, higher proteolytic activity was observed in the samples fermented with co-culture of *S. thermophilus* and *Lb. plantarum*. These amino acids could be the precursors of volatile compounds with positive notes. Hence, the purpose of this study was to identify the volatile compounds of albumin and globulin fractions obtained without (control) or with fermentation by *S. thermophilus* (ST) and *Lb. plantarum* (LP) or their co-culture (STLP). Only one cultivar (AS) was selected in this study for the higher production of small peptides in albumin fraction obtained by fermentation in this cultivar compared to the two others. Volatile compounds were isolated by Solvent Assisted Flavor Evaporation (SAFE) method since the method enable the maximum isolation of volatile compounds from matrix. The isolated volatile compounds were subsequently characterized by Gas Chromatography-Mass Spectrometry (GC-MS). The identification of volatile compounds was completed by detection of odor active compounds by Gas chromatography-Olfactometry (GC-O).

VI.2. Results

VI.2.1. Identification of volatile compounds by GC-MS

Volatile compounds of albumin (A) and globulin (G) fractions obtained without (control) or with fermentation by LP, ST and STLP from pea flour, were characterized by GC-MS (Table VI-1). A total of 96 volatile compounds in pea protein albumin and globulin fractions were identified by GC-MS. The most representative chemical classes in all the samples included ketones, alcohols, acids, aldehydes, esters and lactones. While only a few terpenes, pyrazines and thiazoles were detected. 68 compounds have been already reported for pea protein or pea flour, 10 have been reported in other pulses and to the best of authors knowledge16 have not been detected in pea neither in the other pulses (Table VI-1). The majority of volatile compounds were the products of fatty acids and amino acid degradation (Frankel, 1983). While

a few of them were originated from carotenoids belonging to the ketones. Some of these volatiles were present in all the samples with no significant differences in their concentration, i.e. butanoic acid, dodeca-1-ol, nonanal, dodecanal, isopropyl palmitate, pentan-2-one, 4-methylpentan-2-one, 6-methylheptan-2-one, 5-methylheptan-2-one, nonan-2-one, undecan-2-one and γ-decalactone. Some of the volatile compounds were only detected in fermented samples, i.e. 3-methylbut-3-en-1-ol, (*E*)-oct-2-en-1-ol, undecan-2-ol, ethyl 2-hydroxypropanoate, ethyl 2-hydroxy-4-methylpentanoate, dimethyl glutarate, δ-decalactone, geraniol, 2,5-dimethylpyrazine. While some others were only detected in controls, i.e. methional and octan-2-one.

Comparing the acid volatile compounds in albumin and globulin fractions of control samples, A-control (2.3 µg/mL of sample) had higher content of total acids compared to G-control (1.3 µg/mL of sample), although significant difference was only observed in pentanoic acid quantity. Fermented samples (both albumin and globulin samples) had higher content in acids compared to the controls. Among fermented samples, albumin fractions (~5.4-14.2 µg/mL of sample) had higher content of total acids compared to globulin fractions (~1-8 µg/mL of sample). A-ST had the highest value in acetic acid, hexanoic acid, decanoic acid and octanoic acid. In globulin fermented samples, G-STLP had the highest content of propanoic acid, pentanoic acid and nonanoic acid. 2-methylbutanoic acid was not found in G-control and fermented globulin samples (Table VI-1).

G-control (6.8 µg/mL of sample) had higher content of total alcohols compared to A-control (3.9 µg/mL of sample). 1-methoxypropan-2-ol, pent-1-en-3-ol, pentan-1-ol, (*Z*)-pent-2-en-1-ol, oct-1-en-3-ol, 2-ethyl-1-hexanol and decan-1-ol, had the highest value in G-control compared to A-control and fermented samples. 3-methylbut-3-en-1-ol was not detected in G-control and A-control and it had the highest value in A-ST. (*E*)-oct-2-en-1-ol was only present in G-ST and G-LP. undecan-2-ol was only detected in G-ST and G-STLP. 3-methyl-butan-1-ol was not present in A-control, while it had higher value in all the albumin fermented samples. 2-methylpropan-1-ol, hexan-1-ol, (*Z*)-hex-3-en-1-ol and phenol had the highest value in A-LP. benzenemethanol had the highest value in A-STLP and A-LP.

Esters were mainly detected in albumin fermented samples in particular A-STLP. Ethyl 2-hydroxypropanoate was not found in G-control and A-control and it had the highest value in A-STLP. isopropyl myristate had the highest content in A-LP, A-ST and A-STLP. ethyl

butanoate had the highest value in A-ST. dimethyl glutarate was only found in A-ST and A-STLP. ethyl 2-hydroxy-4-methylpentanoate was only found in A-STLP and A-LP.

There was no significant difference in the concentration of lactones between albumin and globulin controls, except for γ-pentalactone which was higher in A-control. Same as esters, the total content of lactones was higher in fermented samples (~0.2-0.5 µg/mL of sample) compared to controls (0.1 µg/mL of sample) especially the ones fermented with STLP and A-LP. γ-decalactone was only detected in fermented samples. γ-pentalactone was mainly detected in albumin fraction and it had the highest value in A-STLP. γ-octalactone had the highest value in A-STLP and γ-butyrolactone had the highest value in A-STLP, G-STLP and A-ST.

Aldehydes seemed to be more abundant in globulin fractions (~3.6-6 µg/mL of sample). G-control had the highest value in hexanal, octanal, 2-ethylhexanal and tetradecanal, compared to A-control and fermented samples. G-ST seemed to have the highest concentration in (*E*)-hexen-2-al, (*E*)-octen-2-al and benzaldehyde. (2*E*,4*E*)-nona-2,4-dienal and (2*E*,4*Z*)-deca-2,4-dienal were not detected in albumin fractions and it had only a trace amount in G-control, G-LP and G-ST. 3-methyl-buten-2-al was mainly detected in fermented samples especially the ones fermented with ST.

G-control represented a higher content in majority of ketones i.e. pentane-2,3-dione, heptan-3-one, octan-2-one, (3*E*,5*E*)-octa-3,5-dien-2-one and acetophenone compared to A-control, and albumin and globulin fermented samples. (3*E*,5*E*)-octa-3,5-dien-2-one is one of the typical aroma compounds responsible for green notes and it is often reported in pea products (Ebert et al., 2022; Shi et al., 2021; Utz et al., 2022; Xiang et al., 2022). This compound was not found in A-control and it had small quantity in albumin and globulin fermented samples. pentane-2,3-dione was mainly detected in globulin fractions. On the other hand, there was an increase in the content of some other ketones in fermented samples. For instance, butane-2,3-dione which is an important product of glycolysis metabolism of LAB (Chen et al., 2017), had the highest concentration in A-STLP and it was followed by G-STLP. 1-hydroxypropan-2-one and decan-2-one had the highest concentration in fermented albumin fractions. 3-hydroxybutan-2-one had the highest content in A-ST, A-STLP and G-STLP.

There were only two terpenes detected i.e. verbenone and (*E*)-geraniol. (*E*)-geraniol was not detected in controls and G-STLP samples while it showed the highest concentration in G-LP. Pyrazines generally have lower concentration in pea protein and they can barely be detected.

Here 2,5-dimethylpyrazine was not detected in controls and it had trace quantity in the other samples.

Table VI-1. Volatile compounds in pea albumin (A) and globulin (G) fractions obtained without (control) or with fermentation by *S. thermophilus* (ST) and *Lb. plantarum* (LP) or their co-culture (STLP) identified by GC-MS.

Aroma compounds	CAS*	LRI_{exp}*	LRI_{lit}*	Origin	Standard equivalent concentration (µg/mL of sample) **								P value	log Pow***	Reliability****	References
					A-control	G-control	A-LP	G-LP	A-ST	G-ST	A-STLP	G-STLP				
Acids																
acetic acid	64-19-7	1450	1452	AA[7]	0.287bc ±0.037	0.048c ±0.028	0.653bc ±0.388	0.064c ±0.007	7.194a ±0.359	0.075c ±0.015	0.793bc ±0.154	1.267b ±0.734	<0.0001	-0.1	MS, LRI, I	1,2, 3
propanoic acid	79-09-4	1540	1556	AA[11]	0.415ab ±0.030	0.106b ±0.062	0.125b ±0.0199	0.182b ±0.0196	0.316ab ±0.021	0.137b ±0.0159	0.326ab ±0.0257	0.957a ±0.561	0.004	0.3	MS, LRI, I	1
butanoic acid	107-92-6	1635	1628		0.245a ±0.007	0.295a ±0.18	0.637a ±0.30	0.288a ±0.05	0.618a ±0.073	0.311a ±0.0129	0.276a ±0.007	0.633a ±0.371	0.135	0.7	MS, LRI, I	4
2-methylbutanoic acid	116-53-0	1670	1674	AA[3]	0.097b ±0.007	ND	1.871a ±1.082	ND	1.430ab± 0.037	ND	0.323ab ±0.241	ND	0.030	1.4	MS, LRI	2, 5,6
pentanoic acid	109-52-4	1749	1763		0.365a ±0.007	0.011b ±0.006	0.078b ±0.045	0.030b ±0.0007	0.188ab ±0.007	0.044b ±0.0007	0.138ab ±0.007	0.384a ±0.230	0.001	1.3	MS, LRI, I	1, 4, 5
hexanoic acid	142-62-1	1869	1866	FA[5]	0.355bcd ±0.005	0.201cd ±0.120	0.785b ±0.457	0.127d ±0.023	1.423a ±0.0739	0.136d ±0.030	0.778bc ±0.0739	0.242bcd ±0.139	<0.0001	1.9	MS, LRI, I	2, 4, 5, 7
octanoic acid	124-07-2	2081	2084	FA[3]	0.287abc ±0.040	0.050cd ±0.029	0.288ab ±0.174	0.110bcd ±0.009	0.516a ±0.088	0.135bcd ±0.020	0.416a ±0.056	0.018d ±0.010	<0.0001	3	MS, LRI, I, O	1,2, 4, 11,5
nonanoic acid	112-05-0	2180	2180	FA[3]	0.227b ±0.007	0.581ab ±0.336	3.605ab ±2.141	0.387ab ±0.009	2.073ab ±0.073	0.105b ±0.008	2.271ab ±0.073	4.354a ±2.594	0.007	3.1	MS, LRI, O	1,2, 4,5
decanoic acid	334-48-5	2301	2303		0.098b ±0.024	0.041b ±0.024	0.137b ±0.079	0.020b ±0.002	0.469a ±0.059	0.093b ±0.018	0.157b ±0.001	0.159b ±0.097	<0.0001	4	MS, LRI, I	2,4,
Total:					**2.3**	**1.3**	**8.1**	**1.2**	**14.2**	**1**	**5.4**	**8**				
Alcohols																
2-methylpropan-1-ol	78-83-1	1091	1092	AA[1]	0.027c ±0.002	0.022c ±0.012	0.133a ±0.016	0.030c ±0.002	0.047c ±0.002	Trace	0.085b ±0.001	0.028c ±0.016	<0.0001	0.7	MS, LRI, I, O	9,10
1-methoxypropan-2-ol	107-98-2	1129	1135		0.061c ±0.003	0.379a ±0.052	0.216b ±0.0432	0.100c ±0.019	0.041c ±0.002	0.116bc ±0.017	0.096c ±0.018	0.081c ±0.047	<0.0001	-0.4	MS, LRI	
pent-1-en-3-ol	616-25-1	1160	1161	FA[1]	0.066de ±0.009	0.458a ±0.08	0.145cd ±0.01	0.146cd ±0.02	0.188bc ±0.019	0.291b ±0.033	0.094cde ±0.0142	0.034e ±0.008	<0.0001	1.1	MS, LRI, I	4, 5, 7, 8,9
3-methylbutan-1-ol	123-51-3	1210	1211	AA[1]	ND	0.117b ±0.018	0.650a ±0.110	0.216b ±0.028	0.523a ±0.046	0.198b ±0.015	0.493a ±0.066	0.221b ±0.038	<0.0001	1.4	MS, LRI, O	5,9
3-methylbut-3-en-1-ol	763-32-6	1250	1250		ND	ND	0.020b ±0.012	0.006b ±0.003	1.244a ±0.045	0.016b ±0.003	0.024b ±0.003	0.001b ±0.001	<0.0001	1	MS, LRI	5
pentan-1-ol	71-41-0	1253	12.52	FA[2]	Trace	1.235a ±0.163	0.659c ±0.157	0.709c ±0.0806	1.166ab ±0.028	0.719c ±0.68	0.835bc ±0.042	0.246d ±0.155	<0.0001	1.3	MS, LRI, I	1, 4, 5, 7, 8,9, 11,13
(*Z*)-pent-2-en-1-ol	1576-95-0	1322	1322	FA[1]	0.075d ±0.007	0.883a ±0.115	0.376c ±0.018	0.624b ±0.050	0.610b ±0.020	0.660b ±0.0714	0.526bc ±0.011	0.092d ±0.054	<0.0001	1.1	MS, LRI, I	4,5, 7
hexan-1-ol	111-27-3	1356	1356	FA[1]	0.879c ±0.130	0.769c ±0.148	4.317a ±0.507	2.576b ±0.166	0.392cd ±0.054	0.329cd ±0.018	0.928c ±0.142	0.121d ±0.070	<0.0001	2	MS, LRI, I	1, 4, 5, 7, 8, 9, 13
(*Z*)-hex-3-en-1-ol	928-96-1	1388	1386	FA[1]	0.103d ±0.009	0.038d ±0.005	0.843a ±0.116	0.291c ±0.026	0.314c ±0.021	0.033d ±0.001	0.542b ±0.046	0.034d ±0.019	<0.0001	1.6	MS, LRI, I, O	4,5, 7
oct-1-en-3-ol	3391-86-4	1452	1456	FA[1]	0.003e ±0.000	0.387a ±0.026	0.077cd ±0.45	0.139bc ±0.013	0.022de ±0.0007	0.182b ±0.001	0.021de ±0.0007	0.032de ±0.018	<0.0001	2.5	MS, LRI, I	1, 4, 5, 7, 8,9, 11, 13
2-ethylhexan-1-ol	104-76-7	1492	1492		0.355cd ±0.055	0.198ab ±0.14	0.295d ±0.171	0.854b ±0.092	0.747bc ±0.037	1.305a ±0.147	0.364cd ±0.0168	0.805b ±0.134	<0.0001	2.7	MS, LRI	1, 4, 7
octan-1-ol	111-87-5	1561	1561	FA[1]	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution		3	MS, LRI, I	1, 4, 5, 8,9
(*E*)-oct-2-en-1-ol	18409-17-1	1618	1620	FA[1]	ND	ND	ND	0.124a ±0.022	ND	0.119a ±0.017	ND	ND	0.793	2.7	MS, LRI, I	1,5, 7, 8
α-terpineol	98-55-5	1699	1698		Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution		2.9	MS, LRI, I, O	6
undecan-2-ol	1653-30-1	1721	1717		ND	ND	ND	0.090 ±0.019	ND	ND	ND	Trace		4.7	MS, LRI, I	
decan-1-ol	112-30-1	1765	1769		Trace	0.013a ±0.007	0.045a ±0.026	0.022a ±0.003	0.018a ±0.0004	Trace	0.027a ±0.0004	0.043a ±0.0006	0.043	4.5	MS, LRI, I	3, 7

benzenemethanol	100-51-6	1879	1877	AA[7]	0.122c ±0.025	0.267c ±0.042	0.922a ±0.147	0.384bc ±0.07	0.492ab ±0.353	0.239c ±0.01	0.940a ±0.038	0.435bc ±0.252	<0.0001	1.1	MS, LRI, I, O	1, 4, 5, 13
2-phenylethanol	60-12-8	1915	1912	AA[7]	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution		1.3	MS, LRI, I, O	1, 4,9
dodecan-1-ol	112-53-8	1970	1970		0.136a ±0.021	0.227a ±0.047	0.111a ±0.068	0.227a ±0.045	0.163a ±0.026	0.204a ±0.038	0.144a ±0.022	0.212a ±0.123	0.292	4.3	MS, LRI, I	3, 4, 12
phenol	108-95-2	2003	2004	AA[6]	0.0200b ±0.002	0.017b ±0.01	0.372a ±0.223	0.013b ±0.0007	0.165ab ±0.04	0.013b ±0.007	0.028b ±0.007	Trace	0.003	1.4	MS, LRI, I	1, 5
2-phenoxyethanol	122-99-6	2144	2142		0.101b ±0.0006	0.116b ±0.017	0.096b ±0.056	0.040b ±0.003	0.200b ±0.034	0.044b ±0.003	0.826a ±0.109	0.095b ±0.055	<0.0001	1.1	MS, LRI, I	5, 10
tetradecan-1-ol	112-72-1	2175	2175		0.135a ±0.006	0.125a ±0.007	0.064b ±0.008	0.120a ±0.019	0.111ab ±0.009	0.132a ±0.011	0.092ab ±0.012	0.060b ±0.035	0.000	6	MS, LRI, I	
hexadecan-1-ol	36653-82-4	2381	2379		1.139a ±0.200	0.269b ±0.056	0.236b ±0.137	0.211b ±0.0223	0.263b ±0.034	0.362b ±0.031	0.332b ±0.036	0.390b ±0.087	<0.0001	6.8	MS, LRI, I	10
octadecan-1-ol	112-92-5	2588	2585		0.544ab ±0.098	0.349bc ±0.041	0.393abc ±0.055	0.314c ±0.025	0.512abc ±0.068	0.449abc ±0.042	0.568a ±0.107	0.387abc ±0.045	0.003	8.2	MS, LRI, I	
Total:					**3.9**	**6.8**	**10.1**	**7.2**	**7.4**	**5.4**	**7.1**	**3.4**				
Aldehydes																
hexanal	66-25-1	1085	1083	FA[1]	0.082ab ±0.01	0.361a ±0.222	0.009b ±0.005	0.163ab ±0.024	0.093ab ±0.019	0.239ab ±0.03	0.041b ±0.006	0.130ab ±0.075	0.007	1.7	MS, LRI, I, O	1,2,3, 4, 5, 7, 8, 11,13
heptanal	111-71-7	1187	1188	FA[1]	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution		2.2	MS, LRI, I	2, 4, 5, 7, 8, 11
2-ethylhexanal	123-05-7	1190	1198		0.529b ±0.082	4.068a ±0.414	0.929b ±0.537	2.431ab ±0.261	1.410ab ±0.143	2.465ab ±0.471	0.611b ±0.091	0.731b ±0.423	0.006	2.7	MS, LRI, I	5,8
3-methylbuten-2-al	107-86-8	1201	1206		Trace	Trace	0.025b ±0.015	0.053ab ±0.001	0.072a ±0.016	0.079a ±0.008	0.024b ±0.004	0.048ab ±0.007	0.000	0.9	MS, LRI	10
(*E*)-hexen-2-al	6728-26-3	1221	1219	FA[1]	Trace	0.017b ±0.01	Trace	0.068a ±0.003	Trace	0.071a ±0.005	Trace	Trace	0.000	1.7	MS, LRI	1, 4,5, 7, 8,9, 14
octanal	124-13-0	1292	1291	FA[1]	0.074ab ±0.004	0.260a ±0.158	0.056b ±0.032	0.188ab ±0.02	0.072ab ±0.008	0.130ab ±0.003	0.044b ±0.003	0.038b ±0.023	0.007	3.5	MS, LRI, I, O	1, 3, 4, 7, 8, 9, 12,13
nonanal	124-19-6	1396	1396	FA[1]	0.218a ±0.009	0.294a ±0.17	0.247a ±0.144	0.330a ±0.0211	0.350a ±0.027	0.353a ±0.072	0.188a ±0.008	0.219a ±0.126	0.531	3.2	MS, LRI, I	1, 3, 4, 5, 7, 9, 11, 12, 13
(*E*)-octen-2-al	2548-87-0	1434	1434	FA[1]	0.012d ±0.007	0.054b ±0.0122	0.006d ±0.003	0.050b ±0.005	0.022cd ±0.002	0.096a ±0.008	0.014d ±0.002	0.039bc ±0.008	<0.0001	2.8	MS, LRI, I, O	1,2, 3, 5, 7,8, 9,11
methional	3268-49-3	1456	1454	AA	Trace	Trace	ND	ND	ND	ND	ND	ND		0.4	MS, LRI, I, O	2, 4, 5, 11
decanal	112-31-2	1501	1502	FA[3]	0.175b ±0.02	0.316ab ±0.048	0.258ab ±0.154	0.229ab ±0.016	0.490a ±0.096	0.290ab ±0.072	0.191ab ±0.039	0.223ab ±0.134	0.049	4.4	MS, LRI, I	3,5,9, 12
benzaldehyde	100-52-7	1526	1520	AA[1]	0.058d ±0.01	0.189bc ±0.03	0.239b ±0.027	0.135c ±0.021	0.048d ±0.003	0.406a ±0.017	0.042d ±0.001	0.461a ±0.03	<0.0001	1.6	MS, LRI, I	1, 2, 4,5, 7,8, 9
(2*E*,4*E*)-nona-2,4-dienal	6750-03-4	1703	1710	FA[1]	ND	Trace	ND	ND	ND	Trace	ND	ND		2.9	MS, LRI, O	1, 3, 4, 7, 8,11, 12
dodecanal	112-54-9	1712	1710		0.053a ±0.009	0.088a ±0.016	0.058a ±0.005	0.025a ±0.004	0.073a ±0.007	0.091a ±0.004	0.057a±0.007	0.100a ±0.062	0.082	5.3	MS, LRI, I	2,9
(2E,4Z)-deca-2,4-dienal	25152-83-4	1768	1767	FA[1]	ND	Trace	ND	Trace	ND	Trace	ND	ND		3.1	MS, LRI	5, 10, 22
(2*E*,4*E*)-deca-2,4-dienal	25152-84-5	1814	1824	FA[1]	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution		3.1	MS, LRI, O	1,2, 3, 4,5,7, 11
tetradecanal	10486-19-8	1924	1924		0.055b ±0.006	0.099a ±0.024	Trace	Trace	Trace	Trace	Trace	Trace	0.050	6	MS, LRI, I	5,7
4,5-epoxy-(*E*)-dec-2-enal	134454-31-2	2005	2006	FA[4]	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace		1.7	MS, LRI, O	16, 10
pentadecanal	2765_11_9	2030	2042		0.258b ±0.003	0.201b ±0.005	0.183b ±0.014	ND	0.574a ±0.092	ND	ND	ND	<0.0001	6.5	MS, LRI	7
Total:					**1.5**	**6**	**2**	**3.6**	**3.2**	**4.2**	**1.2**	**2**				
Esters																
ethyl butanoate	105-54-4	1038	1035		0.010abc ±0.003	0.003c ±0.002	0.021ab ±0.013	0.006bc ±0.0003	0.026a ±0.0003	0.005bc ±0.0003	0.022ab ±0.0003	0.008bc ±0.00003	0.000	1.4	MS, LRI, I	18
ethyl 2-hydroxypropanoate	97-64-3	1345	1349		ND	ND	2.039b ±0.248	0.896c ±0.102	0.451c ±0.056	0.252c ±0.045	3.201a ±0.648	0.977c ±0.104	<0.0001	0.1	MS, LRI, I	5
6-methylheptyl prop-2-enoate	54774-91-3	1487	1480		0.023b ±0.003	0.358a ±0.078	Trace	0.189ab ±0.029	Trace	0.124b ±0.018	Trace	0.238ab ±0.14	0.007	3.9	MS, LRI	19
ethyl 2-hydroxy-4-methylpentanoate	10348-47-7	1545	1545		ND	ND	0.009b ±0.005	ND	ND	ND	0.064a ±0.003	ND	<0.0001	1.3	MS, LRI	
dimethyl glutarate	1119-40-0	1700	1699		ND	ND	ND	ND	2.103a ±0.036	ND	1.44b ±0.007	ND	<0.0001	0.4	MS, LRI	20

isopropyl myristate	110-27-0	2036	2041		0.237b ±0.02	0.448ab ±0.092	0.727a ±0.105	0.098b ±0.018	0.729a ±0.172	0.419ab ±0.0723	0.753a ±0.088	0.327b ±0.198	<0.0001	7.7	MS, LRI, I	10, 21
isopropyl palmitate	142-91-6	2239	2232		0.0647a ±0.006	0.114a ±0.066	0.052a ±0.008	0.058a ±0.002	0.078a ±0.0001	0.031a ±0.0001	0.095a ±0.009	0.120a ±0.07	0.165	8.2	MS, LRI, I	
methyl dihydrojasmonate	24851-98-7	2290	2276		0.078b ±0.004	0.058b ±0.036	0.023b ±0.014	0.06b ±0.006	0.043b ±0.008	0.070b ±0.008	0.040b ±0.0003	0.180a ±0.039	<0.0001	2.6	MS, LRI, I, O	
Total:					**0.4**	**0.9**	**2.8**	**1.3**	**3.4**	**0.9**	**6**	**1.8**				
Ketones																
3,3-dimethylbutan-2-one	75-97-8	964	978		0.177cd ±0.031	0.291ab ±0.045	0.204bc ±0.003	0.335a ±0.046	0.157cd ±0.014	0.183c ±0.011	0.087d ±0.003	0.152cd ±0.023	<0.0001	1.2	MS, LRI	
butane-2,3-dione	431-03-8	980	977	CAR[6]	0.050d ±0.0003	0.013d ±0.001	0.082d ±0.011	0.014d ±0.002	10.383c ±0.586	0.479d ±0.032	30.507a ±2.04	14.404b ±2.42	<0.0001	-1.3	MS, LRI, I, O	7, 9
pentan-2-one	107-87-9	985	986		0.130a ±0.004	0.098a ±0.059	0.105a ±0.064	0.136a ±0.001	0.104a ±0.003	0.170a ±0.016	0.092a ±0.003	0.112a ±0.065	0.597	0.9	MS, LRI, I	9, 10, 14
4-methylpentan-2-one	108-10-1	1009	1008		0.037a ±0.002	0.054a ±0.032	0.017a ±0.01	0.048a ±0.005	0.023a ±0.004	0.041a ±0.0003	0.012a ±0.0004	0.014a ±0.0008	0.014	1.3	MS, LRI, I	
pentane-2,3-dione	600-14-6	1057	1058	FA[3]	ND	0.098a ±0.009	Trace	0.005b ±0.0003	Trace	0.013b ±0.001	Trace	0.0072b ±0.0003	<0.0001	-0.8	MS, LRI, I, O	4,5, 7,9
heptan-3-one	106-35-4	1156	1163		0.194ab ±0.0242	0.720a ±0.434	0.140b ±0.089	0.403ab ±0.01	0.268ab ±0.034	0.386ab ±0.04	0.147ab ±0.004	0.331ab ±0.191	0.039	1.7	MS, LRI	
heptan-2-one	110-43-0	1185	1185		0.088bc ±0.01717	0.066c ±0.038	0.273a ±0.068	0.204ab ±0.014	0.250a ±0.0145	0.238a ±0.02	0.237a ±0.027	0.276a ±0.063	<0.0001	1.9	MS, LRI, I	1, 4,5, 7, 8,9, 11, 13
6-methylheptan-2-one	928-68-7	1241	1237	CAR[2]	0.110a ±0.008	0.319a ±0.186	0.150a ±0.135	0.217a ±0.014	0.187a ±0.023	0.261a ±0.051	0.145a ±0.03	0.106a ±0.075	0.201	2.3	MS, LRI, I, O	10
5-methylheptan-2-one	18217-12-4	1259	1256		0.049a ±0.0005	0.179a ±0.107	0.097a ±0.016	0.107a ±0.013	0.085a ±0.009	0.075a ±0.013	0.058a ±0.007	0.075a ±0.01	0.070	2.3	MS, LRI	
3-hydroxybutan-2-one	513-86-0	1287	1287		0.107c ±0.007	0.284c ±0.164	4.792bc ±0.706	1.058c ±0.068	66.915a ±5.56	16.159b ±1.007	53.874a ±7.96	54.682a ±5.872	<0.0001	-0.3	MS, LRI, I, O	5, 11
octan-2-one	111-13-7	1288	1283		0.023b ±0.001	0.0679a ±0.014	ND	ND	ND	ND	ND	ND	0.006	2.3	MS, LRI, I	1, 4, 5, 7, 8,9
1-hydroxypropan-2-one	116-09-6	1301	1290		0.074b ±0.014	0.046b ±0.003	0.220a ±0.038	0.054b ±0.009	0.205a ±0.013	0.070b ±0.007	0.206a ±0.017	0.056b ±0.033	<0.0001	-0.7	MS, LRI, I	
oct-1-en-3-one	4312-99-6	1304	1306	FA[2]	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution		2.1	MS, LRI, I, O	8, 17
3-methylcyclohexanone	591-24-2	1334	1333		0.022b ±0.004	0.047a ±0.005	Trace	0.024b ±0.002	0.023b ±0.003	0.036ab ±0.001	Trace	0.021b ±0.012	0.002	1.7	MS, LRI, I	
nonan-2-one	821-55-6	1392	1391		0.057a ±0.0007	0.0798a ±0.048	0.055a ±0.033	0.135a ±0.015	0.131a ±0.017	0.145a ±0.02	0.116a ±0.015	0.176a ±0.106	0.093	3.1	MS, LRI, I	1, 4, 8,9, 13
Oct-3-en-2-one	1669-44-9	1411	1414		Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution		2.5	MS, LRI, O	3, 4, 5, 8
decan-2-one	693-54-9	1496	1493		0.047bc ±0.001	0.037c ±0.004	0.124a ±0.027	0.031c ±0.006	0.087ab ±0.0133	0.028c ±0.004	0.120a ±0.014	0.016c ±0.0098	<0.0001	3.7	MS, LRI, I	1, 5, 7, 8, 13
(3*E*,5*E*)-octa-3,5-dien-2-one	30086-02-3	1574	1570	FA[1]	Trace	0.116a ±0.012	Trace	0.028c ±0.001	Trace	0.0915b ±0.008	Trace	Trace	<0.0001	1.7	MS, LRI, I	1,2, 3, 5, 7, 8,9, 13
undecan-2-one	112-12-9	1601	1599		0.037a ±0.005	0.070a ±0.011	0.140a ±0.081	0.127a ±0.014	0.032a ±0.001	0.173a ±0.035	0.041a ±0.006	0.129a ±0.077	0.018	4	MS, LRI, I	1,2, 3,5, 7, 8
acetophenone	98-86-2	1655	1652	AA[1]	0.0415b ±0.008	0.147a ±0.032	0.085ab ±0.017	0.045b ±0.002	0.059ab ±0.0002	0.120ab ±0.02	0.081ab ±0.005	0.125ab ±0.073	0.014	1.5	MS, LRI, I	1, 9
tridecan-2-one	593-08-8	1812	1814		0.051b ±0.004	0.034b ±0.0198	0.065b ±0.011	0.195a ±0.0319	0.050b ±0.005	0.095b ±0.0138	0.037b ±0.004	0.099b ±0.058	<0.0001	5	MS, LRI, I	4, 7
geranylacetone	3796-70-1	1857	1859	CAR[3]	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution		3.6	MS, LRI, I, O	4, 7, 12
pentadecan-2-one	2345-28-0	2021	2021		0.034ab ±0.0005	0.017b ±0.011	0.019b ±0.011	0.097ab ±0.019	0.017b ±0.0002	0.053ab ±0.006	0.033ab ±0.001	0.102a ±0.063	0.005	6	MS, LRI, I	
2-aminoacetophenone	551-93-9	2220	2218		Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace		1.6	MS, LRI, O	
Total:					**1.3**	**9.3**	**8.8**	**5.3**	**80.5**	**20.5**	**86.7**	**71.7**				
Lactones																
γ-pentalactone	108-29-2	1612	1600		0.033b ±0.0007	Trace	0.028b ±0.016	Trace	0.035b ±0.006	ND	0.076a ±0.014	Trace	0.009	-0.2	MS, LRI, I	5, 10
γ-butyrolactone	96-48-0	1630	1626		0.06c ±0.008	0.056c ±0.0126	0.227bc ±0.028	0.091c ±0.003	0.095a ±0.003	0.117b ±0.003	0.128a ±0.013	0.180a ±0.036	<0.0001	-0.7	MS, LRI, I	5, 10
γ-hexalactone	695-06-7	1705	1708		Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution		0.4	MS, LRI, I	5, 4,10
γ-crotonolactone	497-23-4	1755	1745		0.020b	0.0432b	0.082a	0.053ab	0.0165b	0.082a	0.037b	0.043b	<0.0001	-0.6	MS, LRI	10

					±0.002	±0.002	±0.005	±0.01	±0.002	±0.009	±0.004	±0.026				
γ-octalactone	104-50-7	1922	1923	FA[3]	0.010b ±0.0007	0.010b ±0.006	0.019b ±0.0112	0.012b ±0.0007	0.019b ±0.0007	0.012b ±0.0007	0.015b ±0.0007	0.097a ±0.016	<0.0001	1.43	MS, LRI, I, O	2,5,8
γ-nonanolactone	104-61-0	2033	2037	FA[3]	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution		2	MS, LRI, I, O	3, 4, 5,7,8
γ-decalactone	706-14-9	2150	2152		0.009a ±0.000	0.011a ±0.003	0.014a ±0.002	0.037a ±0.001	0.016a ±0.004	0.017a ±0.001	0.016a ±0.0001	0.017a ±0.0003	0.05	2.7	MS, LRI, I, O	10, 16
δ-decalactone	705-86-2	2202	2201	FA[10]	ND	ND	0.139a ±0.086	0.038a ±0.003	0.143a ±0.02	0.044a ±0.005	0.160a ±0.019	0.091a ±0.053	0.06	2.4	MS, LRI, I, O	15
δ-Jasmine lactone	25524-95-2	2266	2272	FA[9]	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution	Coelution		2.1	MS, LRI, O	
Total:					**0.1**	**0.1**	**0.5**	**0.2**	**0.3**	**0.2**	**0.4**	**0.4**				
Terpenes																
verbenone	80-57-9	1718	1710		Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace		2.1	MS, LRI, I, O	10
(*E*)-geraniol	106-24-1	1850	1853		ND	ND	0.143ab ±0.084	0.253a ±0.038	0.144ab ±0.025	0.095b ±0.005	0.107a b±0.023	ND	0.045	3.2	MS, LRI, I, O	3,7, 10
Thiazole																
benzothiazole	95-16-9	1963	1968		0.0201b ±0.0007	0.027b ±0.015	0.016b ±0.009	0.025b ±0.002	0.047b ±0.007	0.037b ±0.007	0.178a ±0.033	0.0451b ±0.026	<0.0001	2	MS, LRI, I	4,5
Pyrazines																
2,5-dimethylpyrazine	123-32-0	1327	1332	AA[8]	ND	ND	Trace	Trace	Trace	Trace	Trace	Trace		-0.2	MS, LRI, I, O	7, 8

FA: fatty acids, AA: amino acids, CAR: carotenoids, ND: not detected by GC–MS

*CAS and DB-Wax linear retention index (LRI) experimental and from the literature (NIST, 2023). **Concentration of aroma compound (presented as µg of internal standard/mL of sample) are shown as mean ± standard deviation, n (n = 3) (different letters represent significant difference between samples considering the same analysis ($p < 0.05$, Tukey's test)). Concentration (expressed as µg of internal standard/g of sample). *** log Pow, octanol/water partition coefficient expressed in logarithmic form and extracted from literature (PubChem, 2023). ****Reliability of identification: MS = mass spectrometry, LRI = linear retention index and O = odour identical to published data and/or data found in databases, and I = confirmation by injection of standards on equivalent DB-Wax.

[1](Murray et al., 1976) [2](Jakobsen et al., 1998) [3](Karolkowski et al., 2021) [4](Bader et al., 2009) [5](Bail et al., 2009) [6](Chen et al., 2017) [7](Akkad et al., 2019) [8](Zhang et al., 2020) [9](Haffner et al., 1996) [10](Albrecht et al., 1992) [11](Nakae & Elliott, 1965)

1(Xiang et al., 2022), 2(Utz et al., 2022), 3(Ebert et al., 2022), 4(Benavides-Paz et al., 2022a), 5(Murat et al., 2013), 6(Wang et al., 2020), 7(Schindler et al., 2012), 8(Fischer et al., 2022a), 9(El Youssef et al., 2020), 10(Karolkowski et al., 2023), 11(Trikusuma et al., 2020), 12(Pei et al., 2022), 13(Shi et al., 2021), 14 (Ben-Harb et al., 2022), 15(Masiá et al., 2021), 16(Bader et al., 2009), 17(Jakobsen et al., 1998), 18(Akkad et al., 2022), 19(Rajhi et al., 2022), 20(Xu et al., 2020), 21(Zhang et al., 2021), 22(Kaneko et al., 2011)

PCA analysis (Fig. VI-1) was performed in order to clarify the complex data analysis of volatile compounds among all the samples. The volatile compounds were considered as variable and the chemical class as quantitative supplementary variable. The volatile compounds which did not have significant differences (P value≥0.05; Tukey's test) were not considered in PCA analysis. Also, some of the volatile compounds were coeluted and the quantity could not be evaluated. Hence these compounds also were not considered in PCA.

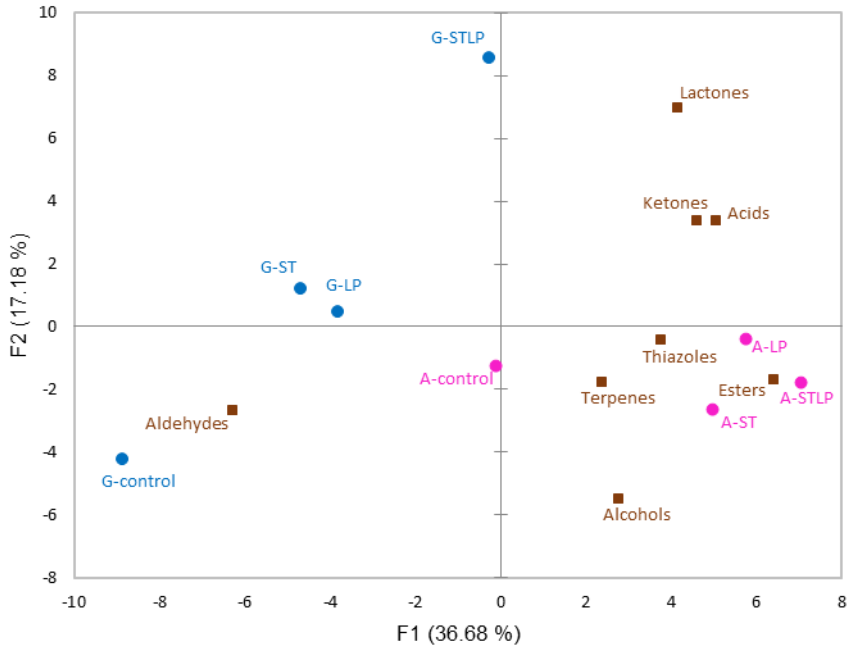


Figure VI-1. Principal component analysis (PCA – Pearson correlation) performed on the volatile compounds ($p<0.05$) of pea protein albumin (A) and globulin (G) fractions obtained without (control) or with fermentation by *S. thermophilus* (ST) and *Lb. plantarum* (LP) or their co-culture (STLP) (●). The 8 chemical class (■) also shown as the supplementary variable. The volatile compounds with significant differences in equivalent concentration were considered for this analysis (P value≥0.05; Tukey's test).

First component, represented 36.68% of the total variance and second component represented 17.10%. First component separated albumin from globulin fractions, and fermented samples from controls. A-ST, A-LP and A-STLP were characterized by high content of terpenes, thiazoles and esters, and low quantity of aldehydes. Globulin fractions (except G-STLP) were characterized by high quantities of aldehydes, especially G-control. The reason could be the degradation of aldehydes by microorganism to the other volatile compounds such as alcohols and acids (Shi et al., 2021). It is also possible, that during protein extraction, aldehydes which

are non-polar volatile compounds (log Pow>1) would be retained preferentially in the more hydrophobic fraction (globulin). Second component separated G-STLP from other globulin fractions. G-ST and G-LP showed similar aromatic profile and differentiated from G-STLP. This last sample had higher content of lactones, acids and ketones compared to the two other fermented globulin fractions.

Agglomerative hierarchical clustering (AHC) was performed to group the samples based on their dissimilarities in volatile compounds (Fig IV-2). The dissimilarity matrix was performed on the basis of the Euclidean distance with a Ward's method of aggregation.

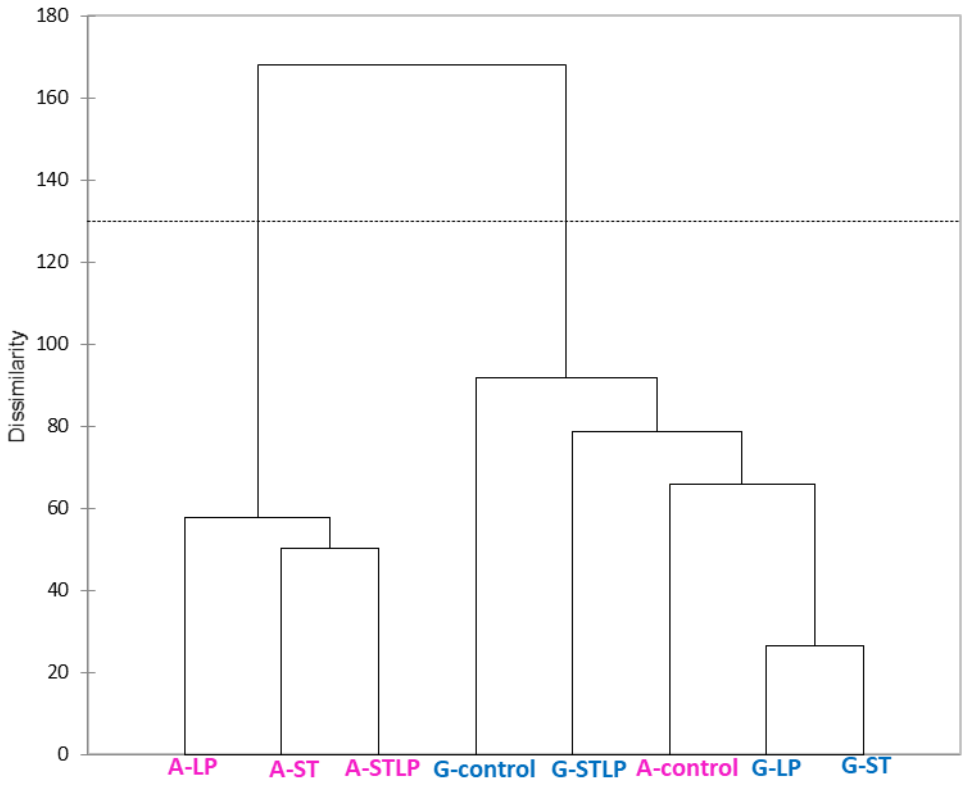


Figure VI-2. Agglomerative hierarchical clustering (AHC) of volatile compounds in pea protein albumin (A) and globulin (G) fractions obtained without (control) or with fermentation by *S. thermophilus* (ST) and *Lb. plantarum* (LP) or their co-culture (STLP). Only the volatile compounds with significant differences between the samples have been considered ($p<0.05$; Tukey's test)

The dendrogram represented two main clusters. A good separation of fermented albumin samples from the other samples, could be observed. One cluster subdivided into four classes. It consists of G-control, G-STLP, A-control, G-ST and G-LP, respectively to their distance

from the centroid. The least dissimilarity in this cluster belonged to G-ST and G-LP. The second cluster included, A-LP, A-ST and A-STLP. The Euclidean distance between A-STLP and A-ST was closer than with A-LP. AHC results showed that the variables had sufficient explanatory power to differentiate between the samples. This analysis corroborated the results already observed by PCA. Two different groups of samples were obtained. Albumin samples obtained by fermentation would differentiate clearly from the globulin samples and controls.

To have a better idea on the differences between the samples regarding the strains (mono- and co-culture), a second PCA analysis was performed for albumin and globulin fractions separately. For albumin fractions (Fig VI-3. a), first and second component represented 48.70% and 28.14% of the variance, respectively. First component separated the fermented albumin samples from control. Clearly, fermented samples contained higher quantity of volatile compounds compared to control. The second component separated albumin fermented samples. A-LP contained higher content of lactones and alcohols compared to the two other fermented samples. While, A-ST represented higher quantity of acids, aldehydes and ketones. In globulin samples (Fig VI-3. b), the first and second component represented 53.19% and 29.23% of the variance, respectively. The first component separated G-STLP, from G-control. G-STLP had higher quantity of lactones, ketones, acids, thiazoles and esters compared to the other samples. On the contrary G-control contained higher quantity of aldehydes and alcohols. G-ST and G-LP seemed to have closer aroma composition to control sample, although they had higher content of terpenes compared to the other samples (representing only 2 volatile compounds identified as already mentioned).

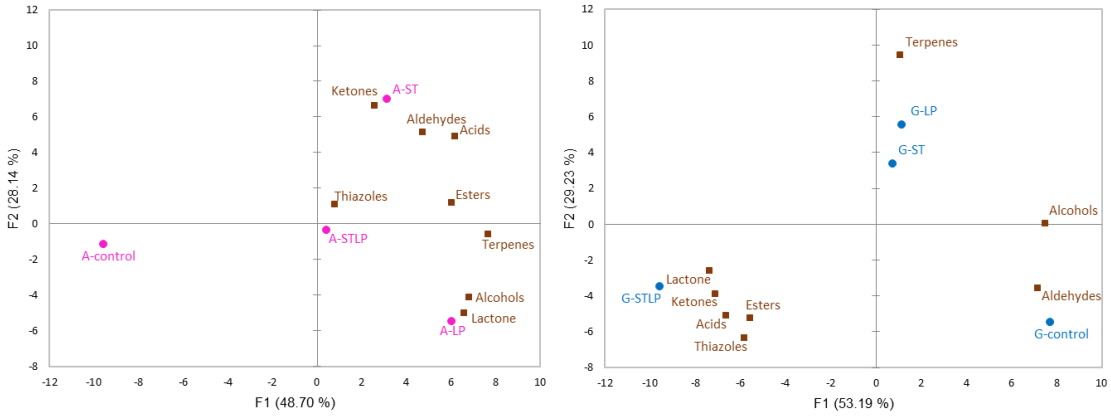


Figure VI-3. Principal component analysis (PCA – Pearson correlation) performed on the volatile compounds ($p<0.05$) of pea protein albumin (A) (a) and globulin (G) (b) fractions (●) obtained without (control) or with fermentation by *S. thermophilus* (ST) and *Lb. plantarum* (LP) or their co-culture (STLP). The 8 chemical class (■) also shown as the supplementary variable. The volatile compounds with significant differences in equivalent concentration were considered for this analysis (P value≥0.05; Tukey's test).

VI.2.2. Odor active compounds

36 odor active compounds (OACs) were detected by GC-O within the control and fermented samples (Table VI-2). 7 of these compounds (i.e. ethyl-3-methylbutanoate or ethyl-2-methylbutanoate, hex-1-en-3-one, (*Z*)-non-2-enal, 2-isobutyl-3-methoxypyrazine, (*E*)-non-2-enal, 2-acetylpyrazine, 2,6-dimethoxyphenol) were not detected by GC-MS which means that the intense odour active zones are not necessarily correlated with the highest peak signals. In other words, volatile compounds with trace quantity could be an important aroma contributor while one with higher quantity may not have direct impact on aroma perception (Table 1) (Schlegel et al., 2021). For instance, 2-isobutyl-3-methoxypyrazine was not detected in GC-MS but it was perceived in GC-O with its distinctive "bell pepper" odour. Also, the LRI was in agreement with those found in the database and those obtained by injecting pure molecules. Indeed, pyrazines with their low olfactory threshold (0.001–0.002 g/L in water) contribute significantly to the aroma profile of pea protein. However, the low concentration of pyrazines in pea protein make it difficult to extract this compound sufficiently for detection (Benavides-Paz et al., 2022a). It was supposed that the LRI at 1056 with odour attribute of fruity could correspond to any of ethyl-3-methylbutanoate or ethyl-2-methylbutanoate since LRIs were in accordance with those in the database.

The 36 OACs were classified in 9 odour classes i.e. lactic, fruity, floral, metallic, chemical, green, grilled, potato, mushroom. Although, green, potato, mushroom and metallic could be all considered as beany note in pea protein (Chigwedere et al., 2022), here they were separated to better distinguish between the samples. In this study, the volatile compounds were considered as OACs if they had detection frequency of ≥45% (Pollien et al., 1997). Some OACs were detected in all the samples including, hex-1-en-3-one (chemical), oct-1-en-3-one (mushroom), oct-3-en-2-one (mushroom), (*E*)-oct-2-enal (green) and 4,5-epoxy-(*E*)-dec-2-enal (metallic).

Comparing the albumin and globulin fractions obtained as controls, ethyl-3methylbutanoate or ethyl-2methylbutanoate (fruity), 2-acetylpyrazine (grilled), geranylacetone (chemical), benzenemethanol (fruity), γ-nonanolactone (fruity), octanoic acid (chemical) were detected in A-control. (*Z*)-hex-3-en-1-ol (metallic), 2-isobutyl-3-methoxypyrazine (green), (2*E*,4*E*)-deca-2,4-dienal (grilled) were detected in G-control.6-methylheptan-2-one (chemical) and (2*E*,4*E*)-nona-2,4-dienal (grilled) were only perceived in G-control, and not in other samples.

Comparing fermented and control samples, some OACs were only detected in fermented samples, i.e. butane-2,3-dione (lactic), 2-methylpropan-1-ol (fruity), 3-methylbutan-1-ol (metallic), 3-hydroxybutan-2-one (lactic), 2,5-dimethylpyrazine (grilled), (*Z*)-non-2-enal (green), (*E*)-non-2-enal (green), geraniol (fruity), 2-phenylethanol (floral), γ-octalactone (fruity), γ-decalactone (fruity), δ-decalactone (fruity), 2-aminoacetophenone (floral), δ-jasmin lactone (fruity) and methyl dihydrojasmonate (floral). Among which butane-2,3-dione (lactic), 3-methylbutan-1-ol (metallic) were only detected in A-STLP, γ-octalactone (fruity) in G-STLP, 2,5-dimethylpyrazine (grilled) in G-ST, (*Z*)-non-2-enal (green) in A-ST and α-terpineol (floral) and geraniol (fruity) in A-LP. 4,5-epoxy-(*E*)-dec-2-enal (metallic), oct-1-en-3-one (mushroom) had higher detection frequency in G-LP and they were detected by all the assessors in this sample. geranylacetone (chemical), was only detected in all the albumin fraction samples (fermented and control).

Table VI-2. Odor active compounds (OACs) found in pea albumin (A) and globulin (G) fractions obtained without (control) or with fermentation by *S. thermophilus* (ST) and *Lb. plantarum* (LP) or their co-culture (STLP). The compounds shown here had a detection frequency ≥4 assessors.

Aroma compouds	**LRI(FID)***	**LRI(MS)**	**Odour Attributes**	**Odour Classes**	**Detection frequency (DF) (%)**							
					A-control	**G-control**	**A-LP**	**G-LP**	**A-ST**	**G-ST**	**A- STLP**	**G-STLP**
butane-2,3-dione	994	980	Butter	Lactic	11	ND	ND	11	22	11	**78**	22
ethyl-3-methylbutanoate or ethyl-2-methylbutanoate	1056	X	Fruity	Fruity	34	ND	11	ND	ND	ND	**45**	11
2-methylpropan-1-ol	1091	1092	Solvent, fruity, acid	Fruity	11	ND	11	ND	ND	ND	11	**56**
hex-1-en-3-one	1104	X	Plastic, dust, warm cloth, leather	Chemical	**67**	**56**	**78**	**56**	**67**	**67**	**67**	**67**
3-methylbutan-1-ol	1202	1209	Herbaceous, metallic, plastic, grilled, green wood	Metallic	ND	11	ND	11	ND	34	**56**	34
6-methylheptan-2-one	1241	1241	Rancid, phenol, dust, sulfur	Chemical	22	**45**	22	34	11	22	22	11
3-hydroxybutan-2-one	1287	1287	Butter, milk, cream	Lactic	ND	ND	**67**	34	**89**	**78**	**78**	**78**
oct-1-en-3-one	1302	1304	Mushroom	Mushroom	**89**	**89**	**89**	**100**	**89**	**89**	**89**	**89**
2,5-dimethylpyrazine	1340	1327	Roasted peanuts, cereals, rusk, toasted bread	Grilled	ND	11	ND	22	ND	**67**	ND	ND
(*Z*)-hex-3-en-1-ol	1384	1388	Vegetal, Garlic, metallic, burnt hair, Unpleasant	Metallic	22	**67**	**56**	ND	34	**56**	22	ND
oct-3-en-2-one	1405	1411	Mushroom	Mushroom	**89**	**56**	**89**	**89**	**78**	**67**	**78**	**67**
(*E*)-oct-2-enal	1435	1434	Vegetal, nutty, poppy, dust, green	Green	**78**	**89**	**67**	**67**	**67**	**78**	**67**	**45**
methional	1454	1456	Potato	Potato	**89**	**89**	**78**	**89**	ND	ND	ND	ND
(*Z*)-non-2-enal	1509	X	Green, leaves, herb, wood bark, dust	Green	11	ND	11	11	**56**	22	34	22
2-isobutyl-3-methoxypyrazine	1528	X	Vegetable , bell pepper, green, carrot	Green	11	**67**	ND	ND	ND	34	ND	**45**
(*E*)-non-2-enal	1537	X	Vegetal, grass, vegetable, faba bean, soup	Green	22	34	34	**56**	**45**	34	34	34
2-acetylpyrazine	1652	X	Cereal, bread, grilled, roasted	Grilled	**45**	34	34	34	**56**	**45**	**45**	**56**
(2*E*,4*E*)-nona-2,4-dienal	1702	trace	Meat broth, cereals, grilled chicken, bread	Grilled	11	**56**	ND	11	11	34	ND	ND
α-terpineol	1719	1699	Anise, floral, menthol	Floral	22	34	**56**	34	22	22	11	22
verbenone	1726	1717	Anise, flower, caramel, violet, fennel	Floral	**56**	**45**	34	34	34	**56**	**78**	**45**
(2*E*,4*E*)-deca-2,4-dienal	1810	1814	Grilled meat, cereals, toasted bread, spice	Grilled	34	**56**	11	**78**	**45**	**89**	ND	ND

Aroma compouds	LRI(FID)*	LRI(MS)	Odour Attributes	Odour Classes	Detection frequency (DF) (%)							
					A-control	G-control	A-LP	G-LP	A-ST	G-ST	A- STLP	G-STLP
geraniol	1848	1850	Citrus, orange blossom, verbena, fruity	Fruity	ND	ND	**45**	34	ND	22	ND	ND
geranylacetone	1858	1857	Phenolic, pharmaceutical, smoky, burned	Chemical	**45**	34	**67**	34	**56**	ND	**56**	ND
benzenemethanol	1870	1879	Sweet, vanilla, coconut, peach, candy-floss	Fruity	**56**	11	**45**	11	**56**	34	**45**	**56**
2-phenylethanol	1913	1915	Floral, rose, grape juice	Floral	22	22	**89**	**78**	**56**	**67**	**78**	**56**
γ-octalactone	1917	1921	Sugared almo0s, rose, candy-floss, floral	Fruity	11	11	ND	ND	11	ND	22	**67**
4,5-epoxy-(*E*)-dec-2-enal	2004	trace	Meat broth, metallic, beany, Green	Metallic	**56**	**78**	**45**	**100**	**78**	**78**	**67**	**67**
γ-nonanolactone	2026	2033	Fruity, strawberry, raspberry, candy-floss	Fruity	**45**	22	**45**	**67**	**56**	**45**	**56**	**67**
octanoic acid	2085	2081	Animal, fatty, candle	Chemical	**45**	34	**67**	34	**67**	34	34	**56**
γ-decalactone	2150	2151	Applesauce, fruity, apricot, peach	Fruity	11	22	11	22	ND	11	45	**56**
nonanoic acid	2186	2191	Leather, animal, rubber	Chemical	**67**	**67**	34	**78**	**45**	**45**	**45**	34
δ-decalactone	2202	2203	candy-floss, fruity, apricot, peach	Fruity	11	ND	**45**	**45**	**67**	11	**67**	**56**
2-aminoacetophenone	2221	2218	Floral, honey, candy, orange blossom	Floral	34	11	**78**	**56**	**67**	**78**	**78**	**56**
δ-Jasmine lactone	2268	2266	Sugar, fruity, peach, oil	Fruity	34	11	**45**	22	**78**	34	34	**45**
2,6-dimethoxyphenol	2274	X	Grilled, chemical, leather, dust, beany, cauliflower	Chemical	**56**	**67**	**56**	**56**	11	22	ND	11
methyl dihydrojasmonate	2306	2290	Fruity, mint, anise, fennel	Floral	ND	ND	**67**	11	**56**	**67**	**67**	**56**

ND: not detected by GCO, X: not detected by GC-MS

*LRI(FID)linear retention index obtained by flame ionization detector in GCO and LRI(MS) obtained by GC-MS

PCA was performed on the average of DFs (≥4 assessors) of the 10 odour classes defined in the samples (Fig. VI-4). The first and second components accounted for 78.97% of the total variance. First component axis separated the samples fermented with ST and STLP from the others. Lactic, floral and fruity notes were mainly attributed to A-STLP, G-STLP and A-ST. At the opposite, G-LP, A-LP, G-control and A-control were classified by potato, chemical and mushroom attributes. LP sample seemed to have a more similar characteristic to A-control rather than the other fermented samples. Methional is the volatile compound responsible for potato. Indeed, the potato descriptor was only recorded for LP and control samples. A-LP was mainly described by mushroom which is for the presence of oct-1-en-3-one.

The second component axis separated G-ST and G-control from the other samples for having higher value in green, grilled and metallic notes. The dominant grilled note in G-ST is probably related to the 2,5-dimethylpyrazine (grilled), which was detected only in this sample.

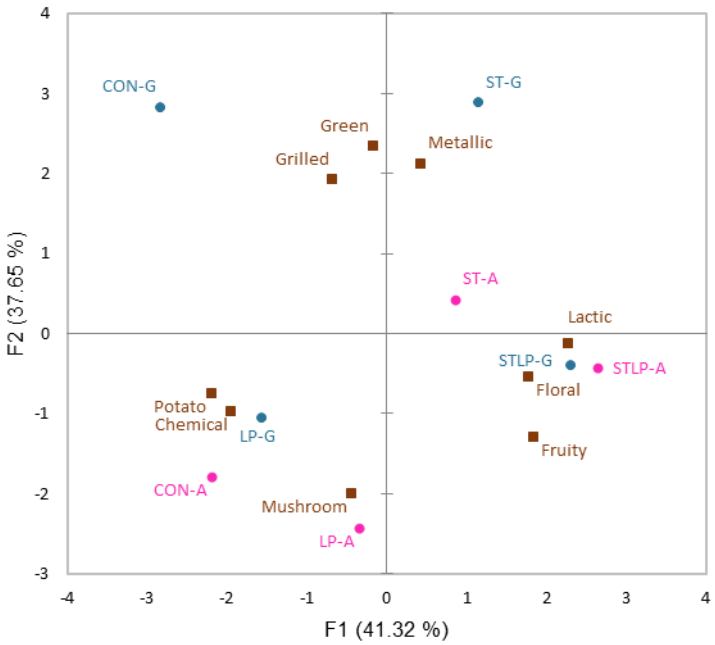


Figure VI-4. Principal component analysis (PCA – Pearson correlation) performed on the 10 odour classes (■) of pea protein albumin (A) and globulin (G) fractions (●) obtained without (control) or with fermentation by *S. thermophilus* (ST) and *Lb. plantarum* (LP) or their co-culture (STLP). The volatile compounds with DF (detection frequency) ≥4 assessors were considered.

The average DF of OACs were applied to AHC analysis and the dissimilarity matrix was performed on the basis of the Euclidean distance with a Ward's method of aggregation (Fig.VI-5). The dendrogram represented a quite good separation between samples fermented with different LAB. Four clusters could be observed in the dendrogram. ST and STLP showed similar OACs profile. The reason why G-ST was separated from the other samples containing ST, could be the presence of more green and metallic notes in this sample. LP samples were similar to control samples. The classification of A-control with LP samples could be for the high DF related to oct-3-en-2-on (mushroom) in these samples. This classification corroborated the results obtained by PCA.

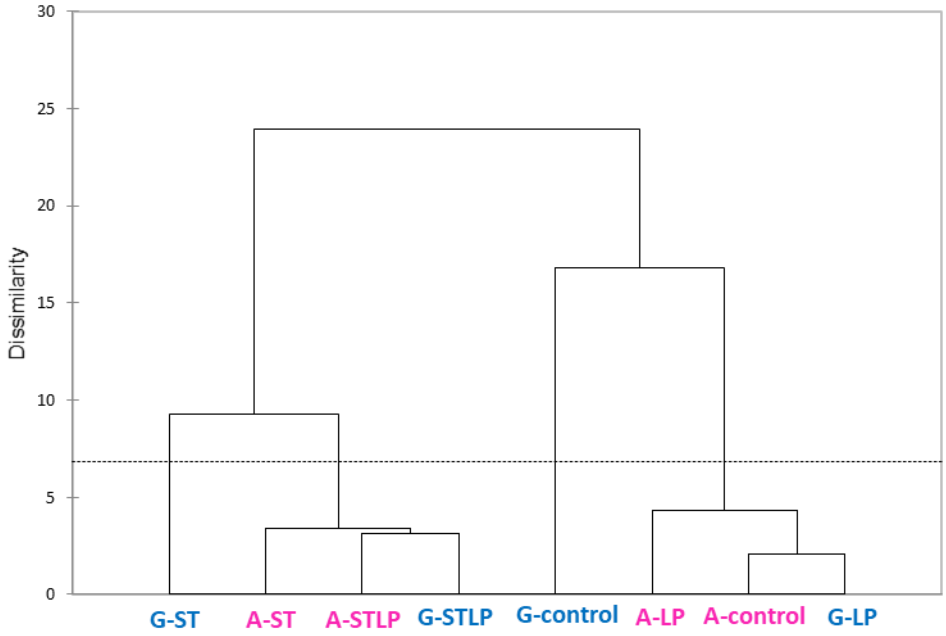


Figure VI-5. Agglomerative hierarchical clustering (AHC) of odour active compounds (OACs) in pea protein albumin (A) and globulin (G) obtained by obtained without (control) or with fermentation by *S. thermophilus* (ST) and *Lb. plantarum* (LP) or their co-culture (STLP). The volatile compounds with DF (detection frequency) of 45% and higher were considered.

To better understand the differences between the samples, PCA analysis was performed for the albumin and globulin fractions separately. The first and second component for albumin fractions (Fig VI-6. a) accounted for 65.38% and 29.60%, respectively. First component separated A-STLP and A-ST, from A-LP and A-control. A-STLP was mainly described by fruity, floral, lactic and metallic notes. The metallic odour attribute in A-STLP was assigned to the presence of "3-methylbutan-1-ol" which was only detected in this sample (Table VI-2). A-

ST is defined mainly by green attributes. The green odour attribute could originate mainly for the presence of (*Z*)-non-2-enal which was detected only in this sample (Table VI-2). A-LP and A-control fractions were characterized mostly by chemical, potato and mushroom notes.

In globulin fractions (Fig VI-6. b), first and second component accounted for 52.47% and 30.93%, respectively. First component separated G-control from fermented samples. G-STLP was mainly characterized by floral, fruity and lactic note. Floral and lactic seemed to be common in G-ST and G-STLP. However, G-ST had green, metallic and grilled note unlike G-STLP.

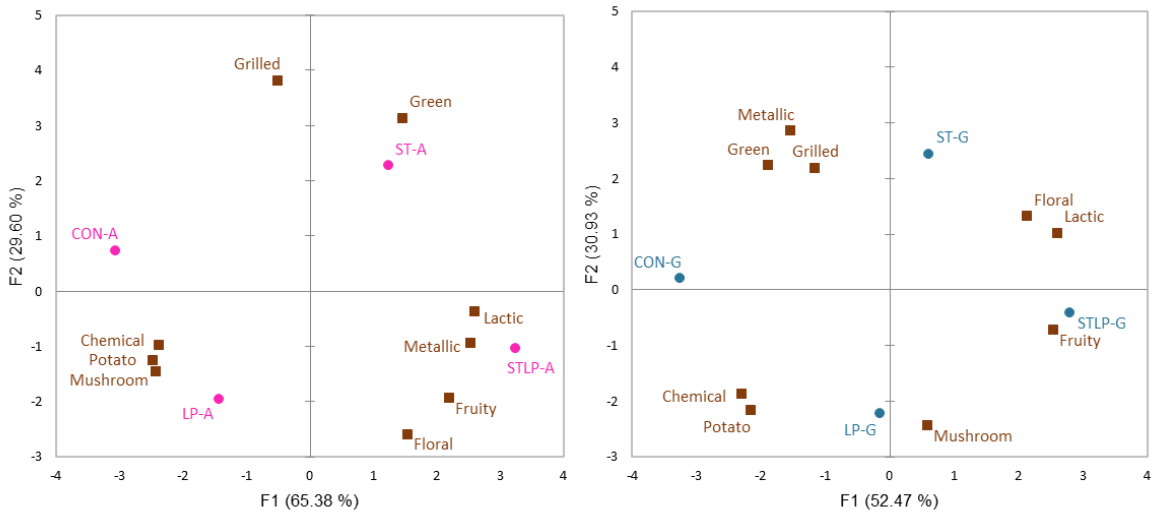


Figure VI-6. Principal component analysis (PCA – Pearson correlation) performed on the 10 odour classes (■) of pea protein albumin (A) (a) and globulin (G) (b) fractions (●) obtained without (control) or with fermentation by *S. thermophilus* (ST) and *Lb. plantarum* (LP) or their co-culture (STLP). The volatile compounds with DF (detection frequency) of 45% and higher were considered. The first and second components accounted for (a) 94.97% and (b) 83.4% of the total variance.

VI.3. Discussion

In this study, the aromatic profile of pea protein albumin and globulin fractions obtained during the lactic acid fermentation or conventional acidification with hydrochloric acid towards the isoelectric point of pea proteins, were characterized by GC-MS and GC-O. The formation and evolution of volatile compounds in pea as well as the effect of fermentation on the recovered fractions is further discussed.

In general, the flavours in pea are originated from different pathways including degradation of lipids, amino acids and carotenoids (Murray et al., 1976). Considering the samples obtained by control, the majority of the volatile compounds in this study were originated from lipid degradation. Lipids broke down to saturated and unsaturated fatty acid by lipase activity. These fatty acids would later be oxidized (by autoxidation or enzymatic pathway) and form different volatile compounds (Trindler et al., 2021). Among samples, G-control had the highest content of volatile compounds originated from fatty acids, such as pent-1-en-3-ol, pentan-1-ol, oct-1-en-3-ol, (3*E*,5*E*)-octa-3,5-dien-2-one, pentane-2,3-dione. The presence of these compounds has been reported previously for unfermented pea protein products (Ben-Harb et al., 2020; Kaczmarska et al., 2018; Murat et al., 2013). They are mainly responsible for the so-called beany note in pea protein (Fischer et al., 2022b). However, it is important to consider that the development of beany note depends on the concentration of the responsible volatile compound (Vara-Ubol et al., 2004). Also the mixture of different volatile compounds at various concentration could alter the perception of certain notes (Bott & Chambers IV, 2006). For instance, “hexanal” has no beany odour but its mixture with a beany volatile could increase the intensity of beany odour (Trindler et al., 2021). In general, aldehydes are the main compounds responsible for pea protein off-flavors and they were mainly present in globulin fractions and in particular G-control which had the highest value in tetradecanal, 2-ethylhexanal, octanal and hexana. This could be explained by the type of protein and their affinity to the volatile compounds in globulin fraction, as changes in protein structure and concentration could alter their flavour-binding affinities (Guichard, 2002). Indeed, these volatile compounds are non-polar (log Pow>1) and they have low solubility in water. Hence they would be retained strongly by the hydrophobic structure of globulin and enriched this fraction during protein precipitation (Heng, 2005). This could be the reason why A-control had lower content of volatile compounds compared to G-control. On the contrary to G-control, higher content of acids and in particular

pentanoic acid were observed in A-control which could be explained by the higher solubility of these compounds and its higher distribution in the soluble fraction.

The volatile compounds responsible for off-flavors were significantly reduced after fermentation in albumin and globulin fractions which could be related to the enzymatic activity of LAB. A good example of enzymatic activity of LAB, could be converting the aliphatic aldehydes either to alcohols by alcohol dehydrogenase (ADH) or to carboxylic acids by aldehyde dehydrogenase (ALDH) enzymes (Fischer et al., 2022b). For instance, hexanal, octanal, with high concentration in G-control, probably were converted to hexanoic acid in A-ST, octanoic acid in A-ST, A-STLP and A-LP, respectively, through the activity of ALDH. Ben-Harb et al. (2019), reported the reduction of aliphatic aldehydes after fermentation of pea protein products with *Lb. plantarum.* In fermented samples, LP and STLP had lower content of aldehydes compared to ST. Harlé et al. (2020) suggested that the heterofermentative LAB strains showed higher ability to reduce aldehydes than homofermentative strains. It is reported previously that heterofermentative strains have more ADH and ALDH compared to homofermentative strains (Fischer et al., 2022b).

Some ketones responsible for green notes such as pentane-2,3-dione and (3*E*,5*E*)-octa-3,5-dien-2-one (Ebert et al., 2022; Shi et al., 2021; Utz et al., 2022; Xiang et al., 2022), were not detected in albumin fermented or decreased in globulin fermented fractions comparing to control. While, the content of some other ketones such as heptan-2-one, 3-hydroxybutan-2-one, 1-hydroxypropan-2-one, decan-2-one, butane-2,3-dione were increased in fermented samples. The increase observed in the content of some volatile compounds after fermentation could be explained by the formation of volatile compounds during the metabolic pathways of bacteria i.e. glycolysis, proteolysis and lipolysis (Emkani et al., 2022). Lactate is the main product of sugar metabolism (glycolysis) which can be alternatively converted into different volatile compounds (Emkani et al., 2022). For instance, butane-2,3-dione, also known as diacetyl, was an important product of glycolysis metabolism of LAB (Cogan & Hill, 1993). The highest concentration of this compound belonged to the samples fermented with STLP which presented the highest rate of acidification (see chapter V or 5). It is worth noting that butane-2,3-dione with lactic note was only detected in A-STLP in analysis of GC-O. The increase in the content of butane-2,3-dione after fermentation has been already reported for fermented pea protein products with LAB strains (Ben-Harb et al., 2020); for lupin flour with commercial yogurt culture (*Lactobacillus delbrueckii and S. thermophillus*) (Kaczmarska et

al., 2018); for pea protein isolate with different LAB, including *Lb. plantarum* (Arteaga et al., 2021). Harlé et al. (2020) reported that in fermentation of soy with different LAB, butane-2,3-dione was mainly produced by *S. thermophilus* strains. 3-hydroxybutan-2-one, known as acetoin, is another metabolite of glycolysis which was found in A-ST, A-STLP and G-STLP with high content. Mefleh et al. (2022) observed that 3-hydroxybutan-2-one had higher content in samples fermented with *S. thermophilus* mono-culture and its co-culture with *Lb. plantarum* compare to unfermented and the ones fermented with co-culture of *S. thermophilus* and *L. lactis*. Harlé et al. (2020) have also reported the high content of 3-hydroxybutan-2-one for fermented soy with different strains of *S. thermophilus* and *Lb. plantarum.*

Proteolysis is another important metabolic activity of LAB. The respective activity of proteases, amino carboxypeptidases and decarboxylases produce different peptides, amino acids and α-keto-acids, which can be converted to various volatile compounds including, aldehydes, acids, esters, alcohols, pyrazines (Ben-Harb et al., 2019). For instance, 3-methylbutan-1-ol and 2-methylpropanol were originated from the metabolism of the branched amino acids, leucine and isoleucine (Morales et al., 2003). 3-methylbutan-1-ol had the highest value in albumin fermented samples compare to all the samples with any difference between globulin fermented samples and globulin control. This compound was not detected in A-control. This represented the hydrolysis of branched chain amino acids during fermentation which were then the precursors of volatile compounds with lower partition coefficient (log Pow <2) and they had less affinities with hydrophobic sites of the protein hence they were found in the soluble fraction (albumin) (Heng, 2005). High proteolytic activity of these bacteria especially STLP and production of small peptides and amino acids in fermented pea protein fractions has been shown in previous chapter. Indeed, 3-methylbutan-1-ol with metalic note was only detected in A-STLP in analysis by GC-O. This volatile compound is responsible for malty aroma in cheese and it can be formed during the enzymatic activity of certain lactic acid bacteria (Meng et al., 2021). Here it was classified in metallic odour class because of the odour attributes described by some assessors. benzenemethanol was derived from the degradation of phenylalanine. It had the highest content on A-STLP, A-LP and it followed by A-ST. In analysis of GC-O, benzenemethanol with fruity note was also detected in all the fermented albumin fractions, also G-STLP and A-control. benzaldehyde which was also obtained from the degradation of phenylalanine, had the highest value in G-STLP and G-ST. Fermented samples had higher content of acids. Acids are the main products of the enzymatic activity in LAB. acetic acid and 2-methylbutanoic acid are originated from amino acids (Nakae & Elliott,

1965). acetic acid was originated from alanine and it had more eminent value in A-ST compare to the all the other samples. The lowest value of this compound was found in G-control, G-ST and G-LP. However, this compound was not detected in GC-O analysis for any of the samples. While, 2-methylbutanoic acid was originated from isoleucine and it had the highest value in A-LP. The high concentration of these acids in fermented samples could indicate the high proteolytic activity of these strains. The high content of acid acetic in ST could be also related to the homofermentative nature of this strain. In fact, homofermentative LAB, produce lactic acid as a sole product of hexose fermentation. However, at slow growth or substrate limitation, homofermentative strains would change to mixed acid fermentation in which they produce lactic acid, acetic acid and formic acid (Nuryana et al., 2019). As it was shown in previous chapter (V), it took longer for ST to reach to pH 4.8 compared to LP and STLP.

The majority of LAB have lipase and esterase activity. The activity of lipase during fermentation produces various volatile compounds such as lactones, esters, acids and alcohols. γ- and δ-lactones are produced by β-oxidation of unsaturated fatty acids (Chen et al., 2017). Indeed, lactones are considered as cyclic esters (Silva et al., 2021). The contents of these compounds were higher in fermented samples especially the ones fermented with STLP. There are some evidence that some LAB have high activity toward hydroxylation of fatty acids which are subsequently converted to the corresponding γ- or δ-lactones (Suzuki et al., 2016). Probably the higher enzymatic activity of STLP toward the generation of lactones could be related to the synergetic effect of the bacteria. Esters which contribute to the floral and fruity notes of the products, are formed by esterification of an alcohol with an organic acid through the metabolism of protein, sugar and fat. For instance, ethyl butanoate had higher concentration in fermented albumin samples compare to the other samples and the highest value belonged to A-ST. Liu et al. (1998) reported the highest production of ethyl butanoate in dairy products belonged to *S. thermophilus* strains. This volatile compound is formed by esterification of ethanol with butyric acid (Liu et al., 1998). The intracellular esterase of LAB is able to synthesize or hydrolysis esters. Pei et al. (2022) also reported an increase in ester volatile compounds for pea flour fermented with *Lb. rhamnosus*. The highest concentration of esters in analysis of GC-MS, mainly belonged to A-ST and A-STLP. In GC-O analysis also esters were mainly detected for the samples obtained by ST and STLP. In general, albumin samples obtained by fermentation and in particular STLP had higher content of volatile compounds compared to the fermented globulin and control fractions. This could be for the generation of

compounds during fermentation with higher solubility and higher affinity with albumin fraction.

As for the OACs, G-control and A-control were mainly characterized by chemical, metallic, mushroom, green and grilled. Samples fermented with LP had more similar characteristics to samples obtained by control. This is probably for the presence of volatiles such as, methional (potato), 2,6-dimethoxyphenol (chemical), (*Z*)-hex-3-en-1-ol (metallic), (*Z*)-non-2-enal (green), geranylacetone (chemical). The mushroom odour corresponding to oct-1-en-3-one and oct-3-en-2-one had a high detection frequency in all the samples. Schindler et al. (2011) reported the presence of these two compounds for lupin protein fermented with LP. Schindler et al. (2012) also detected oct-1-en-3-one in both fermented and unfermented pea protien. While oct-3-en-2-one in their study, was only detected in fermented pea protein (Schindler et al., 2012). This could suggest that the fermentation has almost no effect on reduction of these compounds.

The samples fermented by STLP and ST, were characterized by lactic, floral and fruity odour. Yousseef et al. (2016) also have reported that pea protein isolates fermented with *S. thermophilus* had higher intensity of positive attributes as creamy, dairy and sweet, and lower intensities for negative descriptors such as vegetal, earth and vinegar. Here, the fruity perception in fermented samples corresponded to lactones, esters and alcohols as observed in GC-MS compare to aldehyde. Generally, lactones have low perception thresholds in pea protein (Karolkowski et al., 2021). However, the production of these compounds with LAB would increase their perception (Suzuki et al., 2016). Here, δ-decalactone (fruity), was detected only in all the fermented samples; γ-decalactone (fruity) was perceived only in A-STLP and G-STLP; γ-octalactone only in G-STLP; jasmin lactone (fruity) only in A-LP, A-ST and G-STLP. It seems γ-lactones (i.e. γ-octalactone, γ-nonanolactone and γ-decalactone) have the highest detection percentage in the fermented globulin fractions. While, δ-lactone (i.e. δ-decalactone and δ-Jasmine lactone) have higher percentage in fermented albumin fractions. Although, their log Pow is so close, they do not share similarly between two fractions of albumin and globulin. methyl dihydrojasmonate with floral note was not detected in control fractions and it had the highest detection in samples fermented with ST and STLP and A-LP. ethyl-3methylbutanoate or ethyl-2methylbutanoate with fruity note was only detected in A-STLP. This compound might have been originated from the esterification of 3-methylbutan-1-ol (Murray et al., 1976). Lactic attribute in ST and STLP samples was related to the presence

of 3-hydroxybutan-2-one (lactic). Also, butane-2,3-dione (lactic) was only detected in A-STLP as found by GC-MS.

Some OACs known to be pea protein off-flavours such as (2*E*,4*E*)-nona-2,4-dienal (grilled), (2*E*,4*E*)-deca-2,4-dienal (grilled), 2,6-dimethoxyphenol (chemical), 2,5-dimethylpyrazine (grilled), (*Z*)-hex-3-en-1-ol (metallic) and 6-methylheptan-2-one (chemical) wasn't detected in STLP. Probably the absence of (2*E*,4*E*)-deca-2,4-dienal (grilled) and (2*E*,4*E*)-nona-2,4-dienal (grilled) in samples fermented with STLP, could be related to the double bond hydration and retro-aldol degradations of these compounds which yielded other aldehyde such as (*E*)-oct-2-enal (green) (Josephson & Lindsay, 1987) or degraded to other non-volatile compounds by microbial metabolism. All these considered, it could be said that the odor of the samples fermented with STLP could be more pleasant since they had higher intensity of positive attributes compare to ST and LP alone. This might be for the synergetic effect of the bacteria that would favour the formation of volatile compounds in the samples obtained by co-culture, which were not detected in mono-cultures such as 2-methylpropan-1-ol (fruity), butane-2,3-dione (lactic), γ-octalactone (fruity). Madjirebaye et al. (2022) reported excellent sensorial properties for soymilk fermented with co-culture of *S. thermophilus* and *Lb. plantarum* rather than *S. thermophilus* and *Lb. plantarum* alone. Mefleh et al. (2022) observed a higher score of sweat and creamy attribute for the chickpea fermented with co-culture of *S. thermophilus* and *Lb. plantarum* compare to *S. thermophilus*. Huang et al. (2020) reported an improvement in flavor properties for yogurt fermented with LAB co-culture.

VI.3. Conclusion

To conclude, high concentration of some pea off-flavours was observed in the aroma profile of pea proteins obtained by control (such as (2*E*,4*E*)-deca-2,4-dienal (grilled), (2*E*,4*E*)-nona-2,4-dienal (grilled)). These compounds were mainly retained in the globulin fraction compared to the albumin fraction owing to the hydrophobicity of globulins and the non-polar nature of these volatile compounds. The quantity of these volatile compounds was reduced after fermentation through the enzymatic activity of LAB. Furthermore, the quantity of some other volatiles with positive perceptions (such as 2-methylpropan-1-ol (fruity)) increased through the metabolic pathway of the LAB. Albumin fermented samples presented a higher content of these volatile compounds compared to globulin fermented samples which is probably for the solubility (log Pow<1) and the affinity of the aroma between two fractions. The samples obtained by STLP were described by fruity, floral and lactic attributes. Clearly the synergies between ST and LP

permitted to obtain the positive notes related to the presence of compounds such as γ-octalactone (fruity), γ-decalactone (fruity), butane-2,3-dione (lactic), 2-methylpropan-1-ol (fruity) which were not observed in samples obtained by mono-cultures. Obviously, the changes in volatile profile depended on the strains. Hence, selection of the right strain is highly important for achieving the desired aroma. It would be interesting to complete these results with sensorial analysis to have a real perception of the protein systems.

VII.Conclusion and perspectives

Conclusion

The objective of this work was to explore a green alternative extraction method of pea proteins to the conventional alkaline solubilization/isoelectric precipitation in which the reduction of pH achieved during lactic acid fermentation instead of mineral acid addition. The effect of extraction assisted by fermentation on protein profile, antinutritional properties and aroma profile was further characterized considering resulting albumin and globulin fractions alone or together.

The study was initiated with the examination on the feasibility of the method which generated further questions. The first attempts were performed by application of commercial pea flour and commercial starters. The next part was dedicated to characterize the protein profile and antinutritional properties of albumin residue. Different LAB strains were used here either alone or in co-culture, for their proteolytic activity and their contribution to organoleptic properties of legume protein which bring about further questions on characterisation of volatile compounds and volatile active compounds.

To obtain albumin and globulin fractions, commercial pea flour was initially suspended in water and solubilized at alkaline pH (~7.5). Thereafter, the reduction of pH to ~4.8 was achieved during the fermentation by commercial starters either alone or in co-cultures. The commercial starters including *S. thermophilus* (S), *S. thermophilus* and *Lb. acidophilus* (SL), *S. thermophilus, Lb. acidophilus* and *B. lactis* (SLB), were all able to grow in pea protein extract and reduce the pH to 4.8. To compare the results with conventional method, the reduction of pH was also achieved by addition of a mineral acid (hydrochloric acid) and an organic acid (lactic acid). The results showed that it was possible to obtain albumin and globulin fractions during fermentation. Compared to the conventional alkaline extraction/isoelectric precipitation method, the samples obtained by fermentation had higher content of total nitrogen in albumin fraction. The majority of this increase was related to non-protein nitrogen meaning that the proteolytic activity of the bacteria led to the hydrolysis of protein. Produced small peptides and amino acids had higher solubility and thus they were liberated to the soluble fraction (albumin). An increase in the content of free amino groups and the disappearance of some vicilin subunits (such as Vγ, ~12-16 kDa) bands in polypeptide profile of globulin fraction; convicilin (~70 kDa) and some bands in the range of 40-80 kDa in albumin fraction obtained by fermentation, could explain the proteolysis occured in these fractions. At the same time, slight increase was observed in the denaturation temperature of the

albumin and globulin samples obtained by fermentation compared to the control. Also calculating the vicilin/legumin energy area ratio, could indicated that fermented samples had lower vicilin/legumin ratio compared to control. This may explain two findings; (i) the increase observed in thermal denaturation temperature of fermented samples could be for the presence of more legumin as this fraction has more complex structure, (ii) some vicilin polypeptides have been released in albumin fraction and/or have been consumed by the bacteria explaining why some bands related to vicilin was disappeared in protein profile of the samples.

The higher protein content in albumin fraction obtained by fermentation challenged further analysis on this fraction which is normally discarded in industry for the presence of antinutritional compounds and low protein content. Three pea cultivars (CAR, AC, AS) were used for their contrasted compositions in protein fractions to examine whether fermentation could lead to the enrichment of certain fractions or not. Four LAB were chosen including *Lb. helveticus* (LH), *Lb. rhamnosus* (LR), *Streptococcus thermophilus* (ST) and *Lb. plantarum* (LP), for their proteolytic activity and their contribution to organoleptic properties. Among these strains, only ST and LP were selected since they were both able to growth under aerobic conditions. Therefore, albumin and globulin fractions were obtained without fermentation (control) or with fermentation with ST, LP and their co-culture (STLP). All the strains were able to grow on the protein extracts prepared from the three pea cultivars and reduce the pH to 4.8. Despite the low concentration of mono-, di-saccharides and α-galactosides (~0.04 M glucose, i.e ~7 g/L; 0.02 M sucrose, i.e. ~7.8 g/L; and 0.05 M RFOs) in the alkaline extracts, all the LAB were able to produce acid in a quantity that permitted to reduce the pH significantly. It is worth noting that these sugars concentrations are lower compared to the lactose content in milk (~50 g/L) where these strains are usually able to growth. Probably that's why the addition of glucose (10 g/L) led to the faster pH reduction by LAB in the different protein extracts. However, the fermented samples obtained in presence of glucose were not further studied due to the lower production of peptides in these samples compared to the ones obtained in absence of glucose (data not shown). Still, the kinetics of acid reduction and the time required to reach to pH 4.8 was faster for the samples obtained by co-culture compared to the mono-cultures. This was probably related to the synergetic effect of the bacteria leading to the faster growth and acid production. The results of this study indicated the positive effect of extraction assisted by fermentation on nutritional quality and antinutritional compounds of the albumin fraction without damaging the protein profile of globulin fraction which is usually used as protein isolate in industry. Extraction assisted by fermentation with ST, LP and their

co-culture modified the composition of albumin fraction by increasing the nitrogen and small peptides content, antioxidant activity and reduction of antinutritional compounds such as trypsin inhibitor, α-galactosides and phytic acid. These changes in protein composition is certainly related to the enzymatic activity of the bacteria during fermentation which varied depending on the strains, though the enzymatic activity of the bacteria were not studied directly in this work. For instance, the albumin fraction obtained by LP had the lowest content of α-galactosides compared to the other strains. While, the samples obtained by co-culture had the lowest trypsin inhibitor activity and the highest production of small peptides which is mainly related to the proteolytic activity of bacteria. Probably the synergy between the bacteria in co-culture lead to the strong proteolytic activity of this starter. Among pea varieties studied, extraction assisted by fermentation led to more enrichment of nitrogen and higher peptide production in albumin fraction obtained from cultivar AS. This cultivar was initially richer in albumin (2S) and legumin (11S). However, the other properties studied in this report did not seem to be pea genotype-dependent.

The last question in this study was the effect of extraction assisted by fermentation on aromatic profile of the albumin and globulin fractions. Cultivar AS was selected here since in the previous chapter the highest proteolysis activity was observed in this cultivar compared to the others. The volatile compounds of the samples obtained without (control) or with fermentation by ST, LP and their co-culture STLP were first characterized by GC-MS. The analysis of odor active compounds was further characterized by GC-O. The samples obtained without fermentation were mainly described by chemical, potato, mushroom, green, metallic and grilled. Some volatile compounds such as pent-1-en-3-ol, pentan-1-ol, oct-1-en-3-ol, (3*E*,5*E*)-octa-3,5-dien-2-one, pentane-2,3-dione, acetophenon had the highest value in globulin fraction obtained by control. These compounds are known for being responsible for beany note in pea protein isolate and they are mainly originated from the degradation of fatty acids. Globulin fraction and in particular the one obtained by control, had the highest content of aldehydes such as hexanal, 2-ethylhexanal, tetradecanal, octanal. These compounds have low solubility in water (log Pow>1) and they could be preferentially retained in the hydrophobic structure of globulins. This could explain the lowest content of volatile compounds in albumin fraction obtained by control. Both, albumin and globulin fractions obtained by control were associated with pea off-flavor related compounds. The quantity of these compounds was reduced in fermented samples probably by the enzymatic activity of the LAB and/or they were replaced by production of other volatile compounds generated during the metabolic pathway of the

LAB. For instance, some aldehydes such as hexanal with high concentration in globulin control were converted to hexanoic acid in albumin obtained by ST by the activity of ALDH or to hexanol in albumin obtained by LP by activity of ADH; or 2-ethylhexanal with high concentration in globulin control to 2-ethylhaxanol in globulin obtained by ST by ADH. Moreover, the content of some other volatile compounds produced through the main metabolic pathways of LAB were increased in fermented samples such as 3-hydroxybutan-2-one (lactic) which is the product of glycolysis or benzenemethanol (fruity), the product of proteolysis in LAB. Fermented albumin fractions, especially the one fermented with STLP, had higher content of volatile compounds compared to fermented globulin and control samples. In chapter 5 or V, it has been shown that STLP had higher enzymatic activity in reduction of antinutritional compounds and in production of small peptides and amino acids, compared to the ST and LP. The higher perception of positive attributes such as lactic, floral and fruity in co-culture compared to the other samples could be also related to their enzymatic activity. Probably, the synergies between ST and LP lead to the production of some odour active compounds such as γ-octalactone (fruity), γ-decalactone (fruity), butane-2,3-dione (lactic), 2-methylpropan-1-ol (fruity) which were not observed in samples obtained by mono-cultures.

Perspectives

This study presented the possibility of application an alternative extraction method to the conventional alkaline solubilization/isoelectric precipitation. The method could be promising for environment, human health, industrial approaches and it opens a large possibility of research and analysis on different subjects. However, the acceptance of a new extraction method requires trust on safety of protein, environmental impact, techno-functional, nutritional quality of the product and economic aspect. The results obtained in this study are obtained in laboratory scale, therefore it is needed to examine this method in larger scale for industrial interests.

In this study, it has been shown that extraction assisted by fermentation represented the possibility to recover albumin fraction with high yield without damaging the structure and denaturation of globulin fraction which is used as protein isolate in industry. However, one of the main limitations in commercialization of the pea protein is their low techno-functional properties. It is still necessary to obtain a complete view on their techno-functional properties such as solubility, foaming and emulsifying properties of both albumin and globulin fractions. It was also shown that extraction assisted by fermentation improved the aromatic profile of the

protein fractions. However, to obtain a complete view on aromatic properties, it is required to perform the sensory analysis of food products obtained from the protein fractions. Moreover, the protein extracts in industry go through different processing such as heat stabilization (pasteurization, sterilization) and drying which can possibly affect the techno-functional, sensorial and aromatic profile of the samples. Hence, it could be interesting to observe the effect of further drying or other heat treatments on the aromatic profile and techno-functional properties of the protein isolates prepared from the present process.

In this study, it was shown that albumin fraction obtained by fermentation was richer in nitrogen, it contained lower amount of antinutritional compounds and higher content of small peptides which could have metabolic activity (such as anticancer, anti-inflammatory, antimicrobial, immunomodulatory, antihypertensive, etc). Hence, the production of bioactive peptides in albumin fraction should be considered for the industrial valorisation of this fraction. On the other hand, the proteolytic activity of the bacteria could lead to the production of bitter peptides which should be further examined and controlled in this extraction process.

The proposed method is promising not only for production of protein but also for the production of biomass. Indeed, the bacteria growth during fermentation in pea protein were separated and discarded at the end of the process. While, LAB are considered as safe and they have so many biological activities such as probiotic which can contribute to health benefits. It is also possible that these bacteria have developed different beneficial properties during their growth and adaptation in pea protein substrate. Therefore, it is interesting to harvest the discarded bacteria and analysis their properties and their enzymatic activities that they might have developed and finally re-use them in the process. Moreover, the selection of the bacteria could be optimized based on the interest enzymatic activity (phytate, tannins, etc) in protein fractions. The conditions of the fermentation such as temperature, time or added nutriments could be also optimized in terms of enzymatic activity of the bacteria in protein fractions. In this study, it was shown that the addition of glucose could increase the kinetics of acidification for mono-cultures. The addition of sugar can be further applied in the process of extraction assisted by fermentation in order to facilitate the growth of the bacteria and probably to obtain different results in terms of microbial activity. Also, in this study the fermentation was not extended further than ~10h (which is the time required to reach pH 4.8) and it will be interesting to assess the effect of longer fermentation time on enzymatic activity (proteolytic enzymes, phytase, etc) of LAB.

In this method there are still some aspects to optimize, such as the selection of varieties and the consumption of water and energy. Indeed, the method is sustainable in which the lessen amount of chemical is used. However, the amount of water and energy consumption need to be considered in future Life Cycle Assessment studies of such process.

VIII.Résumé

Au cours de la dernière décennie, il est apparu un questionnement concernant la consommation de protéines d'origine animale. Les préoccupations nutritionnelles augmentent avec l'augmentation de la population mondiale de plus d'un milliard de personnes au cours des 15 prochaines années (atteignant 8,5 milliards en 2030 selon les projections des Nations Unies), ce qui entraîne une augmentation drastique de la demande alimentaire (Goldstein & Reifen, 2022). La consommation de protéines d'origine animale est déjà associée à de nombreux problèmes environnementaux et de santé humaine. En effet, la production de bétail est responsable d'au moins 14,5 % des émissions de gaz à effet de serre (GES) (Lemken et al., 2019), de la pollution de l'air, de la consommation élevée d'eau, de la diminution de la qualité des sols et de l'eau, de la destruction de la biodiversité et de la dégradation des zones de pâturage (Leip et al., 2015). De plus, du point de vue de la santé humaine, la consommation de protéines d'origine animale augmente le risque de maladies coronariennes, de diabète de type 2 et de certains types de cancer (Pihlanto et al., 2017). Par conséquent, il est urgent de trouver des sources alternatives de protéines qui soient disponibles, rentables et plus prometteuses tant pour la santé humaine que pour l'environnement.

Les légumineuses sont la solution possible qui contribue à une alimentation durable et plus saine. En effet, les légumineuses sont capables de fixer l'azote atmosphérique car elles ont une interaction biologique avec les bactéries du sol fixatrices d'azote (rhizobia), ce qui entraîne la formation de nodules racinaires (Wang et al., 2018). La production de légumineuses ne nécessite pas d'engrais azoté supplémentaire pour la croissance. En effet, les légumineuses fournissent à d'autres plantes l'azote excédentaire dans la rotation des cultures, ce qui permet de réduire indirectement les émissions de GES (Foyer et al., 2016). En outre, les légumineuses sont faciles à cultiver et très tolérantes à la sécheresse grâce à leur système racinaire profond (Didinger & Thompson, 2020). Par conséquent, la production de légumineuses peut prévenir les dommages causés à l'écosystème ; elle nécessite une consommation d'eau plus faible et elle peut libérer des terres cultivées (Henn et al., 2022). Ces avantages environnementaux sont de plus en plus pris en compte dans le choix des consommateurs de légumineuses.

Les légumineuses présentent de nombreux avantages pour la santé humaine, notamment le maintien du poids, la santé intestinale et la réduction du risque de maladies cardiovasculaires et de cancers. Elles appartiennent à la famille des Fabaceae ou Leguminosae et représentent la deuxième culture alimentaire la plus produite après les céréales. Les légumineuses comprennent les légumineuses à graines oléagineuses (c'est-à-dire le soja) et les légumineuses à graines telles que les lentilles, les pois chiches, les pois, les lupins et les haricots (Semba et

al., 2021). Parmi eux, le pois présente un grand intérêt pour sa valeur nutritionnelle élevée, qui peut motiver sa consommation. Le pois est riche en protéines et en hydrates de carbone, pauvre en graisses et contient des vitamines et des minéraux importants. Le pois contient environ 20 à 30 % de protéines par rapport à la quantité totale de graines sèches. La protéine de pois contient des acides aminés de haute qualité avec des niveaux élevés de lysine et des niveaux plus faibles de méthionine et de tryptophane (Lam et al., 2018). Elle présente une faible allergénicité et une meilleure digestibilité que les autres légumineuses. Il peut être utilisé soit sous forme de composants issus des grains (par exemple, farine moulue à partir de grains), soit sous forme d'ingrédients protéiques enrichis tels que le concentré de protéine (50-55 %) et l'isolat de protéines (80-90 %) (Barać et al., 2015 ; Boye et al., 2010). Les deux principales protéines du pois sont les albumines (10-20%) et les globulines (70-80%). Les fractions d'albumine et de globuline peuvent être purifiées, car elles ont une solubilité différente. Les globulines sont solubles dans le sel, tandis que les albumines sont solubles dans l'eau. Les albumines possèdent de meilleures propriétés nutritionnelles que les globulines, car elles contiennent une concentration plus élevée d'acides aminés essentiels tels que le tryptophane, la lysine, la thréonine et la méthionine (Kumari et al., 2022).

Malgré les propriétés prometteuses des protéines de pois, leur application dans l'industrie alimentaire reste un défi en raison de leurs propriétés techno-fonctionnelles insuffisantes, notamment leur faible solubilité et leurs propriétés émulsifiantes, leurs propriétés sensorielles imparfaites et la présence de certains facteurs antinutritionnels (Emkani et al., 2021). Toutes ces déficiences dépendent de la structure et de la composition des protéines, qui sont influencées par différents paramètres tels que le cultivar et le processus d'extraction. Les conditions génétiques et environnementales de croissance constituent la première étape du contrôle de la structure des protéines du pois, car elles peuvent affecter la composition et la teneur en protéines, en acides aminés, en composés volatils et en composés antinutritionnels (Daba & Morris, 2022 ; Jakobsen et al., 1998 ; Nikolopoulou et al., 2007).

Différentes méthodes ont été proposées pour l'extraction des protéines de la farine de pois, chacune pouvant avoir un impact sur le rendement et la qualité des protéines. Les méthodes conventionnelles d'extraction par voie humide sont l'extraction alcaline avec précipitation isoélectrique (AEIEP), l'extraction saline avec dialyse, la précipitation micellaire et l'extraction aqueuse (pH > 7) (Lam et al., 2018 ; Owusu-Ansah & McCurdy, 1991). L'AEIEP est la technique la plus populaire avec un rendement élevé pour produire des isolats de protéines de pois dans l'industrie alimentaire, car elle est facile à utiliser et la protéine obtenue par cette

méthode est d'une grande pureté. Le processus d'extraction utilise la manipulation du pH pour valoriser la globuline sous forme d'isolat (Tanger et al., 2020). La méthode sépare les albumines et les globulines car la fraction de globuline a une solubilité élevée à un pH alcalin, et une solubilité minimale à leur point isoélectrique (pI), tandis que les fractions d'albumine restent solubles à presque toutes les valeurs de pH (Djemaoune et al., 2019). Actuellement, la fraction globuline obtenue par cette méthode est exploitée sous forme d'isolats par l'industrie, alors qu'à notre connaissance, la fraction albumine est généralement écartée en raison d'une teneur en protéines plus faible que celle des globulines et de la présence de certains composés antinutritionnels. Les méthodes conventionnelles d'extraction des protéines présentent également certains inconvénients, tels que la forte demande en produits chimiques, les empreintes alimentaires des solvants sur l'environnement, la dénaturation des protéines dans certains cas et, surtout, les techniques actuelles n'ont pas permis de réduire la teneur en composés antinutritionnels et en arômes indésirables (Eze et al., 2022). Ces défis soulignent la nécessité de modifier et d'hybrider les méthodes conventionnelles afin de développer une méthode d'extraction innovante, facile à utiliser, verte, respectueuse de l'environnement et rentable qui, en même temps, augmente la pureté et la qualité des protéines (Eze et al., 2022 ; Wen et al., 2022). Les techniques récentes telles que l'extraction assistée par micro-ondes, l'extraction assistée par ultrasons, la séparation électrostatique ont attiré plus d'attention car elles sont considérées comme vertes, mais elles posent toujours des problèmes de pureté et de dénaturation des protéines (Hewage et al., 2022 ; Wen et al., 2022).

Entre-temps, l'application des micro-organismes dans les produits alimentaires a suscité un grand intérêt, car elle peut améliorer les propriétés nutritionnelles et sensorielles des protéines de légumineuses, ainsi que la teneur et la qualité des protéines (Knez et al., 2023). La fermentation est l'une des plus anciennes techniques de transformation des aliments, utilisée traditionnellement dans certains pays (Knez et al., 2023). Il existe différents types de fermentation, parmi lesquels la fermentation lactique gagne de plus en plus en popularité et est largement utilisée dans l'industrie laitière et alimentaire car elle est considérée comme sûre et contribue à la saveur, à la texture et à la valeur nutritionnelle des aliments fermentés. En effet, l'activité enzymatique des bactéries pendant la fermentation conduit à la production de composants aromatiques et/ou à la réduction des mauvais goûts, à la production d'exopolysaccharides, à l'hydrolyse des protéines en peptides plus petits et en acides aminés ayant des propriétés bioactives, à la modification des propriétés techno-fonctionnelles, à la modification de la digestibilité des protéines par la réduction des composés antinutritionnels et

à la production de composants nutritionnels tels que les vitamines, à la conservation des aliments par la production de bactériocines, ou à la promotion des avantages pour la santé lorsqu'ils sont utilisés comme probiotiques (Fischer et al., 2022 ; Harlé et al., 2020 ; Reuben et al., 2020 ; Xing et al., 2020). Toutefois, l'efficacité et la fonctionnalité de la fermentation lactique dépendent fortement du type de souche de bactéries lactiques (LAB), de la technique de fermentation, de la composition de l'ingrédient protéique et, dans une moindre mesure, de la variété génétique (Bartkiene et al., 2015 ; Rui et al., 2017).

Compte tenu des effets positifs de la fermentation lactique sur les propriétés des légumineuses et de la baisse du pH due à la formation d'acide lactique, l'objectif principal de cette thèse était d'explorer une méthode d'extraction alternative basée sur la solubilisation alcaline/précipitation isoélectrique dans laquelle l'utilisation de produits chimiques serait partiellement réduite. La méthode proposée pourrait améliorer la pureté et la quantité de protéines ainsi que les propriétés nutritionnelles et le profil aromatique des protéines de légumineuses.

L'idée est ici de diminuer le pH par l'activité des bactéries lactiques pendant la fermentation au lieu de l'acidification conventionnelle qui consiste à ajouter des acides minéraux (Figure VIII-1.). Pour ce faire, la farine de pois (commerciale ou cultivars) a été mélangée à de l'eau (10 % p/p) et le pH de la solution a été ajusté à 7,5 avec du NaOH (0,5 M). La suspension a ensuite été agitée pendant une nuit à 4 °C et le pH a été réajusté à 7,5. Les matières insolubles ont été éliminées par centrifugation (10 000xg, 30 min, 20 °C) et le surnageant a été recueilli. La précipitation acide a été utilisée pour séparer les fractions de globuline et d'albumine de pois. L'acidification a été réalisée par fermentation. Les bactéries lactiques sélectionnées ont été ajoutées à la solution protéique à un pH de 7,5 en monoculture ou en coculture (*Streptococcus thermophilus* (S), *Streptococcus thermophilus* + *Lactobacillus acidophilus* (SL) et *Streptococcus thermophilus* + *Lactobacillus acidophilus* + *Bifidobacterium lactis* (SLB), *S. thermophilus* (ST), *Lb. Plantarum* (LP), *S. thermophilus* (ST) + *Lb. Plantarum* (STLP)). Les échantillons ont ensuite été placés dans un incubateur (Sanyo incubator MIR-153 w, Osaka, Japon) à une température optimale (37 °C pour les starters, 37 °C pour LP, 43 °C pour ST et 40 °C pour STLP) sous agitation modérée. Chaque fermentation a été préparée en trois exemplaires. Le pH de la protéine de pois incubée a été mesuré en continu et enregistré automatiquement à intervalles de 5 minutes (pH-mètre 3310, WTW GmbH, Weilheim, Allemagne). L'acidification a été arrêtée à pH 4,8 qui est proche du point isoélectrique des globulines. Les suspensions obtenues ont ensuite été centrifugées (10 000×g, 30 min, 4 °C) et la partie soluble correspondant à la fraction d'albumine a été séparée de la partie non soluble

sous forme de culot. Le culot contenait principalement des globulines et de la biomasse. Afin de récupérer la fraction de globuline, le culot a été suspendu dans de l'eau (5% w/v) et le pH a été ajusté à 7,5 par NaOH 0,5 M pour solubiliser les globulines. L'échantillon a été agité pendant une nuit et le pH a été réajusté à 7,5. La suspension a été à nouveau centrifugée (10 000×g, 20 min, 20°C) et le surnageant a été recueilli comme la fraction de globuline, exempte de biomasse qui a décanté. Les fractions de protéines de pois ont également été obtenues par acidification conventionnelle en utilisant de l'acide chlorhydrique (HCl) 0,5 M ou de l'acide lactique (LA) 0,5 M en suivant les mêmes étapes. Les fractions obtenues par acidification conventionnelle ont ensuite été comparées à celles obtenues par fermentation.

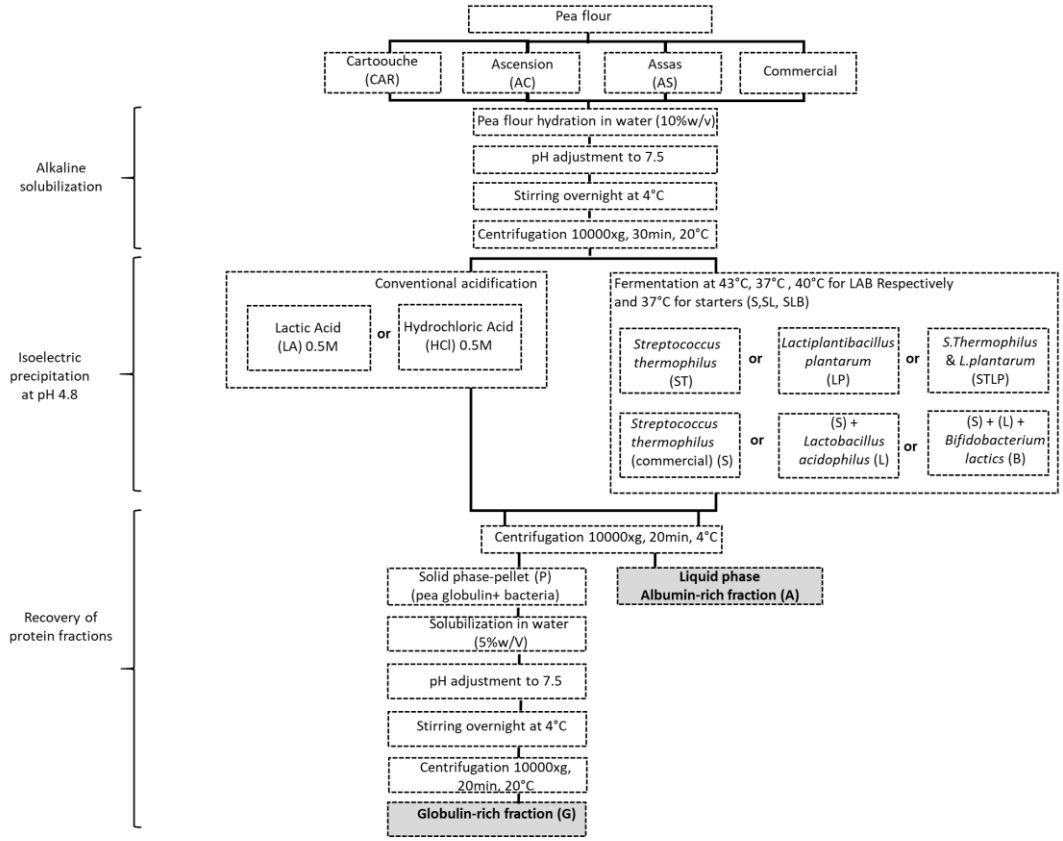


Figure VIII-1. Schéma du processus d'extraction.

Cette étude devait répondre à trois questions principales. La première question était de valider la méthode et la possibilité d'obtenir des isolats de protéines de pois par fermentation. La méthode d'extraction a été réalisée sur de la farine de pois commerciale par l'application de starters commerciaux (*Streptococcus thermophilus* (S), *Streptococcus thermophilus* + *Lactobacillus acidophilus* (SL) et *Streptococcus thermophilus* + *Lactobacillus acidophilus* + *Bifidobacterium lactis* (SLB)). L'effet de la fermentation sur le profil et la dénaturation des protéines a ensuite été évalué. La teneur en azote total et non protéique de la farine de pois, des

extraits de protéines de pois et des isolats de protéines (albumine et globuline) a été mesurée par la méthode Kjeldahl. L'hydrolyse des protéines isolées au cours de la fermentation a été caractérisée par la quantification des groupes aminés libres et la présence ou l'absence de fractions protéiques a été caractérisée par l'électrophorèse par SDS-PAGE. Enfin, l'état de dénaturation des protéines au cours de la fermentation a été mesuré par analyse calorimétrique à balayage différentiel (DSC). Les résultats obtenus pour les échantillons fermentés ont été comparés aux échantillons obtenus par la méthode conventionnelle (ajout d'acide i.e. HCl ou acide lactique).

Les résultats de cette étude indiquent que la fermentation a permis de produire des fractions riches en albumine et en globuline. Par rapport à la méthode conventionnelle d'extraction alcaline/précipitation isoélectrique, les échantillons obtenus par fermentation présentaient une teneur plus élevée en azote total dans la fraction albumine. Parallèlement à cette augmentation, la teneur en azote total a diminué dans la fraction de globuline obtenue par fermentation. Par ailleurs, la teneur en azote non protéique a augmenté dans les fractions d'albumine et de globuline obtenues par fermentation par rapport à celles obtenues par acidification conventionnelle, ce qui pourrait indiquer l'activité protéolytique des bactéries et l'hydrolyse des protéines (Figure VIII-2). Une augmentation de la teneur en groupes aminés libres et la disparition de certaines sous-unités de la viciline (telles que Vγ, ~12-16 kDa) dans le profil polypeptidique de la fraction de globuline ; la conviciline (~70 kDa) et certaines bandes dans la gamme de 40-80 kDa dans la fraction d'albumine obtenue par fermentation, pourraient expliquer la protéolyse qui s'est produite dans cette fraction. En même temps, une légère augmentation a été observée dans la température de dénaturation des échantillons d'albumine et de globuline obtenus par fermentation par rapport au témoin. Le calcul du rapport entre la surface énergétique de la viciline et de la légumine pourrait également indiquer que les échantillons fermentés ont un rapport viciline/légumine inférieur à celui du témoin. Cela peut expliquer deux résultats : (i) l'augmentation observée de la température de dénaturation thermique des échantillons fermentés pourrait être due à la présence d'une plus grande quantité de légumineuses, car cette fraction a une structure plus complexe, (ii) certains polypeptides de la viciline ont été libérés dans la fraction d'albumine et/ou ont été consommés par les bactéries, ce qui explique pourquoi certaines bandes liées à la viciline ont disparu dans le profil protéique des échantillons.

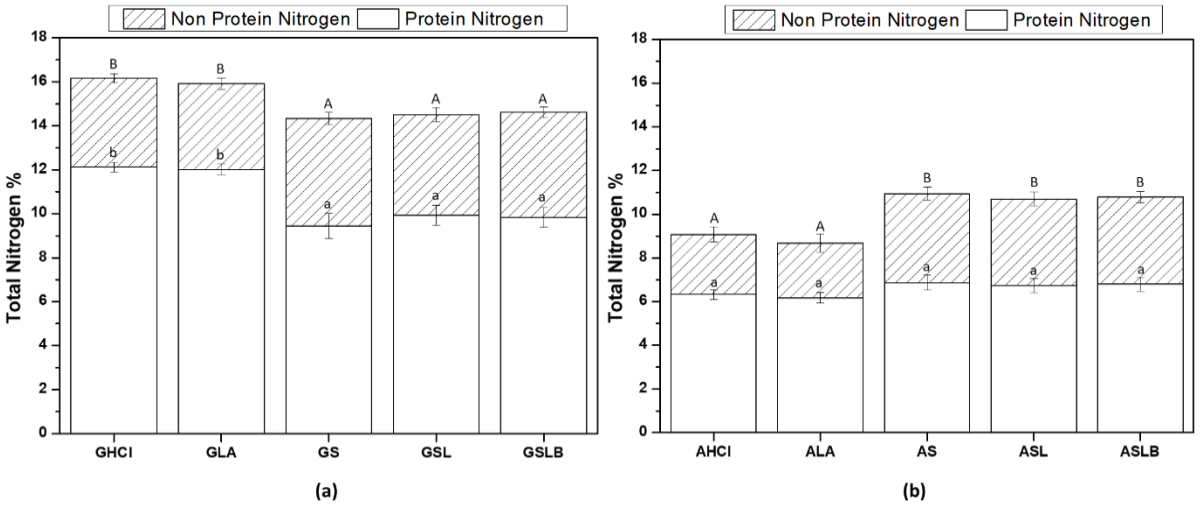


Figure VIII-2. Teneur en azote total (azote non protéique et protéique) des fractions de globuline (G) (a) et d'albumine (A) (b) obtenues par les témoins (HCl, LA) et les fermentations avec *S. thermophilus* (S), *S. thermophilus + Lb. acidophilus* (SL), *S. thermophilus*+ *Lb. acidophilus*+ *B. lactis* (SLB). Les différentes lettres majuscules représentent des différences significatives dans la teneur en azote total et les lettres minuscules représentent des différences significatives dans l'azote protéique entre les différents échantillons (test post hoc de Tukey).

La teneur plus élevée en protéines de la fraction d'albumine obtenue par fermentation a justifié une analyse plus poussée de cette fraction. En effet, la solubilisation alcaline/précipitation isoélectrique est une méthode reconnue dans l'industrie pour la production d'isolats avec un rendement élevé (~60-70%). En revanche, la fraction albumine est généralement rejetée par l'industrie en raison de son profil peu pratique, comme la présence de composants antinutritionnels et une teneur en protéines inférieure à celle de la fraction globuline (Malley et al., 1975 ; Sell et al., 2005 ; Yang et al., 2022). Cependant, cette fraction pourrait présenter des propriétés intéressantes et une teneur élevée en acides aminés contenant du soufre par rapport à la fraction des globulines (Croy et al., 1984 ; Davies, 1976 ; Lu et al., 2000). La question était donc d'évaluer l'effet de la fermentation sur le profil protéique et les propriétés nutritionnelles et antinutritionnelles de la fraction d'albumine fermentée. Des cultivars de pois présentant une composition polypeptidique contrastée ont été utilisés pour observer l'effet de la fermentation sur l'enrichissement éventuel de certaines fractions protéiques. La fermentation a été réalisée par l'application de différentes espèces de LAB, seules ou en co-culture, afin d'observer l'effet des différentes souches sur la composition protéique du pois. Quatre souches LAB ont été sélectionnées : *Lb. plantarum*, *S. thermophilus*, *Lb. helveticus* et *Lb. rhamnosus*.

La sélection des bactéries a été basée sur leur capacité d'acidification, leur activité protéolytique et leur contribution aux propriétés organoleptiques des aliments à base de légumineuses.

Une souche de *Lb. plantarum* a été sélectionnée car elle est bien adaptée aux protéines de pois (Ben-Harb et al., 2019 ; Djeghri-Hocine et al., 2007) et a été largement utilisée dans la fermentation du pois (Arteaga et al., 2021 ; Cabuk et al., 2018a ; Cabuk et al., 2018b ; Czarnecka et al., 1998 ; Li et al., 2021). Son activité protéolytique a également été signalée (Byanju et al., 2021 ; Diana et al., 2014). En outre, de nombreuses études ont fait état de l'amélioration des propriétés organoleptiques et de la réduction notes aromatiques indésirables des protéines de pois fermentées par *Lb. plantarum* (Schindler et al., 2012 ; Shi et al., 2021 ; Xiang et al., 2022). *S. thermophilus* est largement utilisé dans l'industrie laitière pour son taux d'acidification élevé et sa contribution aux propriétés organoleptiques (Galia et al., 2009 ; Iyer et al., 2010). Il existe également des preuves de la croissance et de l'acidification élevées de cette souche dans un substrat à base de pois (Narala et al., 2022 ; Yousseef et al., 2014), ainsi que des attributs organoleptiques positifs de la protéine de pois fermentée, tels que crémeux, laitier et sucré (Yousseef et al., 2016). *Lb. helveticus* est un ferment industriel important car il possède un système protéolytique efficace (Griffiths & Tellez, 2013). Une activité protéolytique élevée de *Lb. helveticus* vsi à vis des protéines de légumineuses a été rapportée précédemment (Meinlschmidt et al., 2016 ; Vermeirssen et al., 2003). Cette souche contribue également à la réduction des attributs négatifs tels que l'acidité, l'amertume et l'astringence (Li et al., 2021 ; Schlegel et al., 2019).

Après des tests préliminaires, parmi quatre souches, seules *S. thermophilus* (ST) et *Lb. plantarum* (LP) ont été sélectionnées pour la fermentation et les expériences ultérieures. La co-culture de ces deux souches (STLP) a également été utilisée pour étudier les synergies possibles sur le profil et les propriétés des protéines. Trois cultivars de pois (Cartouche (CAR), Ascension (AC), Assas (AS)) ont été choisis pour leurs profils polypeptidiques et leur composition protéique contrastés. Cartouche était riche en viciline, Ascension était riche en conviciline et Assas était riche en albumine. L'azote total et l'azote non protéique des farines de trois cultivars de pois, de leurs extraits protéiques alcalins et des isolats protéiques résultants (fractions d'albumine et de globuline) de tous les échantillons obtenus sans ou avec fermentation ont été mesurés par la méthode Kjeldahl. L'activité protéolytique des bactéries sur les isolats de protéines a été évaluée par l'analyse de la teneur en groupes aminés libres de tous les échantillons. Le profil polypeptidique par SDS-PAGE des isolats protéiques a été caractérisé pour observer la pureté et la présence des fractions protéiques. La présence de

peptides (<10kDa) a été mesurée par chromatographie d'exclusion (HPLC-SEC). L'activité de piégeage du DPPH et la capacité antioxydante équivalente au Trolox (TEAC) ont été réalisées pour mesurer l'activité antioxydante des peptides produits par fermentation. Les composés antinutritionnels (inhibiteurs de la trypsine, α-glactosides (raffinose family of oligosaccharides, RFOs) et phytates) ont été mesurés dans tous les échantillons. La teneur en composés antinutritionnels des isolats de protéines a ensuite été comparée à celle de la farine de pois.

Les résultats de cette étude indiquent que l'extrait de protéines de pois est un bon substrat puisque toutes les souches ont pu se développer sur les extraits de protéines préparés à partir des trois cultivars de pois et réduire le pH à 4,8. Malgré la faible concentration de mono-, di-saccharides et α-galactosides (~0,04 M de glucose, soit ~7 g/L ; 0,02 M de saccharose, soit ~7,8 g/L ; et 0,05 M de RFOs) dans les extraits alcalins, toutes les LAB ont été capables de produire de l'acide permettant de réduire le pH de manière significative. Cependant, la cinétique d'acidification et le temps nécessaire pour atteindre un pH de 4,8 étaient plus rapides pour les échantillons obtenus par co-culture que pour les mono-cultures. Ceci est probablement lié à l'effet synergique des bactéries conduisant à l'activation de certaines enzymes nécessaires à la consommation de nutriments pour une croissance et une production d'acide plus rapides. Les résultats de cette étude indiquent l'effet positif de l'extraction assistée par fermentation sur la qualité nutritionnelle et les composés antinutritionnels de la fraction d'albumine sans affecter le profil protéique de la fraction de globuline qui est généralement utilisée comme isolat de protéine dans l'industrie. L'extraction assistée par fermentation avec ST, LP et leur co-culture a modifié la composition de la fraction albumine en augmentant la teneur en azote et en petits peptides, l'activité antioxydante et en réduisant les composés antinutritionnels tels que l'inhibiteur de trypsine, les α-galactosides et l'acide phytique. Ces modifications de la composition protéique sont certainement liées à l'activité enzymatique des bactéries au cours de la fermentation qui varie en fonction des souches, bien que l'activité enzymatique des bactéries n'ait pas été étudiée directement dans ce travail. Par exemple, la fraction d'albumine obtenue par LP avait la plus faible teneur en α-galactosides (RFOs) par rapport aux autres souches (Figure VIII-3 a). En revanche, les échantillons obtenus par co-culture avaient l'activité inhibitrice de la trypsine la plus faible (Figure VIII-3 b) et la production la plus élevée de petits peptides, ce qui est principalement lié à l'activité protéolytique des bactéries. La synergie entre les bactéries en co-culture est probablement à l'origine de la forte activité protéolytique de ce ferment. Parmi les variétés de pois étudiées, l'extraction assistée par la fermentation a conduit à un plus grand enrichissement en azote et à une plus grande production de peptides dans la

fraction d'albumine obtenue à partir du cultivar AS. Ce cultivar était initialement plus riche en albumine (2S) et en légumine (11S). Cependant, les autres propriétés étudiées dans ce rapport ne semblent pas dépendre du génotype du pois.

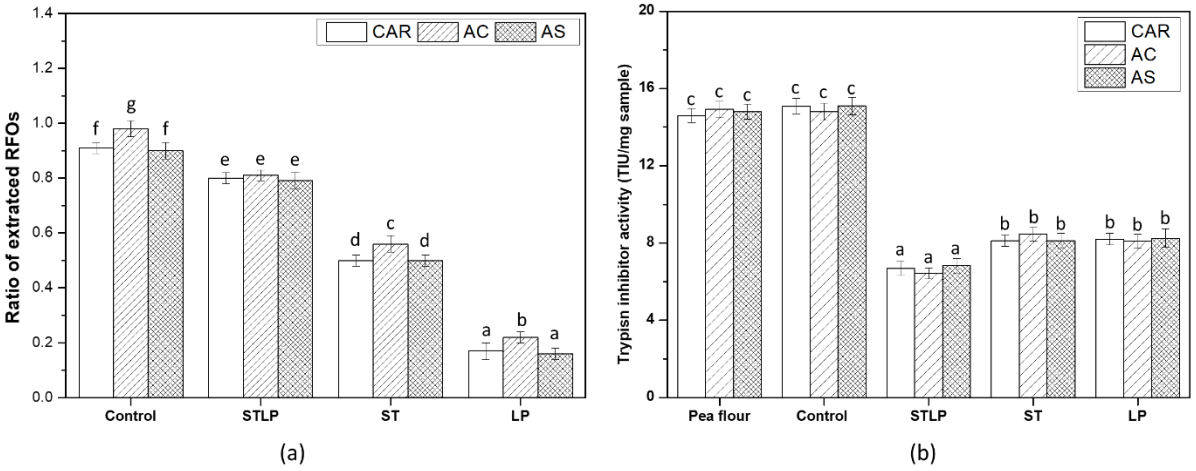


Figure VIII-3. Le rapport des α-galactosides extraits (RFOs) (a) L'activité inhibitrice de la trypsine (unité inhibitrice de la trypsine/mg d'échantillon) (b) des fractions d'albumine obtenues sans (témoin) ou avec ajout de fermentation par *S. thermophilus* (ST), *Lb. plantarum* (LP) ou leur mélange (STLP). Comparaison entre différents cultivars de pois : CAR, AC et AS. Les lettres différentes représentent des différences significatives entre les différents échantillons (test post hoc de Tukey, valeur $P < 0,05$).

Suite aux résultats obtenus révélant l'activité enzymatique des bactéries, la troisième question soulevée concerne l'effet de la fermentation sur le profil aromatique des fractions d'albumine et de globuline. L'activité enzymatique des LAB au cours du métabolisme de différents nutriments produit des précurseurs de composés volatils qui peuvent améliorer positivement le profil aromatique des protéines de légumineuses, soit en réduisant les composés indésirables, soit en en produisant de nouveaux. L'objectif était donc de caractériser l'effet de l'extraction assistée par fermentation sur les composés volatils des fractions d'albumine et de globuline. Un seul cultivar de pois (Assas) a été utilisé pour obtenir des fractions d'albumine et de globuline sans (acidification conventionnelle ou témoin) et avec fermentation en utilisant différentes souches LAB (seules ou en co-culture). Les composés volatils ont été extraits par la technique SAFE, qui permet d'isoler au maximum les composés volatils, et ils ont été identifiés et quantifiés par spectrométrie de masse couplée à la chromatographie en phase gazeuse (GC-MS). Pour obtenir une caractérisation complète du profil aromatique des échantillons, les

composés odorants actifs ont été caractérisés par chromatographie en phase gazeuse et olfactométrie (GC-O).

Les résultats de cette étude indiquent que les échantillons obtenus sans fermentation sont principalement décrits par les termes "chimique", "pomme de terre", "champignon", "vert", "métallique" et "grillé". Certains composés volatils tels que le pent-1-en-3-ol, le pentan-1-ol, l'oct-1-en-3-ol, le (3E,5E)-octa-3,5-diène-2-one, le pentane-2,3-dione, l'acétophénon avaient la valeur la plus élevée dans la fraction de globuline obtenue par le témoin. Ces composés sont connus pour être responsables de la note d'oxydation dans l'isolat de protéines de pois et proviennent principalement de la dégradation des acides gras. La fraction de globuline, et en particulier la fraction témoin, avait la teneur la plus élevée en aldéhydes tels que l'hexanal, le 2-éthylhexanal, le tétradécanal et l'octanal. Ces composés ont une faible solubilité dans l'eau (log Pow>1) et ils pourraient être préférentiellement retenus dans la structure hydrophobe des globulines. Cela pourrait expliquer la plus faible teneur en composés volatils dans la fraction d'albumine témoin. Les fractions d'albumine et de globuline témoins étaient toutes deux associées à des composés liés à l'arôme de pois. La quantité de ces composés a été réduite dans les échantillons fermentés, probablement par l'activité enzymatique des LAB et/ou ils ont été remplacés par la production d'autres composés volatils générés par la voie métabolique des LAB.

Par exemple, certains aldéhydes tels que l'hexanal avec une concentration élevée dans la globuline témoin ont été convertis en acide hexanoïque dans l'albumine obtenue par ST par l'activité de l'ALDH ou en hexanol dans l'albumine obtenue par LP par l'activité de l'ADH ; ou le 2-éthylhexanal avec une concentration élevée dans la globuline témoin en 2-éthylhaxanol dans la globuline obtenue par ST par l'ADH. De plus, la teneur de certains autres composés volatils produits par les principales voies métaboliques des bactéries lactiques (LAB) a augmenté dans les échantillons fermentés, tels que la 3-hydroxybutan-2-one (lactique) qui est le produit de la glycolyse ou le benzénéméthanol (fruité), le produit de la protéolyse dans les LAB. Les fractions d'albumine fermentées, en particulier celle fermentée avec le STLP, avaient une teneur plus élevée en composés volatils par rapport aux globulines fermentées et aux échantillons témoins. Dans le volet expérimental précédent, il a été montré que la STLP avait une activité enzymatique plus élevée pour la réduction des composés antinutritionnels et la production de petits peptides et d'acides aminés, par rapport à la ST et à la LP. L'analyse en composantes principales (ACP - corrélation de Pearson) réalisée sur les 10 classes d'odeurs des fractions d'albumine (a) et de globuline (b) de protéines de pois obtenues sans (témoin) ou avec

fermentation par *S. thermophilus* (ST) et *Lb. plantarum* (LP) ou leur co-culture (STLP), a été présentée dans la figure VIII 4. La perception plus élevée d'attributs positifs tels que lactique, floral et fruité dans la co-culture par rapport aux autres échantillons pourrait également être liée à leur activité enzymatique. Il est probable que les synergies entre ST et LP conduisent à la production de certains composés odorants tels que la γ-octalactone (fruité), la γ-décalactone (fruité), la butane-2,3-dione (lactique), le 2-méthylpropan-1-ol (fruité), qui n'ont pas été observés dans les échantillons obtenus par mono-cultures.

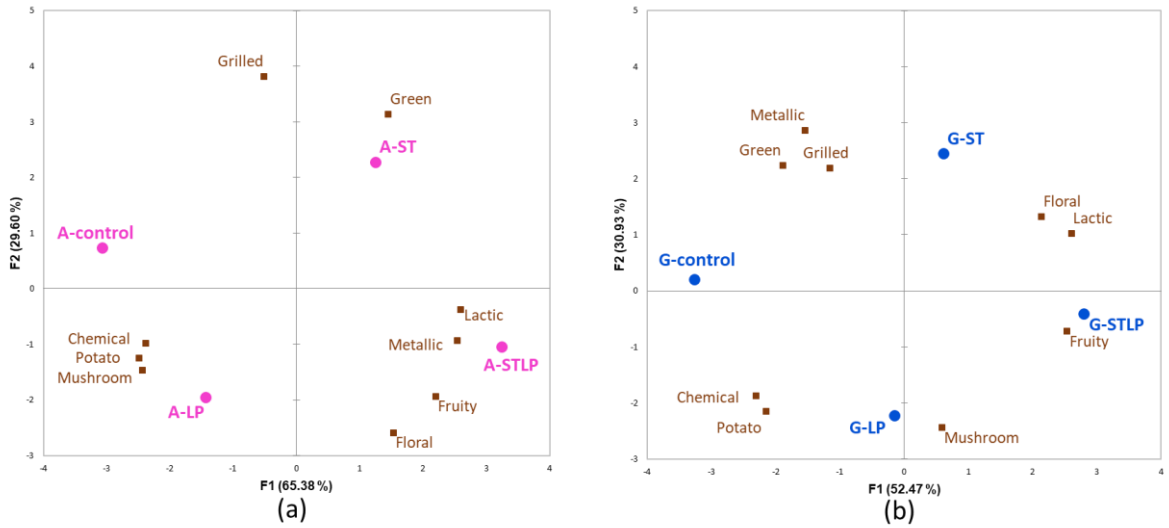


Figure VIII-4. Une analyse en composantes principales (ACP - corrélation de Pearson) a été réalisée sur les 10 classes d'odeurs (■) des fractions d'albumine (A) (a) et de globuline (G) (b) de protéines de pois (●) obtenues sans (témoin) ou avec fermentation par *S. thermophilus* (ST) et *Lb. plantarum* (LP) ou leur co-culture (STLP). Les composés volatils avec DF (fréquence de détection) ≥4 évaluateurs ont été pris en compte.

En conclusion, cette étude visait à explorer une méthode d'extraction des protéines de pois alternative à la solubilisation alcaline/précipitation isoélectrique conventionnelle. La méthode peut être prometteuse car elle peut remédier à certains défauts de l'approche traditionnelle impliquant la précipitation isoélectrique. En effet, ici, l'utilisation de produits chimiques pourrait être partiellement réduite puisque la réduction du pH est obtenue pendant la fermentation grâce à l'acide lactique produit au lieu de l'ajout d'acide minéral. L'extraction assistée par fermentation peut conduire avec succès à la production d'albumine et de fraction de globuline. Cette méthode permet d'envisager de valoriser la fraction d'albumine en tant qu'aliment humain avec une teneur en azote enrichie, une pureté et une teneur en protéines plus élevées sans affecter le profil protéique de la fraction de globuline qui est généralement utilisée

comme isolat de protéine dans l'industrie. La fraction de globuline obtenue par cette méthode avait une teneur élevée en protéines (~87% avec un facteur de conversion de l'azote de 6,25), proche de celle obtenue par l'industrie.

Les résultats de cette étude indiquent l'effet positif de l'extraction assistée par la fermentation sur la qualité nutritionnelle de la fraction d'albumine en augmentant la teneur en azote et en petits peptides avec une activité antioxydante et une réduction des composés antinutritionnels tels que l'inhibiteur de la trypsine, les α-galactosides et l'acide phytique. La fermentation avec *S. thermophilus, Lb. plantarum* et leur co-culture a permis d'améliorer le profil aromatique de l'albumine et de la fraction de globuline en réduisant les composés volatils responsables de la note verte ou en produisant de nouveaux composés avec une note positive. L'amélioration du profil protéique était liée à l'activité enzymatique des bactéries qui dépendait de la souche. En effet, la co-culture avait une activité enzymatique plus élevée dans la réduction des composés antinutritionnels, dans la production de petits peptides et d'acides aminés, par rapport à la ST et à la LP. La perception plus élevée d'attributs positifs tels que lactique, floral et fruité dans la co-culture par rapport aux autres échantillons pourrait également être liée à leur activité enzymatique. Il est probable que les synergies entre *S. thermophilus* et *Lb. plantarum* conduisent à l'activation de certaines enzymes.

Cette méthode pourrait être prometteuse pour l'environnement, la santé humaine, les approches industrielles et elle ouvre de larges possibilités de recherche et d'analyse sur différents sujets. L'une des principales limites à la consommation de protéines de légumineuses reste toutefois leurs propriétés techno-fonctionnelles, qui devraient être examinées de manière plus approfondie. En outre, les protéines obtenues dans l'industrie subissent différents traitements tels que la stabilisation thermique. Par conséquent, l'effet de ces traitements devrait être examiné de manière plus approfondie sur les fractions protéiques. Cette étude a montré que la fraction d'albumine obtenue par fermentation était plus riche en azote et contenait davantage de petits peptides susceptibles d'avoir une activité métabolique (anticancéreuse, anti-inflammatoire, antimicrobienne, immunomodulatrice, antihypertensive, etc.). Par conséquent, la production de peptides bioactifs dans la fraction d'albumine devrait être envisagée pour la valorisation industrielle de cette fraction. D'autre part, l'activité protéolytique des bactéries pourrait conduire à la production de peptides amers qui devraient être examinés et contrôlés plus avant dans ce processus d'extraction. La méthode proposée est prometteuse non seulement pour la production de protéines mais aussi pour la production de biomasse. En effet, les bactéries qui se sont développées pendant la fermentation dans la protéine de pois ont été

séparées et éliminées à la fin du processus. Or, les LAB peuvent avoir développé de nombreuses activités biologiques au cours de leur croissance et de leur adaptation au substrat de protéines de pois. Il est donc intéressant de récolter les bactéries rejetées et d'analyser les activités enzymatiques qu'elles ont pu développer pour finalement les réutiliser dans le processus. De plus, la sélection des bactéries et les conditions de fermentation (telles que la température, le temps ou les nutriments ajoutés) pourraient être optimisées sur la base de l'activité enzymatique d'intérêt (phytate, tanins, etc.) dans les fractions protéiques. Enfin, les résultats obtenus dans le cadre de cette étude ont été obtenus en laboratoire et il conviendrait d'effectuer des études à plus grande échelle en optimisant la consommation d'eau et d'énergie et d'engager l'évaluation du cycle de vie de ce processus.

IX.References

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X.Appendix

Appendix 1. Pea cultivars characteristics

Table X-1. Cultivars characteristics. Data are collected by INRAE (UMR Agroécologie, Dijon, France)

Code_INRA	Genotype	Botanic name	Cultivars type	Culture type	Hull color	Grain color	Weight of the grain (g)	Protein content (db%)	Polypeptides profile						
									11S	7S	7S/11S	PA2	Vic47/30	Vic/Conv	PA2/globulin
DCG0473	CARTOUCHE	Pisum sativum	PROTEAGINEUX	Winter	Yellow	Yellow	0,18 ± 0,03	21,08 ± 0,05	21.52	57.26	2.66	15.12	8.31	4.05	0.19
DCG0677	ASCENSION	Pisum sativum	FOURRAGER	Winter	Green	Green	0,16 ± 0,02	17,76 ± 0,05	33.11	50.75	1.53	13.25	8.97	2.37	0.16
VFD0001	ASSAS	Pisum sativum	FOURRAGER	Winter	Brown	Yellow	0,15 ± 0,04	21,39 ± 0,08	42.30	29.09	0.69	23.43	3.53	5.28	0.33

Appendix 2. The impact of added sugar on acidification kinetics and protein profile of pea cultivars

This study was performed to examine the effect of added sugar on acidification kinetics of the mono-cultures. The long acidification kinetics could be related to the low substrate availibity to the bacteria. This problem can be overcome by adding directly a source of sugar(Heron et al., 1988). Glucose was added in this study since it seemed to be digestable by both ST and LP(Charalampopoulos et al., 2002; Hutkins & Morris, 1987). The reduction of pH was then monitored during fermentation of soluble pea protein extracts obtained from three pea cultivars (CAR, AC, AS) in presence (ST/Glu, LP/Glu) and absence (ST and LP) of glucose 1% (Figure IX-1. and IX-2). Obviously, pH reduction was affected by the sugar added since the diagram presented a high drop in pH for the samples incubated in presence of sugar. The acidification kinetics parameters were characterized by Vmax, tVmax, pHVmax, pHf, tpH, tpH4.8 (Appendix C). Vmax was around ~1.2 pH units/min for the samples incubated with ST/Glu, LP/Glu. tVmax (5.5-6.3) had lower value for the samples fermented with ST/Glu, LP/Glu, compare to the ones fermented with ST, LP and STLP. Samples fermented in presence of sugar also had the lowest pHf (3.8-4.7) compare to the ones fermented with ST, LP and STLP. tpH4.8 was also presented in the table IX-2. It took around 7.1-7.5 hours for ST/Glu and around 6.8-7.2 hours for LP/Glu to reduce the pH to 4.8. Higher Vmax, lower tVmax, pHf and tpH4.8, could be related to the higher acid production capacity of bacteria in presence of glucose compare to the absence of glucose. This supported the theory of faster acidification kinetics in presence of added sugar(Heron et al., 1988). However, the effect of adding sugar on acidification kinetics remained still weak compare to the effect of co-culture (Vmax ranged from 1.5-2.5 pH units/min). Haddadin (Haddadin, 2005) also reported a higher acidification for co-culture compare to the effect of sugar in fermentation of skim milk by different *Lactobacillus* strains.

Regarding the protein profile, the samples fermented in presence of glucose had lower content of total nitrogen in albumin fraction compare to the other fermented samples. While, there was no significant differences between control, ST/Glu and LP/Glu in total nitrogen content of albumin fractions. In globulin fractions, the value of total nitrogen was higher for ST/Glu and LP/Glu compare to the other fermented samples. Though, this value was still lower than control. The content of non-protein nitrogen in albumin and globulin was lower for ST/Glu and LP/Glu. Probably this is related to the low proteolytic activity of the bacteria in presence of

glucose. The lower content of free amino groups in both albumin and globulin for ST/Glu and LP/Glu compared to the other fermented samples, also supported the hypothesis of low proteolysis. This might be related to the fast and sudden decrease of the pH for the samples fermented with ST/Glu and LP/Glu. Indeed, the higher pH reduction could have inhibited the proteolytic activity of bacteria and reduced the degradation of polypeptides(Cabuk et al., 2018a; Heron et al., 1988; Pei et al., 2022; Winters et al., 2000). On the other hand, the samples fermented with ST/Glu and LP/Glu had higher extraction yield of protein nitrogen for both albumin and globulin fractions.

To conclude, the presence of sugar promoted the kinetics of acidification. However, the protein profile of the samples was quite similar to the ones obtained by control. In the future, it could be interesting to assess the effect of added sugar on aromatic profiles of the fermented pea protein samples since the metabolic pathway of LAB could change in presence of glucose leading to the formation of different volatile compounds (Cogan & Hill, 1993; Grujović et al., 2022).

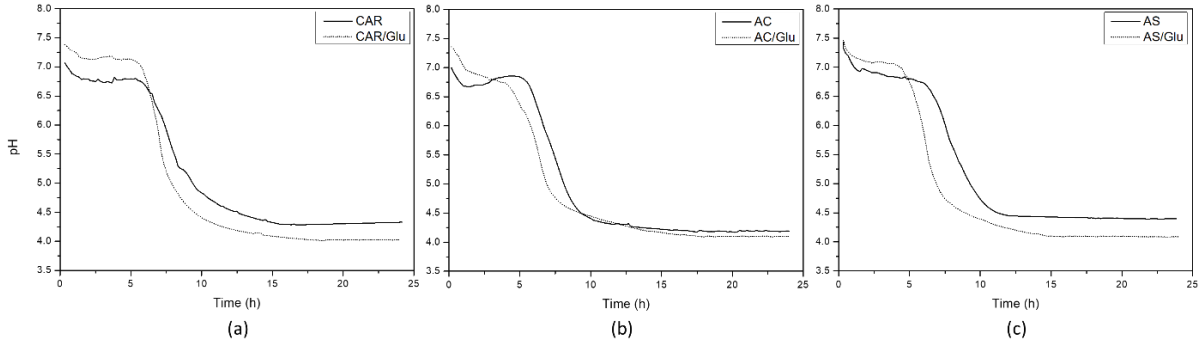

Figure X-1. Acidification kinetics of *S. thermophilus* (ST) in protein extracts obtained from different pea cultivars CAR (a), AC (b), AS (c) without glucose (solid line) and in presence of glucose 1% (short dot line).

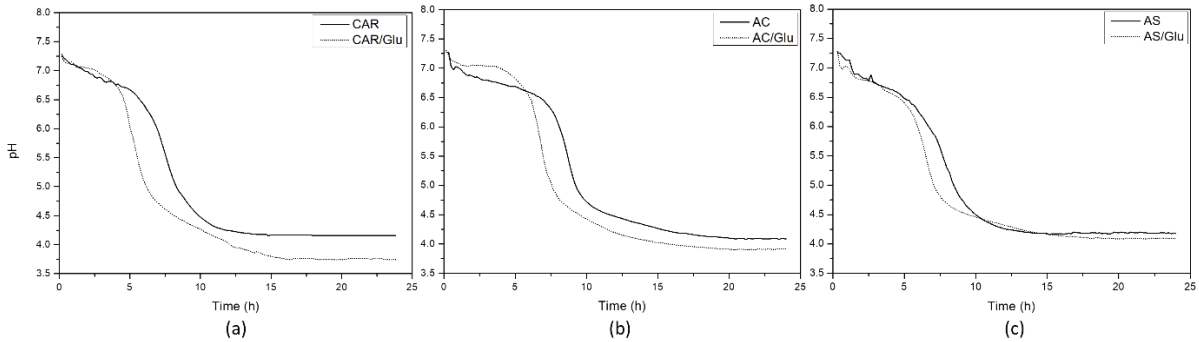

Figure X-2. Acidification kinetics of *Lb. plantarum* (LP) in protein extracts obtained from different pea cultivars CAR (a), AC (b), AS (c) without glucose (solid line) and in presence of glucose 1% (short dot line).

Table X-2. Acidification kinetics parameter of pea varieties flour (CAR, AC and AS) fermented with co-culture (*S. thermophilus* and *Lb. plantarum* (STLP)) and mono-cultures without glucose (*S. thermophilus* (ST), *Lb. plantarum* (LP)) and in presence of glucose (ST/Glu, LP/Glu)

Strain	Pea cultivars	V_{max} (pH units/h)	t_{Vmax} (h)	pH $_{Vmax}$	pH_f	t_{pHf} (h)	$t_{pH\ 4.8}$ (h)
STLP	CAR	1.53± 0.15c[1]	3.75± 0.95a	6.1± 0.5a	4.16± 0.096bc	7.5± 0.2a	4.7±0.6a
	AC	1.2± 0.2bc	3.5± 1.05a	5.5± 0.27a	4.17± 0.12bc	7.03± 0.25a	4.1±0.72a
	AS	2.5± 0.2d	3.6± 1.1a	5.9± 0.3a	4.17±0.09bc	6.8± 0.57a	3.9±0.64a
ST	CAR	0.89±0.05a	8.1± 0.9cd	5.6± 0.4a	4.29±0.10c	15.36± 0.9bc	10.1±0.19d
	AC	0.82±0.03a	7.5± 1.5b	5.7± 0.5a	4.18±0.12bc	15.5± 0.47bc	9.5±0.32cd
	AS	0.84±0.07a	7.5± 1.1b	6.02± 0.7a	4.43±0.13d	14.3± 0.8b	10±0.24d
LP	CAR	0.83±0.12a	8.33± 1.4d	5.5± 0.31a	4.16±0.15bc	14.6± 0.48b	9±0.21c
	AC	0.91±0.10a	8.5± 1.3d	5.5± 0.5a	4.13±0.14bc	16.16± 1.22c	9.5±0.38cd
	AS	0.85±0.07a	8.1± 0.87cd	5.4± 0.3a	4.16±0.16bc	14.1± 0.59b	9±0.26c
ST/Glucose	CAR	1.17±0.27bc	6.1±0.9ab	6.08±0.3a	3.97±0.09ab	14.6±0.38b	7.5±0.34b
	AC	1.12±0.1bc	5.5±0.5ab	5.79±0.23a	4.07±0.05ab	14.3±0.68b	7.1±0.25b
	AS	1.19±0.13bc	6.1±0.2ab	5.78±0.1a	3.95±0.08ab	15±0.7bc	7.1±0.28b
LP/Glucose	CAR	1.21±0.085bc	5.5±0.05ab	5.98±0.26a	3.86±0.06a	13.8±0.87b	6.8±0.4b
	AC	1.2±0.14bc	6.3±0.6ab	5.69±0.36a	3.96±0.07ab	15.16±0.9bc	7.2±0.32b
	AS	1.14±0.16bc	6.3±0.9ab	5.34±0.16a	4.06±0.068ab	14.00±0.83b	7.1±0.27b

[1]The values were presented as average ± standard deviation, n (n=3). Different letters in the same column represent significant differences among different samples (Tukey's post hoc test, P value < 0.05).

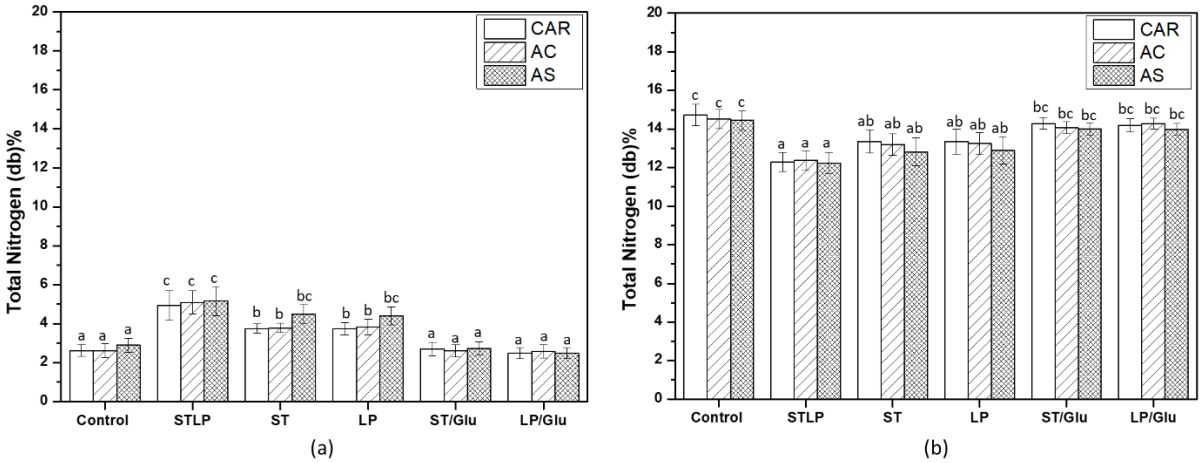

Figure X-3. Total nitrogen content of albumin (a) and globulin (b) fractions obtained without (control) or with added fermentation with *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP) in absence or presence (ST/Glu, LP/Glu) of glucose. Comparison between different pea cultivars: CAR, AC and AS. Different letters represent significant differences in free amino groups content among different samples (Tukey's post hoc test, P value < 0.05).

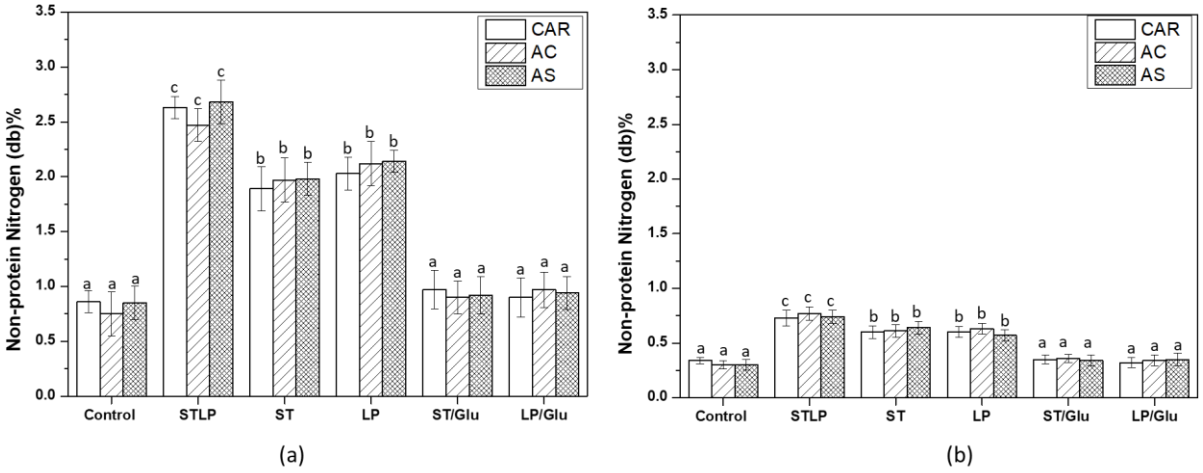

Figure X-4. Non-protein nitrogen content of albumin (a) and globulin (b) fraction obtained without (control) or with added fermentation with *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP) in absence or presence (ST/Glu, LP/Glu) of glucose. Comparison between different pea cultivars: CAR, AC and AS. Different letters represent significant differences in free amino groups content among different samples (Tukey's post hoc test, P value < 0.05).

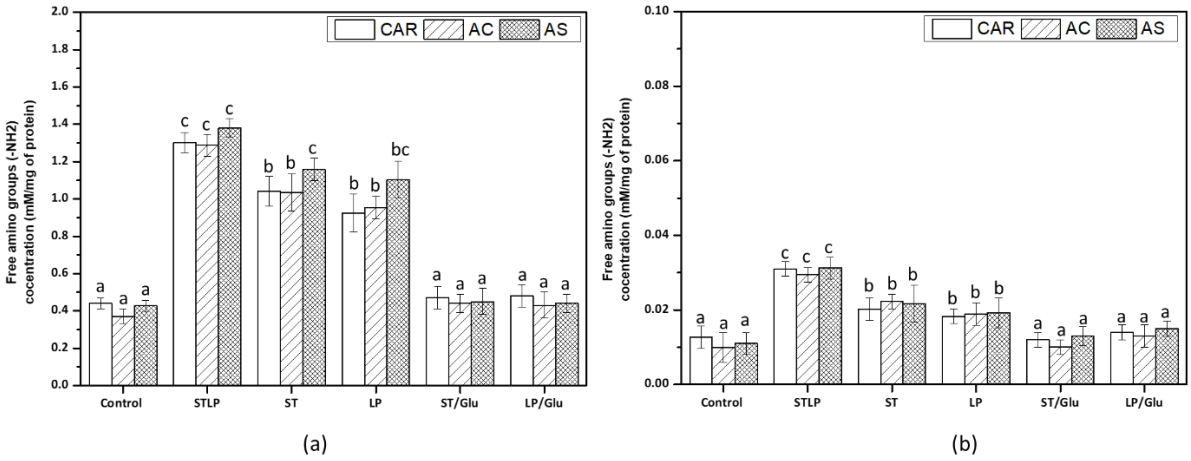

Figure X-5. Free amino groups content of albumin (a) and globulin (b) fraction obtained without (control) or with added fermentation with *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP) in absence or presence (ST/Glu, LP/Glu) of glucose. Comparison between different pea cultivars: CAR, AC and AS. Different letters represent significant differences in free amino groups content among different samples (Tukey's post hoc test, P value < 0.05).

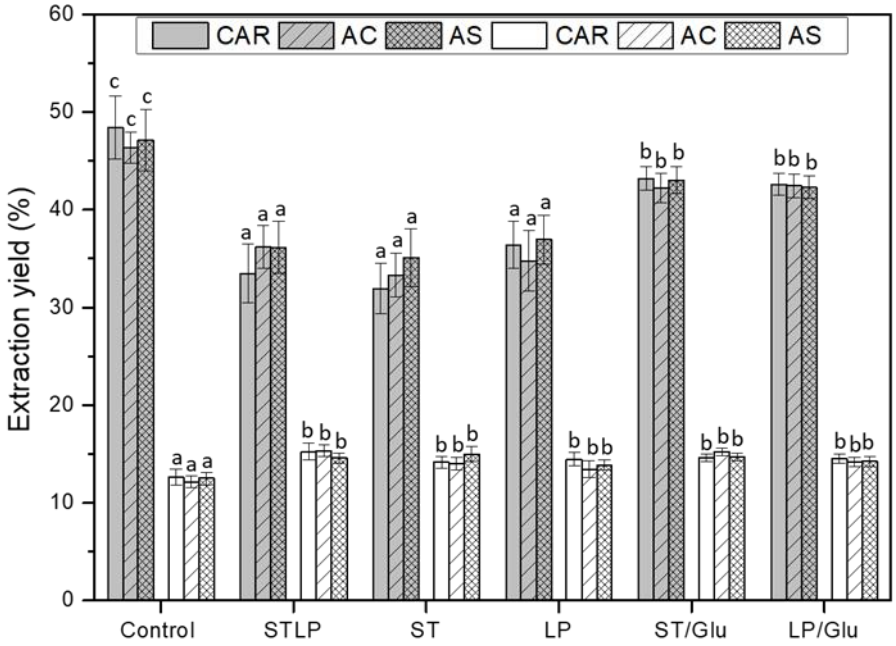

Figure X-6. Extraction yield of albumin (white) and globulin (gray) fractions obtained without (control) or with added fermentation with *S. thermophilus* (ST), *Lb. plantarum* (LP) or their mixture (STLP) in absence or presence (ST/Glu, LP/Glu) of glucose. Comparison between different pea cultivars: CAR, AC and AS. Different letters represent significant differences in free amino groups content among different samples (Tukey's post hoc test, P value < 0.05

Appendix 3. Principal component analysis (PCA – Pearson correlation) performed on the volatile compounds of pea protein albumin and globulin fractions

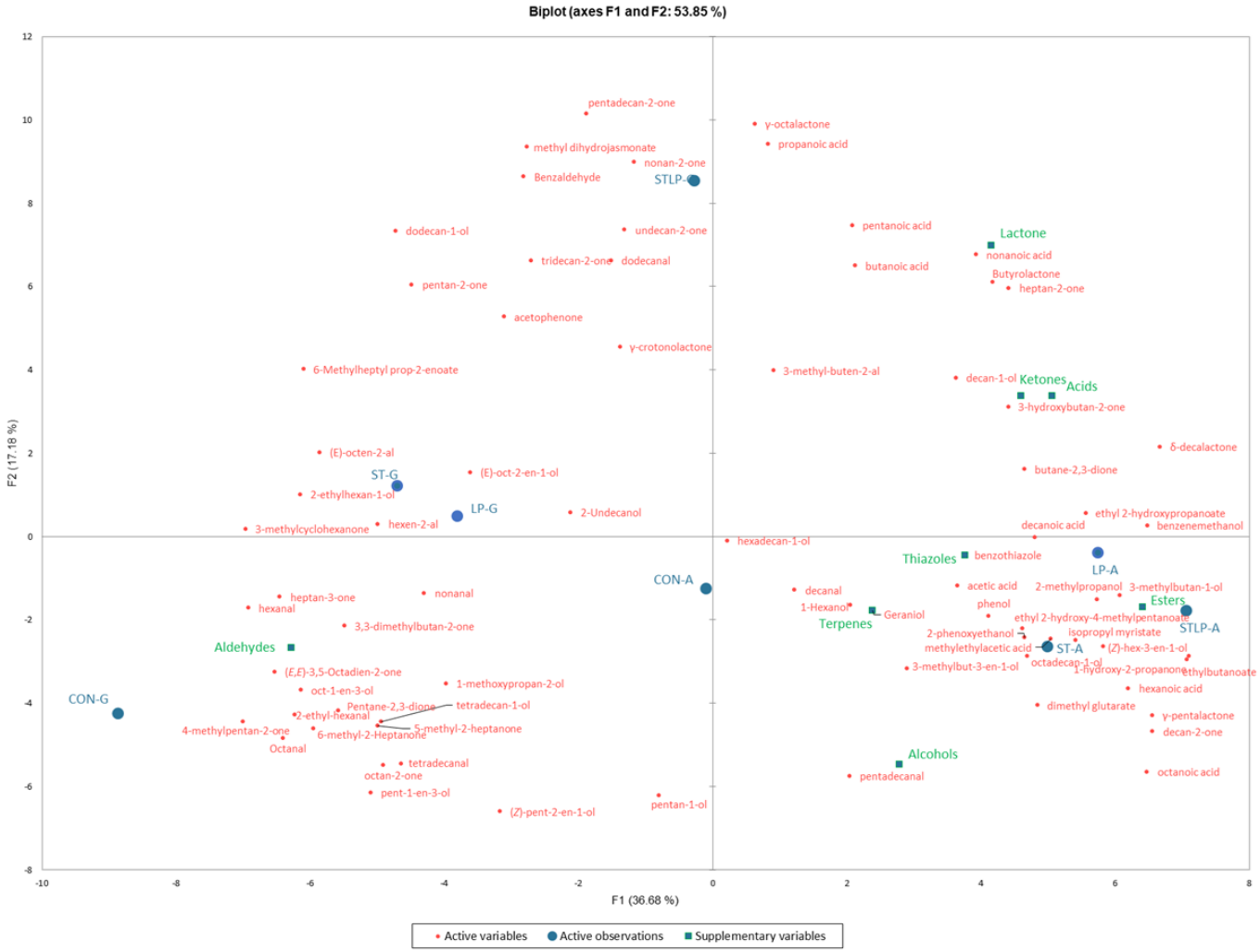

Figure X-7. Principal component analysis (PCA – Pearson correlation) performed on the volatile compounds ($p<0.05$) of pea protein albumin (A) and globulin (G) fractions obtained without (CON) or with fermentation by *S. thermophilus* (ST) and *Lb. plantarum* (LP) or their co-culture (STLP). The 8 chemical class (■) also shown as the supplementary variable. The volatile compounds with significant differences in equivalent concentration were considered for this analysis (P value≥0.05; Tukey's test)