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Characteristics of lacto-fermented whey, milk, hemp and lupine proteins

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ABSTRACT

Lacto-fermentation of proteins not only improves their biological and functional value but also causes nutritional and biochemical alteration as well as the formation of undesirable compounds, which needs to be monitored. The aim of this study was to evaluate the changes in whey, milk, hemp and lupine protein characteristics (acidity, microbiological parameters, color characteristics, free amino acid (AA) profile, biogenic amine (BA) and gamma-aminobutyric acid (GABA) concentrations) during lacto-fermentation with *Pediococcus acidilactici* LUHS29 and *Pediococcus pentosaceus* LUHS183 strains. Greater lactic acid bacteria growth and drop in pH was found in fermented plant proteins than in the animal ones. The contents of free essential and non-essential AAs were increased in all proteins fermented with the LUHS29 strain. This strain also possessed a greater GABA-producing ability in all fermented proteins. Compared to plant proteins, fermented animal proteins exhibited less GABA and total BA contents. Fermented hemp proteins had the highest BA content (on average, 215.8 mg/kg), while milk proteins fermented with LUHS183 for 48 h had the lowest value. *P. acidilactici* LUHS29 strain could be beneficial for a notable enhancement of AA and GABA in proteins, while the monitoring of BA synthesis in fermented hemp proteins needs specific attention.

1. Introduction

A key component of wellbeing and good health is a proper nutrition (Abhari et al., 2019). Thus, eating a diet high in proteins is essential for maintaining and repairing the body's systems (Tapsell, Neale, Satija, & Hu, 2016; van de Noort, 2024). Various protein sources may now be added to a wide variety of foods and beverages in order to improve its biological value. For example, milk proteins, including whey proteins, are recognized as a potential source of functional nutrients for food preparation and health improvement (Goulding, Fox, & O'Mahony, 2020; Horstman & Huppertz, 2023; Minj & Anand, 2020). Whey protein is a highly popular ingredient due to its technical features and the ability to promote satiety and control appetite (Agyei & Danquah, 2011; Shang, Chaplot, & Wu, 2018). Although animal-origin proteins have a wider profile of amino acids, plant-based proteins are receiving greater attention by consumers due to the popularity of vegan lifestyles,

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sustainability, and ethical causes, and that makes the food industry react and create more products of this type (Lonnie et al., 2020). Hemp (*Cannabis sativa* L.) proteins are known for their high digestibility and favorable amino acid composition (Hourfane, Mechqoq, Bekkali, Rocha, & El Aouad, 2023; Shen, Gao, Fang, Rao, & Chen, 2021). Moreover, they contain a markedly greater amount of free sulfhydryl than soy protein (Yano & Fu, 2023). Lupine (*Lupinus* spp.) proteins possess high digestibility but lack cysteine and methionine (van de Noort, 2024). Due to their good technological properties, they can be applied to the main food formula enrichment (van de Noort, 2024). However, the techniques used for producing protein derivatives as isolates, hydrolysates and concentrates, can result in products with a varied quality in terms of amino acid profiles and altered functional properties, as well as biological and nutritional values (Minj & Anand, 2020; Shen et al., 2021). Hydrolysis with enzymes or fermentation by lactic acid bacteria (LAB) is typically used to boost the functional and biological value of some proteins (Morifuji et al., 2010). Because fermentation causes nutritional and biochemical alterations in raw ingredients, further food product formulation can be challenging (Sharma, Garg, Kumar, Bhatia, & Kulshrestha, 2020). Despite the fact that LAB have a generally recognized as safe (GRAS) status, fermentation can lead to biogenic amine (BA) formation (Suzzi & Torriani, 2015). BA are a class of nitrogen-containing compounds with physiological activities. A small concentration of BA can be degraded by amine oxidase in human, but excessive BA can cause human discomfort, poisoning and even death (Gao et al., 2023). Therefore, the research on BA in fermented foods, especially enriched with proteins, is one of the hot spots in the field of food safety.

To date, numerous studies have been carried out on the general role of lacto-fermentation in plant-based foods and dairy products but data on diverse protein changes and BA production during lacto-fermentation of protein food ingredients obtained from milk, hemp and lupine is scarce. We hypothesize that the changes of proteins of different origin can differ during the fermentation process and this knowledge may be useful to predict product formulations in order to avoid non-desirable compounds formation in the end-product. Whey and milk proteins were selected as they are the most common ingredients used for food and beverages production with improved functionality. Hemp and lupine proteins were also chosen because these proteins have been on the rise in recent years as functional ingredients with high nutritional value, good techno-functional properties and health-improving bioactive compounds (Boukid & Pasqualone, 2022; Chukwuejim, Utioh, Choi, & Aluko, 2024; Shen et al., 2021). Whey, milk, hemp and lupine proteins were fermented with *Pediococcus acidilactici* LUHS29 and *Pediococcus. pentosaceus* LUHS183 strains and changes in acidity, lactic acid bacteria viable counts, total bacteria viable count (TBC), mold and yeast (M/Y) viable counts, color characteristics, free amino acid (AA) profile, biogenic amine (BA) and gamma-aminobutyric acid (GABA) concentrations of the non-treated and fermented proteins were evaluated.

2. Materials and methods

2.1. Characteristics of proteins and LAB strains used in the experiment

The composition of native whey protein isolate powder (Pienas LT, Biruliskiu village, Kaunas district, Lithuania), milk protein concentrate powder (Pienas LT, Biruliskiu village, Kaunas district, Lithuania), hemp protein powder (SME Bioproduktas, Tabariskiu village, Kaunas district, Lithuania) and lupine seeds protein powder (Ltd. Bio-Qlinar, Sierakowice, Poland) is given in Supplementary Table S1. Before the experiment, protein samples were stored in a dry, dark and cool (≤20 °C) place. *P. acidilactici* LUHS29 and *P. pentosaceus* LUHS183 strains were selected for protein's fermentation. These strains are high-acid-resistant and possess a wide profile of the carbohydrate fermentation capacity as well as good antibacterial and antifungal activity. In general, because of their bacteriocinogenic characteristics, *Pediococcus pentosaceus* and *Pediococcus acidilactici* are frequently used as commercial starters to prevent the growth of undesirable and harmful microbiota. Characteristics of selected LAB strains are reported by Bartkiene et al. (2020). The LAB strains were stored at −80 °C in a Microbank system (Pro-Lab Diagnostics, Bromborough, UK) and grown in de Man, Rogosa and Sharpe (MRS) broth (CM 0359, Oxoid, Basingstoke, UK) at 30 °C for 48 h prior to use.

2.2. Fermentation of whey, milk, hemp, and lupine proteins

Each protein powder was individually mixed with water (100 g of protein powder with 100 mL of water) and 3 mL of a LAB cell suspension, containing 8.9 $\log_{10}$ colony-forming units (CFU) per mL of the individual LAB strain, was added, mixed and followed by a fermentation (at *30 °C* for 48 h). Analyses of the fermented proteins were performed after 24 and 48 h. The non-fermented protein samples were mixed with the same quantity of water and served as a control. The principal scheme of experiment is depicted in Fig. 1.

2.3. Evaluation of the pH and color characteristics of protein samples

The pH value of protein samples was measured and recorded using a pH electrode (PP – 15, Sartorius, Goettingen, Germany). The color coordinates (L*, a*, b*) were assessed using the CIELAB, or CIE L* a* b*, system (Chromameter CR-400, Konica Minolta, Tokyo, Japan).

2.4. Microbiological analysis of protein samples

For the evaluation of LAB viable count, 10 g of sample was homogenized with 90 mL of saline (9 g/L NaCl solution) (Sigma-Aldrich, St. Louis, Missouri, USA) (ISO 15214:1998). Serial dilutions of 10^{-4} to 10^{-8} with saline solution were used for sample preparation. Sterile MRS agar (Oxoid, Basingstoke, UK) of 5 mm thickness was used for bacterial growth on Petri dishes. The dishes were separately seeded with the sample suspension using the surface sowing technique and were incubated under anaerobic conditions at 30 °C for 72 h. The total bacteria viable count was determined on plate count agar (PCA) (Oxoid, Basingstoke, UK) and were incubated at 32 °C for 24–48 h under aerobic conditions (ISO 4833-2:2013). Molds and yeasts colonies growing on the plates were counted after 5 days of incubation at 25 ± 2 °C under aerobiosis (ISO 21527-1:2008, ISO 21527-2:2008). The number of viable microorganisms was counted and expressed as $\log_{10}$ of colony-forming units per gram (CFU/g).

2.5. Analysis of free amino acid profile and gamma-aminobutyric acid

Sample preparation and dansylation were performed according to the method of Cai and Zhu (2010) with some modifications. Homogenized sample (~1 g) was weighted out in 15 mL tube and analytes were extracted with 10 mL of aqueous 0.1 mol/L HCl solution (Sigma-Aldrich, St. Louis, Missouri, USA) by shaking for 1 h. The resulting mixture was centrifuged (Sigma 2–5, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 1792×*g* for 5 min at room temperature. For derivatization, 200 μL of resultant supernatant was diluted to 500 μL with 0.1 mol/L HCl solution. The resulting mixture was made alkaline by adding 40 μL of 2 mol/L NaOH (Sigma-Aldrich, St. Louis, USA) and 70 μL of saturated $NaHCO_3$ solution (Sigma-Aldrich, St. Louis, USA). Derivatization was performed by adding 0.5 mL of 20 mg/mL dansyl chloride solution (Sigma-Aldrich, St. Louis, USA) in acetonitrile (Sigma-Aldrich, St. Louis, USA) and heating the resulting mixture at 60 °C for 30 min. The reaction mixture was quenched using 50 μL of ammonia solution (0.9 g/mL) (Sigma-Aldrich, St. Louis, USA) the excess ammonia neutralized using 20 μL of formic acid (Sigma-Aldrich, St. Louis, USA) and filtered with 0.22 μm membrane filter to the autosampler vial. The concentration of analytes was determined by using a Varian ProStar HPLC system [(Varian Corp., Palo Alto, California, USA): two ProStar 210 pumps, a ProStar 410 autosampler] and Thermo Scientific LCQ Fleet

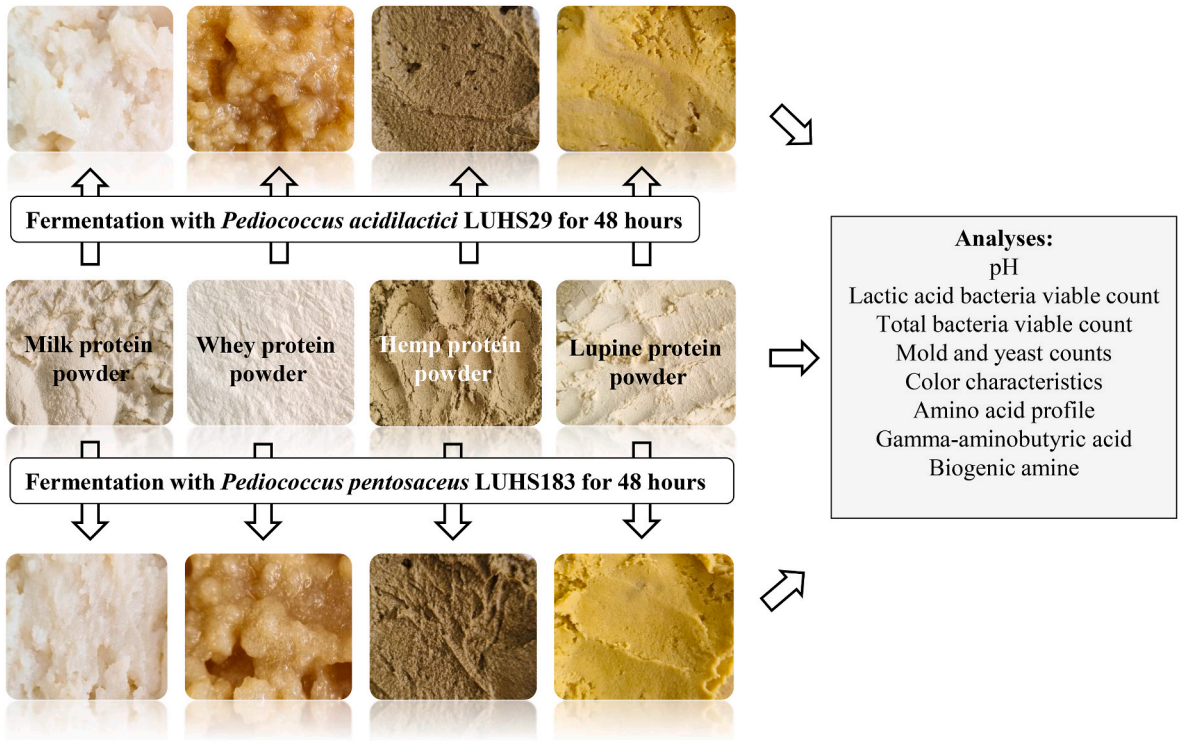

Fig. 1. Principal scheme of whey, milk, hemp and lupine proteins fermentation with *Pediococcus acidilactici* and *Pediococcus pentosaceus*.

Ion trap mass detector (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). For the analyte detection, the mass spectrometer was operated at positive ionization consecutive reaction monitoring mode for specific ions which correspond to derivatized analyte. The concentration of the analyte was determined from the calibration curve which was obtained by derivatizing the analytes at different concentrations. For the separation of derivatives, a Discovery® HS C18 column (150 × 4.6 mm, 5 μm; SupelcoTM Analytical, Bellefonte, Pennsylvania, USA) was used. The mobile phase A was composed of 1 mL of formic acid (Sigma-Aldrich, St. Louis, USA), 950 mL of deionized water, and 50 mL of acetonitrile, while the mobile phase B was prepared by adding 1 mL of formic acid to 1 L of acetonitrile. A 0.3 ml/min flow-rate was used for analysis. A 10 μL injection volume was used. Analytical gradient was as follows: 0–10 min (linear gradient) 50–70% B, 10–15 min (linear gradient) 70–90% B, and 15–30 min 90–95% B, followed by re-equilibration for 10 min with 50% B (flow-rate increased to 0.8 mL/min).

2.6. Evaluation of biogenic amines content in protein samples

Sample preparation and determination of the BA tryptamine (TRY), phenylethylamine (PHE), putrescine (PUT), cadaverine (CAD), histamine (HIS), tyramine (TYR), spermidine (SPRMD), and spermine (SPER) in non-fermented and fermented proteins was performed according to the method described by Ben-Gigirey, Vieites Baptista de Sousa, Villa, & Barros-Velazquez (1999) with some modifications. In 20 mL of deionized water, stock solutions for each BA (1 mg/mL), including the internal standard 1.7-diamino-heptane (Sigma-Aldrich, St. Louis, Missouri, USA), were produced. Ten milliliters of perchloric acid (0.4 mol/L) (Sigma-Aldrich, St. Louis, Missouri, USA) were used 2 times to extract 5 g of the sample. The derivatization of sample extracts and standards was performed with a dansyl chloride solution in acetonitrile (10 mg/mL) (Sigma-Aldrich, St. Louis, Missouri, USA). Varian ProStar HPLC system (Varian Corp., Palo Alto, California, USA) equipped with a ProStar 325 UV/VIS Detector and Galaxy software (Agilent, Santa Clara, California, USA) was employed. A Discovery HS C18 column (150 mm length × 4.6 mm, 5 μm particle size; SupelcoTM Analytical, Bellefonte, Pennsylvania, USA) was used for separation. Ammonium acetate 0.1 mol/L (Sigma-Aldrich, St. Louis, Missouri, USA) (solvent A) and acetonitrile (solvent B) were used as mobile phases at constant flow-rate of 0.8 mL/min. The detection limit of BA was 0.1 mg/kg. The BA were identified based on their retention times in comparison to their corresponding standards.

2.7. Statistical analysis

For data interpretation, the results of protein samples were expressed as mean values (n = 6) ± standard error (SE) of the mean. Two parallel fermentations of each protein sample (whey, milk, hemp and lupine protein) were performed with three replicates analyzed from each group. In total, six samples from each group were analyzed for assessment of the microbiological and physicochemical parameters. A one-way analysis of variance (1 way-ANOVA), followed by the Tukey HSD post-hoc test was used for data analysis. To evaluate the effects of fermentation duration, type of protein and LAB strain used for fermentation, data were analyzed by tests of between-subjects effects using the statistical package IBM SPSS Statistics [28.0.1.0(142), Chicago, Illinois, USA]. In addition, Pearson correlation coefficients were calculated between various parameters. The Pearson's correlation coefficients were interpreted by Evans (1996, p. 600). The results were recognized as statistically significant at $p \leq 0.05$.

3. Results and discussion

3.1. Microbiological parameters of protein samples

Microbiological parameters of protein (whey, milk, hemps and lupine) samples are shown in Table 1. Molds and yeasts were absent in all samples. However, despite that total bacteria viable counts (TBC) in non-fermented protein samples ranged from 2.84 $\log_{10}$ CFU/g (in lupine protein) till, on average, 1.77 $\log_{10}$ CFU/g (in whey and milk protein samples), the LAB growth in non-fermented samples were absent. The

Table 1
Microbiological parameters, pH, and color coordinate values of the non-fermented and fermented whey, milk, hemp and lupine proteins.

Protein samples	LAB, log_{10} CFU/g	TBC, log_{10} CFU/g	pH	Color coordinates, NBS		
				L*	a*	b*
Non-fermented						
Lupine-P	Nd	2.8 ± 0.2^{g}	5.65 ± 0.02^{f}	68±2^{e}	−0.50 ± 0.01^{b}	34±1^{l}
Hemp-P	Nd	2.4 ± 0.1^{f}	6.01 ± 0.03^{g}	43±1^{b}	1.2 ± 0.1^{c}	11.2 ± 0.3^{f}
Whey-P	Nd	1.7 ± 0.1^{c}	6.29 ± 0.02^{g,h}	72±3^{f}	1.2 ± 0.1^{c}	15.5 ± 0.4^{g}
Milk-P	Nd	1.9 ± 0.1^{d}	6.37 ± 0.01^{h}	80±3^{h}	−0.55 ± 0.04^{b}	10.3 ± 0.1^{e}
24 h fermentation with LUHS29						
Lupine-P	5.9 ± 0.2^{e}	1.8 ± 0.2^{c,d}	4.78 ± 0.01^{c}	66±3^{e}	1.6 ± 0.1^{c}	31±1^{k}
Hemp-P	6.2 ± 0.3^{ef}	2.0 ± 0.1^{d}	5.33 ± 0.02^{e}	38±2^{a}	2.0 ± 0.1^{d}	5.5 ± 0.1^{a}
Whey-P	3.2 ± 0.1^{a}	1.6 ± 0.1^{b,c}	6.19 ± 0.03^{g,h}	54±2^{c}	5.0 ± 0.2^{g}	16.5 ± 0.2^{h}
Milk-P	4.1 ± 0.2^{b}	1.4 ± 0.1^{b}	6.31 ± 0.01^{h}	75±3^{f,g}	−0.36 ± 0.03^{c}	9.1 ± 0.2^{d}
24 h fermentation with LUHS183						
Lupine-P	6.0 ± 0.2^{e}	1.8 ± 0.1^{c,d}	4.47 ± 0.01^{b}	67±2^{e}	3.0 ± 0.1^{f}	31±2^{k}
Hemp-P	6.4 ± 0.2^{f}	2.1 ± 0.1^{d}	4.89 ± 0.02^{c,d}	39±2^{a}	2.7 ± 0.1^{e}	7.7 ± 0.2^{c}
Whey-P	3.3 ± 0.1^{a}	1.5 ± 0.1^{b,c}	6.14 ± 0.02^{g,h}	58±2^{d}	6.4 ± 0.2^{h}	19.9 ± 0.3^{j}
Milk-P	3.8 ± 0.1^{b}	1.4 ± 0.1^{b}	6.16 ± 0.03^{g,h}	70±3^{e,f}	−1.0 ± 0.1^{a}	8.0 ± 0.2^{c}
48 h fermentation with LUHS29						
Lupine-P	7.9 ± 0.3^{h}	1.7 ± 0.1^{c,d}	4.37 ± 0.01^{a,b}	68±3^{e}	1.8 ± 0.1^{c}	30±1^{k}
Hemp-P	7.3 ± 0.2^{g}	1.9 ± 0.2^{d}	4.73 ± 0.01^{e}	39±2^{a}	2.2 ± 0.1^{d}	5.9 ± 0.1^{b}
Whey-P	4.9 ± 0.2^{d}	1.4 ± 0.1^{b}	5.79 ± 0.02^{f,g}	55±2^{c}	8.5 ± 0.3^{i}	22.3 ± 0.4^{i}
Milk-P	4.5 ± 0.1^{c}	1.2 ± 0.1^{a}	5.92 ± 0.03^{g}	78±3^{g,h}	−0.60 ± 0.03^{b}	8.3 ± 0.1^{e}
48 h fermentation with LUHS183						
Lupine-P	8.2 ± 0.2^{h}	1.8 ± 0.1^{c,d}	4.23 ± 0.02^{a}	66±2^{e}	2.4 ± 0.3^{d}	31±1^{k}
Hemp-P	8.3 ± 0.3^{h}	1.6 ± 0.1^{c}	4.61 ± 0.03^{b,c}	39.3 ± 0.3^{a}	2.2 ± 0.2^{d}	59±2^{m}
Whey-P	5.2 ± 0.1^{d}	1.2 ± 0.1^{a}	5.74 ± 0.01^{f,g}	56±2^{c,d}	7.7 ± 0.3^{j}	21.4 ± 0.2^{i}
Milk-P	4.9 ± 0.1^{d}	1.2 ± 0.1^{a}	5.99 ± 0.02^{g}	76±2^{g}	−0.68 ± 0.05^{b}	7.9 ± 0.1^{c}

LUHS29 – fermented with *Pediococcus acidilactici*; LUHS183 – fermented with *Pediococcus pentosaceus*; P – protein. LAB – lactic acid bacteria; TBC – total bacteria viable count; CFU – colony-forming units; L* – lightness; a* – redness; -a* – greenness; b* – yellowness; -b* – blueness; NBS – National Bureau of Standards units; nd – not determined. The data was expressed as mean values (n = 6) ± SE; SE – standard error. $^{a–m}$ Mean values between lines with different letters are significantly different ($p \leq 0.05$).

highest LAB viable counts were found in lupine protein at 48 h fermentation with LUHS29 strain and in lupine and hemp protein samples at 48 h fermentation with LUHS183 strain (on average, 8.12 log_{10} CFU/g). The tests of between-subjects effects showed that the type of protein influenced LAB and TBC viable counts in protein samples ($p = 0.002$ and $p \leq 0.001$, respectively). Also, the duration of fermentation influenced LAB viable counts in protein samples ($p = 0.018$). A moderate negative correlation was found between LAB viable counts in protein samples and protein pH values ($r = -0.515$, $p \leq 0.001$).

Differences in LAB growth intensity between different types of tested protein powders could be related with the availability of nutrients as well as with the increasing concentration of organic acids. LAB growth and metabolic activities during fermentation are influenced by both biochemical and biophysical factors (Hayek & Ibrahim, 2013). The composition of different raw materials varies, affecting the availability of nutrients for LAB growth and the synthesis of metabolites. Free amino acids, sugars, peptides, nucleic acid derivatives, fatty acids esters, minerals (Mn, Mg, and Ca) and vitamins (mainly vitamin B5, B2, and niacin) in raw materials play an important role in LAB growth (Peng, Koubaa, Bals, & Vorobiev, 2020). LAB strains differ in their capacity to ferment different sugars, which may have an impact on their growth and functionality (Fuso et al., 2023). Peptides and amino acids are also required for LAB growth, and the availability of these compounds in raw materials is increased by the proteolytic activity of LAB (Gammoh et al., 2020; Savijoki, Ingmer, & Varmanen, 2006). The profile and amount of amino acids necessary for LAB varies greatly between species and stains and is depending on the strain (Hayek & Ibrahim, 2013). Although there is little information on the fatty acid requirements for LAB development, it has been suggested that minimal amounts of fat may actually promote bacterial growth (Jenkins & Courtney, 2003). Moreover, LAB lipases participate in such fatty acid conversion reaction as hydration, saturation, omerization and dehydration (Ogawa et al., 2005). Milk proteins fermented for 24 h had higher LAB viable counts than 24 h fermented whey proteins and that probably occurred due to the fact that caseins have higher fermentative capacity than whey proteins due to the greater content of proline residues and the porosity nature of micelles (Głąb & Boratyński, 2017). The same tendency is observed in fermented camel milk whey protein and casein protein (Gammoh et al., 2020). LAB viable counts of fermented plant proteins were similar to those reported for soy protein isolate fermented with *Lactobacillus helveticus* for 48 h (Meinlschmidt, Ueberham, Lehmann, Schweiggert-Weisz, & Eisner, 2016). The primary energy source for LAB proliferation is carbohydrates (hexoses, disaccharides, and pentoses), which are mainly used to produce lactic acid (Peng et al., 2020). The fact that hemp protein and lupin protein powders tested in this study contained a certain amount of carbohydrates (0.5 and 13 g/100g, respectively) and dietary fibers (18.5 and 20 g/100 g, respectively) that were absent in milk protein and whey protein powders accounts for the considerably greater LAB growth in plant proteins. The antibacterial activity of LAB, which produces antimicrobial metabolites, such organic acids, bacteriocins, bacteriocin-like inhibitory substances, hydrogen peroxide, ethanol, *etc.*, contributes to the lower total bacterial viable counts in all fermented protein powders (König & Fröhlich, 2017).

3.2. pH and color characteristics of protein samples

The pH values of protein (whey, milk, hemp and lupine) samples are displayed in Table 1. After 24 h of fermentation, the highest pH reduction was observed in lupine protein samples (the pH values of 24 h samples fermented with LUHS29 and LUHS183 strains were, on average, by 1.18 and 1.26 times lower, respectively). Similar tendencies were found in pH of the hemp protein samples after 24 h of fermentation. However, pH of non-fermented and 24 h fermented whey and milk protein samples was similar. After 48 h fermentation with LUHS29, the pH of lupine, hemp and milk proteins decreased even more. The pH values of 24 and 48 h fermented whey proteins were similar. Moreover, contrasting the pH values of samples fermented 24 and 48 h with LUHS183 strain, only lupine protein samples showed lower pH values after 48 h of fermentation, whereas the pH of the other protein samples was similar regardless the time of fermentation (24 and 48 h). The tests of between-subjects effects showed that the type of protein influenced the pH of samples ($p \leq 0.001$).

The pH of the proteins dropped during the fermentation process due to the production of organic acids, mainly lactic acid, by LAB and it moved closer to the isoelectric point for lupine and hemp proteins (El-Sohaimy, Androsova, Toshev, & El Enshasy, 2022; Lo, Kasapis, & Farahnaky, 2021; Shi, Singh, Kitts, & Pratap-Singh, 2021). The higher

values of pH in fermented milk and whey proteins is related with the slower growth of the bacterial starter in these substrates, which greatly affects the production of organic acids (Gammoh et al., 2020). The microbial hydrolysis and acid-induced hydrolysis during fermentation lead to the release of the hydrophobic groups and the alteration of the structure of the protein, which affects the solubility and functional properties of the protein (Emkani, Oliete, & Saurel, 2022). The decrease in pH during pea protein fermentation by *Lactiplantibacillus plantarum,* soy protein isolate fermentation by *Lactobacillus helveticus*, as well as casein and whey protein fermentation by *Lactobacillus delbrueckii* subsp. *lactis* was also observed (Gammoh et al., 2020; Meinlschmidt et al., 2016; Shi et al., 2021).

Color coordinates of protein samples are shown in Table 1. The lightness (L*) was similar between non-treated and fermented lupine proteins. However, L* value was lower, on average, by 11 and 22% in all fermented hemp and whey proteins, compared to non-fermented ones. The fermentation reduced L* values in all milk protein samples, except 48 h fermented with LUHS29 samples. The analyzed factors (fermentation duration, type of protein and LAB strain used for fermentation) had no influence on the L* values of proteins. All milk protein and non-fermented lupine protein samples showed more greenness than redness. In all cases, the fermentation increased a* values of hemp, lupine and whey proteins. The tests of between-subjects effects showed that the type of protein and the interaction between the type of protein and LAB strain used for fermentation, as well as between the type of protein and fermentation duration had an influence on the a* values of the protein samples ($p \leq 0.001$, $p = 0.042$ and $p = 0.021$, respectively). Fermentation reduced yellowness (b*) of lupine (on average, by 11%), hemp, and milk proteins (on average, by 12%). Conversely, b* values of fermented whey proteins were higher than non-fermented ones. The tests of between-subject effects showed that the type of protein influenced b* values of samples ($p = 0.007$).

Shi et al. (2021) also report a reduced lightness and an increased yellowness of pea protein isolates fermented with *Lp. plantarum* for 30 h. In general, fermented protein powders may change color due to changes in protein structure induced by a raise in acidity and alcohol concentration, the oxidation of native pigments and the formation of new ones during fermentation (Degrain, Manhivi, Remize, Garcia, & Sivakumar, 2020; Shi et al., 2021). The greenish color of hemp protein appears because phenolic compounds, which are abundant in hemp seed meal, are also extracted during protein production (Hadnađev et al., 2018). These phenolic compounds are typically found attached to proteins and other molecules but they are released by the enzymatic and metabolic activities of fermenting microorganisms (Melini & Melini, 2021). However, free phenolics might by degraded or hydrolyzed again by certain microbial strains and enzymes (Adebo & Gabriela Medina-Meza, 2020).

The slightly yellow shade of lupine protein powder is related to the presence of carotenoids in seeds (Estivi et al., 2023). The fermentation process may favors release of carotenoids from the substrate and even induce the formation of aroma compounds derived from carotenoids (Mapelli-Brahm et al., 2020). It was also reported that in acidic pH, carotenoids show a brighter yellow color (Yadav & Prabha, 2014). The yellow color of fermented whey protein powder may also be caused by the browning reaction, in which lysine, as an abundant amino acid in whey, participates at relatively elevated temperatures (35 °C) when moisture is present (Tunick et al., 2016).

3.3. Free amino acid profile of protein samples

Essential amino acid (histidine, leucine, isoleucine, lysine, methionine, phenylalanine, threonine and valine) concentrations in non-fermented and fermented protein (whey, milk, hemps and lupine) samples are shown in Table 2, while non-essential amino acid (arginine, glutamine, asparagine, glutamic acid, serine, aspartic acid, glycine, alanine and proline) concentrations are given in Table 3. Non-fermented

Table 2
Essential amino acid concentrations (μmol/g) in the non-fermented and fermented whey, milk, hemp and lupine proteins.

Protein sample	Essential amino acid concentration, μmol/g						
	Histidine	Leucine and isoleucine	Lysine	Methionine	Phenylalanine	Threonine	Valine
Non-fermented							
Lupine-P	0.39 ± 0.03^{e}	0.62 ± 0.05^{b}	0.67 ± 0.06^{e}	nd	0.22 ± 0.02^{a}	0.19 ± 0.02^{b}	0.42 ± 0.04^{b}
Hemp-P	0.24 ± 0.02^{c}	0.83 ± 0.03^{c}	nd	nd	0.40 ± 0.03^{c}	0.15 ± 0.01^{a}	0.51 ± 0.04^{c}
Whey-P	nd	nd	nd	nd	nd	nd	nd
Milk-P	nd	nd	nd	nd	nd	0.10 ± 0.01^{a}	0.22 ± 0.02^{a}
24 h fermentation with LUHS29							
Lupine-P	0.60 ± 0.05^{h}	$5.6 \pm 0.5^{g,h}$	2.2 ± 0.2^{k}	0.78 ± 0.05^{g}	1.1 ± 0.1^{f}	2.3 ± 0.2^{g}	2.7 ± 0.2^{i}
Hemp-P	0.47 ± 0.03^{f}	4.6 ± 0.4^{f}	0.84 ± 0.06^{g}	0.34 ± 0.03^{c}	1.4 ± 0.1^{e}	1.7 ± 0.1^{e}	1.9 ± 0.2^{f}
Whey-P	0.53 ± 0.04^{g}	5.7 ± 0.3^{h}	1.2 ± 0.1^{i}	0.64 ± 0.05^{f}	2.2 ± 0.2^{g}	1.6 ± 0.1^{e}	2.4 ± 0.2^{h}
Milk-P	0.31 ± 0.03^{d}	$6.0 \pm 0.5^{h,i}$	0.36 ± 0.03^{b}	0.58 ± 0.04^{e}	2.4 ± 0.2^{h}	1.2 ± 0.1^{d}	2.2 ± 0.2^{g}
24 h fermentation with LUHS183							
Lupine-P	nd	1.1 ± 0.1^{d}	0.36 ± 0.03^{b}	nd	0.26 ± 0.02^{a}	nd	0.56 ± 0.04^{d}
Hemp-P	nd	0.86 ± 0.05^{c}	nd	nd	0.24 ± 0.02^{a}	nd	0.46 ± 0.04^{b}
Whey-P	nd	0.39 ± 0.03^{a}	nd	nd	nd	nd	0.19 ± 0.02^{a}
Milk-P	nd	1.6 ± 0.1^{e}	0.63 ± 0.05^{e}	nd	0.47 ± 0.04^{d}	0.28 ± 0.03^{c}	0.86 ± 0.05^{e}
48 h fermentation with LUHS29							
Lupine-P	0.63 ± 0.04^{h}	6.2 ± 0.5^{i}	1.6 ± 0.1^{j}	0.76 ± 0.05^{g}	2.0 ± 0.2^{g}	2.7 ± 0.2^{h}	3.0 ± 0.3^{j}
Hemp-P	0.53 ± 0.03^{g}	5.3 ± 0.4^{g}	0.74 ± 0.04^{f}	0.53 ± 0.04^{d}	1.7 ± 0.2^{f}	1.9 ± 0.1^{f}	2.1 ± 0.2^{g}
Whey-P	0.51 ± 0.03^{g}	8.0 ± 0.6^{j}	0.91 ± 0.05^{h}	1.2 ± 0.1^{h}	3.2 ± 0.3^{i}	2.2 ± 0.2^{g}	3.1 ± 0.2^{j}
Milk-P	0.49 ± 0.02^{g}	9.3 ± 0.6^{k}	0.70 ± 0.05^{f}	1.5 ± 0.1^{i}	4.1 ± 0.4^{j}	1.8 ± 0.2^{f}	3.0 ± 0.3^{j}
48 h fermentation with LUHS183							
Lupine-P	nd	1.1 ± 0.1^{d}	0.45 ± 0.04^{c}	0.13 ± 0.01^{a}	0.37 ± 0.03^{b}	0.16 ± 0.01^{a}	0.58 ± 0.05^{d}
Hemp-P	0.16 ± 0.01^{a}	0.91 ± 054^{d}	0.31 ± 0.03^{a}	nd	0.33 ± 0.03^{b}	0.12 ± 0.01^{a}	0.45 ± 0.04^{b}
Whey-P	nd	0.99 ± 0.07^{d}	0.50 ± 0.04^{d}	0.24 ± 0.02^{b}	0.52 ± 0.05^{d}	0.16 ± 0.01^{a}	0.51 ± 0.04^{c}
Milk-P	0.20 ± 0.01^{b}	1.1 ± 0.1^{d}	0.40 ± 0.03^{b}	nd	0.39 ± 0.03^{b}	0.25 ± 0.02^{c}	0.61 ± 0.05^{d}

LUHS29 – fermented with *Pediococcus acidilactici*; LUHS183 – fermented with *Pediococcus pentosaceus*; P – protein. The data was expressed as mean values (n = 6) ± SE; SE – standard error. $^{a–k}$ Mean values between lines with different letters are significantly different ($p \leq 0.05$).

Table 3
Non-essential amino acid concentrations (μmol/g) of the non-fermented and fermented whey, milk, hemp, and lupine proteins.

Protein sample	Non-essential amino acid concentration, μmol/g									GABA, μmol/g
	Arginine	Glutamine	Asparagine	Glutamic acid	Serine	Aspartic acid	Glycine	Alanine	Proline	
Non-fermented										
Lupine-P	4.7 ± 0.3^{f}	1.4 ± 0.1^{d}	0.32 ± 0.02^{a}	1.0 ± 0.1^{d}	0.26 ± 0.02^{a}	1.0 ± 0.1^{c}	0.45 ± 0.04^{d}	0.59 ± 0.05^{c}	0.29 ± 0.03^{b}	3.5 ± 0.3^{d}
Hemp-P	0.99 ± 0.03^{d}	0.71 ± 0.05^{b}	0.92 ± 0.04^{b}	2.4 ± 0.2^{e}	0.23 ± 0.02^{a}	0.50 ± 0.03^{b}	0.27 ± 0.02^{b}	0.89 ± 0.04^{e}	1.9 ± 0.2^{e}	0.88 ± 0.06^{b}
Whey-P	0.20 ± 0.01^{b}	nd	nd	0.15 ± 0.01^{a}	nd	nd	0.11 ± 0.01^{a}	nd	nd	nd
Milk-P	nd	nd	nd	0.17 ± 0.02^{a}	nd	nd	0.24 ± 0.02^{b}	0.17 ± 0.01^{a}	nd	nd
24 h fermentation with LUHS29										
Lupine-P	6.2 ± 0.1^{g}	3.7 ± 0.3^{g}	1.4 ± 0.1^{c}	0.66 ± 0.04^{c}	2.8 ± 0.2^{e}	2.0 ± 0.1^{f}	2.4 ± 0.2^{j}	4.1 ± 0.3^{h,i}	1.5 ± 0.1^{d}	8.6 ± 0.8^{g}
Hemp-P	nd	0.99 ± 0.05^{c}	1.1 ± 0.1^{b}	2.6 ± 0.2^{e}	0.70 ± 0.04^{b}	1.5 ± 0.1^{e}	1.5 ± 0.1^{g}	1.5 ± 0.1^{f}	1.1 ± 0.1^{c}	5.9 ± 0.6^{e}
Whey-P	2.8 ± 0.1^{e}	1.3 ± 0.1^{d}	2.3 ± 0.1^{e}	4.2 ± 0.4^{f}	2.1 ± 0.2^{d}	1.2 ± 0.1^{d}	1.6 ± 0.1^{g,h}	3.8 ± 0.3^{h}	3.7 ± 0.3^{g}	2.1 ± 0.1^{c}
Milk-P	nd	0.18 ± 0.01^{a}	2.1 ± 0.2^{e}	5.4 ± 0.5^{g}	0.73 ± 0.04^{b}	1.2 ± 0.1^{d}	1.1 ± 0.1^{f}	2.7 ± 0.3^{g}	3.3 ± 0.3^{f}	2.0 ± 0.2^{c}
24 h fermentation with LUHS183										
Lupine-P	0.13 ± 0.01^{a}	nd	nd	0.93 ± 0.04^{d}	nd	nd	0.43 ± 0.02^{d}	0.72 ± 0.02^{d}	nd	nd
Hemp-P	0.12 ± 0.01^{a}	nd	nd	0.60 ± 0.03^{c}	nd	nd	0.33 ± 0.03^{c}	0.54 ± 0.04^{c}	nd	nd
Whey-P	0.22 ± 0.02^{b}	nd	nd	0.24 ± 0.03^{a}	nd	nd	0.09 ± 0.02^{a}	0.20 ± 0.02^{a}	nd	nd
Milk-P	nd	nd	nd	1.0 ± 0.1^{d}	nd	0.23 ± 0.02^{a}	0.65 ± 0.03^{e}	0.88 ± 0.05^{e}	0.22 ± 0.02^{a}	nd
48 h fermentation with LUHS29										
Lupine-P	0.55 ± 0.04^{c}	2.8 ± 0.2^{f}	2.7 ± 0.2^{f}	4.0 ± 0.2^{f}	1.7 ± 0.1^{c}	2.8 ± 0.2^{h}	2.6 ± 0.2^{k}	3.8 ± 0.4^{h,i}	1.9 ± 0.1^{e}	7.3 ± 0.7^{f}
Hemp-P	nd	0.94 ± 0.03^{c}	1.7 ± 0.2^{d}	4.3 ± 0.3^{f}	0.69 ± 0.05^{b}	1.9 ± 0.2^{f}	1.7 ± 0.6^{h}	1.6 ± 0.2^{f}	1.3 ± 0.1^{c}	5.3 ± 0.5^{e}
Whey-P	nd	1.1 ± 0.1^{c}	3.8 ± 0.3^{g}	7.3 ± 0.3^{h}	1.6 ± 0.1^{c}	1.9 ± 0.2^{f}	2.3 ± 0.2^{i,j}	4.3 ± 0.4^{i}	4.3 ± 0.3^{h}	2.3 ± 0.2^{c}
Milk-P	0.10 ± 0.01^{a}	1.6 ± 0.1^{e}	5.1 ± 0.3^{h}	7.8 ± 0.3^{h}	2.0 ± 0.2^{d}	2.4 ± 0.2^{g}	2.1 ± 0.2^{i}	5.1 ± 0.5^{j}	3.8 ± 0.3^{g}	2.0 ± 0.2^{c}
48 h fermentation with LUHS183										
Lupine-P	nd	nd	nd	0.92 ± 0.02^{d}	nd	nd	0.47 ± 0.04^{d}	0.61 ± 0.06^{c}	0.13 ± 0.01^{a}	0.30 ± 0.02^{a}
Hemp-P	0.11 ± 0.01^{a}	nd	nd	0.73 ± 0.02^{c}	nd	nd	0.42 ± 0.04^{d}	0.40 ± 0.03^{b}	nd	0.21 ± 0.02^{a}
Whey-P	0.17 ± 0.02^{a}	nd	nd	0.53 ± 0.02^{b}	nd	nd	0.34 ± 0.03^{c}	0.47 ± 0.03^{c}	nd	0.30 ± 0.02^{a}
Milk-P	nd	nd	nd	0.80 ± 0.03^{c}	nd	nd	0.68 ± 0.04^{e}	0.54 ± 0.04^{c}	0.13 ± 0.01^{a}	0.31 ± 0.02^{a}

LUHS29 – fermented with *Pediococcus acidilactici*; LUHS183 – fermented with *Pediococcus pentosaceus*; P – protein; GABA – gamma-aminobutyric acid. The data was expressed as mean values (n = 6) ± SE; SE – standard error. $^{a–i}$ Mean values between lines with different letters are significantly different ($p \leq 0.05$).

plant proteins contained small amounts of histidine, lysine, leucine and isoleucine, phenylalanine, threonine, and valine. The latter two amino acids were also found in non-fermented milk proteins. In general, all protein powders fermented with LUHS29 had a noticeably higher concentrations of essential and non-essential amino acids than those fermented with LUHS183. After 24 h fermentation with LUHS29, leucine and isoleucine, methionine, and phenylalanine concentrations in fermented whey and milk proteins were higher than those in fermented plant proteins and even increased with prolonged fermentation. After 48 h of fermentation with LUHS29 strain, histidine content in lupine and whey protein samples was similar to those with 24 h of fermentation, while its content increased, on average, by 12 and 38%, in hemp and milk protein samples, respectively. The highest concentration of lysine was found in lupine proteins fermented with LUHS29 for 48 h. In all proteins, except milk, lysine concentration showed a tendency to decrease after 48 h of fermentation. Threonine and valine concentrations increased with prolonged fermentation with LUHS29 strain in all proteins.

Glutamine, asparagine, serine, aspartic acid and proline were absent in non-treated milk and whey proteins. Arginine was absent in non-treated milk proteins, while alanine was not found in non-treated whey proteins. However, glutamine, serine and asparagine were not found in all protein samples fermented for 24 and 48 h with LUHS183 strain. The highest concentrations of arginine, glutamine and serine were found in lupine proteins fermented with LUHS29 for 24 h, while longer fermentation of these proteins resulted in the highest concentrations of aspartic acid and glycine (Table 3). Fermentation with LUHS29 for 48 h resulted in the greatest concentrations of the following amino acids: asparagine and alanine in milk proteins, proline in whey proteins, and glutamic acid in animal proteins (Table 3). The protein type and LAB strain had an influence on histidine, alanine glutamine, serine, aspartic acid and glycine concentration in protein samples ($p \leq 0.001$). Furthermore, LAB strain and fermentation duration influenced phenylalanine and lysine concentration in protein samples ($p \leq 0.001$). The type of protein, LAB strain and fermentation duration had an influence on threonine, methionine, valine, leucine, isoleucine and asparagine concentration in protein samples ($p \leq 0.001$). The fermentation duration influenced glutamic acid content in protein samples ($p \leq 0.001$).

Alterations in protein composition and amount during fermentation are related to the complex proteinase system of LAB, which is composed by extracellular and intracellular proteases, and other metabolites as organic acids (Emkani et al., 2022). The fermentation causes protein to hydrolyze, producing smaller polypeptides, free amino acids and

bioactive peptides, as well as improving the ratio of essential amino acids (Sun, Shahrajabian, & Lin, 2022). It was reported that fermentation of certain cereals increased the content of lysine but it decreased in sorghum-based fermented foods (Sharma et al., 2020; Singh, Rehal, Kaur, & Jyot, 2015). As our results unfolded, the availability of essential and non-essential amino acids was noticeably increased in all tested powders fermented with the LUHS29 strain. This could be explained by the fact that the degree of protein hydrolysis varies with LAB strains and that is mainly due to the diverse proteolytic activities of LAB (Emkani et al., 2022). The study of Toe et al. (2019) showed that different LAB isolates of *Pediococcus acidilactici* and *Pediococcus pentosaceus* had varying potentials for producing extracellular amino acids and extracellular proteolytic activities. Besides, LAB benefit from the catabolism of amino acids, which is diverse among species, because it enhances bacterial proliferation and maintains metabolite synthesis (Fernandez & Zuniga, 2006). Furthermore, variations in the original proteins' polypeptide profiles of each powder could be the reason why differences were noted in hydrolyzed amino acid content between the types tested of protein powders. It was reported that lupin cultivar had an impact on the qualities and amount of protein after fermentation with LAB, while microorganisms could break down both medium and low-molecular weight polypeptides during the fermentation process of legumes (Emkani et al., 2022; Lampart-Szczapa et al., 2006).

3.4. Gamma-aminobutyric acid concentration in protein samples

Gamma-aminobutyric acid (GABA) concentration in non-fermented and fermented protein samples is revealed in Table 3. GABA was already found in non-fermented plant proteins, while it was absent in non-fermented milk and whey proteins. With all proteins, LUHS29 strain produced higher amounts of GABA than LUHS183, which synthesized GABA in small amounts only after 48 h of fermentation of all proteins. Higher concentration of GABA was found in fermented with LUHS29 plant proteins compared to animal proteins. Moreover, longer fermentation with LUHS29 of plant proteins resulted in reduction of GABA concentration, while opposite tendency was observed during whey proteins fermentation. Compared to all protein samples, the highest GABA concentration was found in lupine protein fermented with LUHS29 strain for 24 h (8.62 μmol/g). The type of protein ($p = 0.005$), LAB strain used for fermentation ($p \leq 0.001$) and their interaction ($p = 0.016$) had an influence on GABA concentration in protein samples.

GABA is a bioactive compound synthesized from glutamic acid by the glutamate decarboxylase in yeasts, fungi and bacteria, including LAB (some species of *Lactobacillus* and *Leuconostoc)* (Cui, Miao, Niyaphorn, & Qu, 2020). Its production capacity is influenced by glutamate decarboxylase activity of strain, pH, temperature, glutamic acid content and availability (Li & Cao, 2010). This compound is a neurotransmitter and efficiently participates in the relief of pain and depression, improves memory, prevents the growth of cancer cells and possesses antidiabetic and diuretic properties (Saha Turna, Chung, & McIntyre, 2024). GABA can be found in trace amounts in a variety of foods derived from plants, including cereals and fruits (Quílez & Diana, 2017). This explains the detected GABA content in non-fermented lupine and hemp protein powders. However, fermented cereals, legumes, dairy products and meat products may contain markedly concentrations of GABA (Quílez & Diana, 2017). The LUHS29 strain showed a greater GABA-producing ability in all protein powders and such an evidence confirms the fact that GABA productivity varies substantially between species or even between strains from the same species (Cui et al., 2020). Compared to LUHS183 strain, a higher GABA production in powders fermented with LUHS29 strain can also be related to a higher content of glutamic acid in these substrates. The lower GABA content in fermented milk and whey protein powders compared to plant proteins, can be attributed to the higher pH values because effective GABA synthesis occurs in an optimal pH range of 3.5–5.0 (Cui et al., 2020).

3.5. Biogenic amines formation in protein samples

Biogenic amine concentration (mg/kg) in tested protein samples is shown in Table 4. TRY, CAD and HIS were not detected in protein samples, while TYR was found in only in hemp proteins fermented with LUHS29 and in lupine proteins fermented with LUHS29 for 48 h. Fermented hemp proteins showed a higher concentration of PHE than non-treated. Fermentation reduced PHE content in lupine proteins and most whey proteins (except 48 h fermented with LUHS29 samples). In most of fermented milk proteins, PHE concentration was similar to the non-fermented ones, except samples fermented with LUHS183 for 48 h, in which PHE content was lower by 11%. All hemp proteins (non-fermented and fermented) contained PUT, SPRMD, and SPRM, and fermentation with both LAB strains increased concentrations of these BA. However, fermentation with both strains reduced SPRMD concentration in lupine proteins. The type of protein was a statistically significant factor on PUT, TYR, SPRMD and SPRM concentration in protein samples ($p \leq 0.001$), while the LAB strain used for fermentation was a statistically significant factor on TYR and SPRM concentrations in protein samples ($p \leq 0.001$). In addition, the duration of fermentation was a statistically significant factor on PHE, TYR and SPRM concentration in protein samples ($p = 0.026$, $p \leq 0.001$ and $p = 0.041$, respectively).

Total biogenic amine content is shown in Table 4. Contrasting plant-based protein groups with animal-based ones, a higher total BA content was consistently found in plant protein samples. In all analyzed whey and milk protein samples, BA concentration was lower than 10 mg/kg. In all cases, fermentation increased the total BA content in hemp proteins, on average, by 17–40%, but reduced it, on average, by 35–40% in lupine proteins. The type of protein was a statistically significant factor on total BA content in protein samples ($p \leq 0.001$). Positive moderate correlation was found between total BA concentration and TB viable counts in protein samples ($r = 0.441$, $p \leq 0.001$).

Biogenic amines are produced from certain amino acids by decarboxylase-positive microorganisms (yeast, Gram-negative and Gram-positive bacteria) and the extent of this synthesis highly depends on the substrate composition, pH, water activity and temperature (Suzzi & Torriani, 2015). The fluctuations in BA content of fermented samples could be attributed to different sample composition (e.g. availability of free amino acids), pH (microorganisms produce decarboxylase as a defence mechanism in acidic conditions), the presence of other decarboxylase positive microorganism in raw material and LAB metabolic activity (proteolytic activity or synthesis of such metabolites as antibacterial compounds and GABA to inhibit BA-producing microorganisms) (Omer, Mohammed, Ameen, Abas, & Ekici, 2021).

Because BA possess physiological effects and biological activity, they are necessary for numerous bodily activities at low quantities (Emkani et al., 2022). The most harmful BA's in food are HIS and TYR. The fermented protein samples did not contain histamine, while tyramine was present in very small amounts and only in few fermented plant protein powders. Such polyamines as PUT, SPRMD and SPRM appear to be less harmful but they are able to intensify the harmful responses brought on by PHE, TYR and HIS (Omer et al., 2021). The precursor of PHE is phenylalanine, while PUT is produced from ornithine and agmatine, which are obtained after arginine being hydrolyzed or decarboxylated (Benkerroum, 2016; Gao et al., 2023). PUT can be converted into the polyamines SPRMD and SPRM by adding amino-propyl groups to it one at a time (Saha Turna et al., 2024). As each person's gastrointestinal system's capacity for detoxification of BA varies, the toxicity standard for BA is very difficult to ascertain (Omer et al., 2021). It is recommended by European Food Safety Authority (EFSA) that daily intake of TYR should not exceed 800 mg/kg (Wójcik, Łukasiewicz, & Puppel, 2021). The upper legal limit of 30 mg/kg for PHE are also reported (Omer et al., 2021). The contents of TYR and PHE in samples from this study did not exceed the recommended values. Histamine was not found in samples of this study, but only this BA is covered by specific regulation for fish: maximum histamine level of 200 mg/kg for fish and fish

Table 4
Biogenic amine concentrations (mg/kg) in non-fermented and fermented lupine, hemp, whey and milk proteins.

Protein sample	Biogenic amine concentration, mg/kg								Total BA content
	Tryptamine	Phenyl-ethylamine	Putrescine	Cadaverine	Histamine	Tyramine	Spermidine	Spermine	
Non-fermented									
Lupine-P	<0.1	17.4 ± 1.2^{g}	<0.1	<0.1	<0.1	<0.1	136±8^{c}	<0.1	153^{h}
Hemp-P	<0.1	4.0 ± 0.3^{a}	24.5 ± 1.2^{b}	<0.1	<0.1	<0.1	88±3^{a}	17.4 ± 1.2^{a}	134^{g}
Whey-P	<0.1	7.3 ± 0.2^{d}	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	7.3^{d}
Milk-P	<0.1	6.2 ± 0.2^{b,c}	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	6.2^{c}
24 h fermentation with LUHS29									
Lupine-P	<0.1	5.6 ± 0.4^{b}	<0.1	<0.1	<0.1	<0.1	102±5^{b}	<0.1	108^{f}
Hemp-P	<0.1	8.8 ± 0.4^{e}	42.6 ± 3.4^{c}	<0.1	<0.1	3.2 ± 0.3a	141±4^{c}	25.0 ± 1.9^{b}	221^{i}
Whey-P	<0.1	5.8 ± 0.4^{b}	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	5.8^{b}
Milk-P	<0.1	6.8 ± 0.2^{c}	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	6.8^{c}
24 h fermentation with LUHS183									
Lupine-P	<0.1	6.9 ± 0.6^{c,d}	<0.1	<0.1	<0.1	<0.1	85±6^{a}	<0.1	92^{e}
Hemp-P	<0.1	9.2 ± 0.4^{e}	38.2 ± 2.1^{c}	<0.1	<0.1	<0.1	133±7^{c}	26.0 ± 2.3^{b}	206^{i}
Whey-P	<0.1	5.6 ± 0.4^{b}	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	5.6^{b}
Milk-P	<0.1	6.7 ± 0.3^{c}	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	6.7^{c}
48 h fermentation with LUHS29									
Lupine-P	<0.1	4.6 ± 0.4^{a}	5.7 ± 0.4^{a}	<0.1	<0.1	6.2 ± 0.4^{c}	94±7^{a}	15.8 ± 1.5^{a}	126^{g}
Hemp-P	<0.1	11.7 ± 0.2f	41.5 ± 2.3c	<0.1	<0.1	4.6 ± 0.4b	138±8c	26.1 ± 1.8b	222^{i}
Whey-P	<0.1	7.1 ± 0.2^{d}	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	7.1^{d}
Milk-P	<0.1	5.2 ± 0.4^{b}	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	5.2^{b}
48 h fermentation with LUHS183									
Lupine-P	<0.1	6.8 ± 0.4^{c,d}	<0.1	<0.1	<0.1	<0.1	92±6^{a}	<0.1	99^{e}
Hemp-P	<0.1	11.0 ± 0.2^{e}	43.0 ± 4.0^{c}	<0.1	<0.1	<0.1	135±8^{c}	25.6 ± 1.5^{b}	215^{i}
Whey-P	<0.1	6.5 ± 0.2^{c}	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	6.5^{c}
Milk-P	<0.1	4.3 ± 0.3^{a}	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	4.3^{a}

LUHS29 – fermented with *Pediococcus acidilactici*; LUHS183 – fermented with *Pediococcus pentosaceus*; P – protein; BA – biogenic amine. The data was expressed as mean values (n = 6) ± SE; SE – standard error. $^{a–i}$ Mean values between lines with different letters are significantly different ($p \leq 0.05$).

products and 400 mg/kg in fish sauces (Wójcik et al., 2021). The Food and Drug Administration (FDA) in the United States has set a general limit of 50 mg/kg for histamine in food (Ruiz-Capillas & Herrero, 2019). No standards have been set for other BAs or foods like meat, dairy, etc. Representatives of plant kingdom, including seeds and legumes, naturally contain biogenic amines as polyamines and putrescine (Ekici & Omer, 2018). This explains the higher total BA content of plant proteins compared to animal proteins. BA can be naturally found in the hemp seeds and its profile is impacted by the cultivar and harvesting period (Ciano, Vinci, Rapa, & Ruggieri, 2019). Spermidine and spermine, putrescine are present in lupine seeds (Martínez-Villaluenga, Gulewicz, Pérez, Frías, & Vidal-Valverde, 2006). The further accumulation of BA during fermentation occurs due to bacterial decarboxylation of amino acids. Significant content of PUT, SPRMD and SPRM in fermented hemp protein powder could be due to the high levels of arginine in hemp seed protein (Nionelli et al., 2018). Low quantities of BA are an anticipated characteristic of fermented plant food and dairy products, and they commonly occur in protein foods (Gobbi, Ciano, Rapa, & Ruggieri, 2019). LAB, *e.g.*, *Lactococcus* and *Lactobacillus* spp., also contribute to the BA production but the risk of synthesis is low and the ability to generate these compounds is a trait unique to a particular strain (Yazgan, Kuley, Güven Gökmen, Regenstein, & Özogul, 2021). However, it was reported that BA-producing microorganisms could be inhibited by such LAB metabolites as antibacterial compounds, including GABA (Gao et al., 2023).

4. Conclusions

The extent of changes in the characteristics of protein powders during lacto-fermentation was highly influenced by the *Pediococcus* strain and protein type used for fermentation. Fermentation with *P. acidilactici* LUHS29 could be attributed to the higher concentrations of free amino acids in hemp, lupine, whey, and milk proteins and the more effective production of GABA. This study highlights the beneficial side of plant proteins, such as hemp and lupine, in fermentation: the better growth of used LAB strains, a faster increase in medium acidity, and a higher content of GABA when compared to animal-based proteins, such as milk and whey. However, the use of latter proteins, i.e. milk and whey, for fermentation resulted in low biogenic amines content, while accumulation of these compounds in hemp proteins was elevated. The obtained data provides important insights for the food, beverage, and nutraceuticals industries that could be applied to the sustainable development of higher-value food, beverage, or nutraceutical formulas.

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CRediT authorship contribution statement

Elena Bartkiene: Writing – review & editing, Writing – original draft, Supervision, Methodology, Data curation, Conceptualization. **Dovile Klupsaite:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Vytaute Starkute:** Methodology, Formal analysis, Data curation. **Ernestas Mockus:** Methodology, Formal analysis, Data curation. **Vadims Bartkevics:** Methodology. **Romas Ruibys:** Writing – original draft, Formal analysis. **Gabija Batkeviciute:** Formal analysis. **Fatih Özogul:** Writing – original draft. **Muhammad Usman Khalid:** Writing – original draft. **João Miguel Rocha:** Writing – review & editing.

Declaration of competing interest

None.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2024.116259.

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