Effect of solid-state fermentation on the functionality, digestibility, and volatile profiles of pulse protein isolates

Stone, Andrea K.; Shi, Dai; Liu, Enyu; Jafarian, Zahra; Xu, Caishuang; Bhagwat, Aarti; Lu, Yuping; Akhov, Leonid; Gerein, Jessica; Han, Xiumei; Zaharia, L. Irina; Rajagopalan, Nandhakishore; Tanaka, Takuji; Korber, Darren R.; Nickerson, Michael T.

Publisher's version / Version de l'éditeur:
https://doi.org/10.1016/j.fbio.2024.104580
Food Bioscience, 61, pp. 1-10, 2024-06-19

Food Bioscience 61 (2024) 104580

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Effect of solid-state fermentation on the functionality, digestibility, and volatile profiles of pulse protein isolates

Andrea K. Stone [a], Dai Shi [a], Enyu Liu [a], Zahra Jafarian [a], Caishuang Xu [b], Aarti Bhagwat [b], Yuping Lu [b], Leonid Akhov [b], Jessica Gerein [b], Xiumei Han [b], L. Irina Zaharia [b], Nandhakishore Rajagopalan [b,c], Takuji Tanaka [a], Darren R. Korber [a], Michael T. Nickerson [a,*]

[a] Department of Food and Bioproduct Sciences, University of Saskatchewan, 51 Campus Drive, Saskatoon, SK, S7N 5A8, Canada
[b] National Research Council Canada, 110 Gymnasium Pl, Saskatoon, SK, S7N 0W9, Canada
[c] Department of Chemical and Biological Engineering, University of Saskatchewan, 57 Campus Drive Saskatoon, SK, S7N 5A9, Canada

ARTICLE INFO

Keywords:
Chickpea
Faba bean
Green lentil
Lactobacillus plantarum
Aspergillus niger
Aspergillus oryzae

ABSTRACT

Fermentation, as a clean processing technique, induces structural and compositional modifications to plant proteins, improving their functionality and nutrition. However, the effect varies depending on the level of hydrolysis, the fermenting strain, and the specific substrate used. The present work examined the effect of solid-state fermentation (SSF) by *Lactobacillus plantarum, Aspergillus niger* and *Aspergillus oryzae* of several niche market pulse (chickpea, green lentil, and faba bean) protein isolates on their functional and nutritional properties. The pulse proteins were moderately hydrolyzed to different extents (degree of hydrolysis of 9%–15%) after 48 h of fermentation, enhancing surface charge and solubility while decreasing water holding capacity and emulsion stability. Protein digestibility was reduced for all pulses which was hypothesized to be due to an increase in phenolic content caused by fermentation. Among the strains, only *A. niger* outgrew the natural microbiota for all pulses. Variations relating to the property changes were observed among the inocula and pulses; however, no general trend could be concluded. Fermentation produced a large variety of favourable new volatile compounds in the protein isolates.

1. Introduction

With the increasing global population and the ever-growing consumer awareness for healthy, sustainable products, the plant-based market is growing rapidly (Rivera et al., 2022). The current plant-based market is dominated by soy and wheat (gluten) proteins; however, demand is increasing for non-GMO (genetically modified organism) and low-allergenicity products. Pulses, including peas (*Pisum sativum* L.), lentils (*Lens culinaris*), chickpeas (*Cicer arietinum* L.), and faba beans (*Vicia faba* L.), meet these criteria and are receiving significant interest due to their high production quantity and high protein levels, especially for faba bean (~29%–35% protein on a dry weight basis) (Shi & Nickerson, 2022). Research efforts have been made towards finding food applications for protein ingredients from pulses, including bakery products, beverages, meat analogs, and dairy alternatives (Rivera et al., 2022).

Generally, plant proteins are lower in functionality than those of animal origins due to their less flexible protein structures (Sim et al., 2021). For example, many have poor solubility in water, which is critical for liquid-based applications such as plant-based milks and beverages (Nadeeshani et al., 2022). Nutritional concerns associated with plant proteins relate to their low protein digestibility compared to animal proteins (Nadeeshani et al., 2022). Pulse ingredients specifically often have flavour issues and are perceived as beany tasting/smelling (Nadeeshani et al., 2022). Therefore, improved functionality, digestibility, and flavour are necessary for pulse proteins to be successfully incorporated into more products and compete with those traditionally animal-based.

Numerous processing approaches have been applied to plant proteins to enhance their quality of which fermentation has gained much attention as it is considered a "clean" and natural protein modification technique. Fermentation is a biotransformation process where microorganisms convert nutrients (carbohydrates, proteins, and lipids) in the substrate into new products (enzymes and metabolites) (Christensen

* Corresponding author.
E-mail address: michael.nickerson@usask.ca (M.T. Nickerson).

https://doi.org/10.1016/j.fbio.2024.104580
Received 23 April 2024; Received in revised form 13 June 2024; Accepted 13 June 2024
Available online 19 June 2024

et al., 2022). The process, dependent on the microorganisms involved, can positively modify the substrate's nutritional and flavour profile with various excreted proteases facilitating protein hydrolysis, altering the protein structure and generating unique functionality (Christensen et al., 2022; Patel, Wang, Chandrashekar, Pandiella, & Webb, 2004).

Lactobacillus plantarum, Aspergillus niger and *Aspergillus oryzae* are traditional GRAS (generally recognized as safe) strains commonly used in industrial applications to produce large amounts of enzymes, metabolites, and foods (Christensen et al., 2022). Work has been done highlighting the varying effect of fermentation by these strains on the functional and nutritional quality of air-classified pea protein-enriched flours, which was shown to be dependent on the degree of protein hydrolysis (DH) (Kumitch, Stone, Nickerson, et al., 2020; Kumitch, Stone, Nosworthy, et al., 2020; Çabuk, Nosworthy, et al., 2018; Çabuk, Nosworthy, et al., 2018). Fermentation studies have also been conducted on other pulse flours including chickpea (Xiao et al., 2015), faba bean (Chandra-Hioe et al., 2016), common bean (Granito et al., 2002), and lentil (Byanju et al., 2021). However, fermentation using concentrated proteins in the form of isolates has been less studied and gaps remain in terms of understanding the impact of varying levels of hydrolysis on the functional and nutritional properties of these modified pulse proteins. Substrate composition greatly affects the growth and metabolism of microorganisms (Ben-Harb et al., 2019). The overall goal of this study was to examine the impact of solid-state fermentation (SSF) by *A. oryzae, A. niger*, and *L. plantarum* of chickpea, green lentil, and faba bean protein isolates on their functional, nutritional, and flavour properties. It was hypothesized that SSF would improve protein digestibility and modify protein functionality as a function of DH.

2. Materials and methods

2.1. Materials

Chickpea protein isolate (CPI), lentil protein isolate (LPI), and faba bean protein isolate (FBPI) were obtained from commercial sources. GRAS strains in this study, including *A. oryzae* NRRL 5590, *A. niger* NRRL 334, and *L. plantarum* NRRL B-4496 were obtained from the Agricultural Research Service (ARS) Culture Collection (Peoria, IL, USA). These three specific strains have been used to ferment other protein-rich pulse ingredients, mainly from pea (Batbayar et al., 2023; Khorsandi, Shi, et al., 2024; Khorsandi, Stone, et al., 2024). De Man, Rogosa and Sharpe (MRS) broth was purchased from Fisher Scientific (BD Difco™ Lactobacilli MRS Broth; Ottawa, ON, Canada). Potato Dextrose Agar (PDA) plates were purchased from Becton, Dickinson and Company (Sparks, MD, USA). All other chemicals were of ACS grade and purchased from Fisher Scientific and VWR Canada (Mississauga, ON, Canada).

2.2. Strains and inoculum preparation

Asperigillus oryzae and *A. niger* were cultivated on PDA plates and incubated at 30 °C for one week. Fungus spores were recovered from each PDA plate by suspending them in 20 mL of sterilized deionized water. The concentration of spores was determined using a hemocytometer (Bright-Line, Horsham, PA, USA) with direct microscopy counting. *Lactobacillus plantarum* was cultured at 37 °C for 48 h in MRS broth. The culture's CFU (colony forming unit) was determined through plating on MRS media.

2.3. Solid state fermentation

Fermentation conditions were determined according to our previous works (Batbayar, Kryachko, Nickerson, Korber, & Tanaka, 2023; Khorsandi et al., 2024; Kumitch, Stone, Nickerson, Korber, & Tanaka, 2020) and preliminary growth experiments (data not shown). CPI, LPI, and FBPI (60 g) were each mixed into a dough at a 50% (w/w) moisture content in an aluminum baking pan (13 × 9 × 2 inches) with an aluminum foil cover. The dough was inoculated separately with ~0.6 mL of bacterial culture or spore suspensions to provide 10^7 CFU per g of protein isolate. Fermentation was conducted at the 50% moisture content (w/w) at 30 °C for fungi and at 37 °C for *L. plantarum*. During fermentation, the moisture content of the doughs was adjusted daily by adding sterilized water to maintain the initial weight. After 48 h, the samples were put into an air oven to dry at 63 °C for 24 h. Uninoculated controls were incubated for 0 h and at 30 and 37 °C for 48 h (spontaneous fermentation) under the same conditions. Morphological differences were used to differentiate between indigenous organisms and inoculated strains. Dried samples were ground using a coffee burr mill (Cuisinart DBM-8C, Woodbridge, ON, Canada) into powders and were stored at 4 °C for further analyses. The moisture and protein contents of all samples were determined according to AOAC methods 925.10 and 920.87 (N% × 6.25), respectively (AOAC, 2003).

2.4. Determination of degree of hydrolysis

The DH was determined using Adler-Nissen's (1979) method with slight modification. Briefly, the powdered fermented protein isolates (0.4 g) were suspended in 40 mL of sodium phosphate buffer (pH 7.8). After stirring overnight at 4 °C, the pH was adjusted to 7.8, and the samples were centrifuged at 8230×*g* for 30 min at 4 °C. TNBS (2,4, 6-trinitrobenzenesulfonic acid) reaction with free amino acid groups was used to determine the DH. The light absorption of the supernatant was measured at 340 nm using a spectrophotometer (Genesys 20, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The total acid hydrolysis was also measured as part of the DH calculation by dissolving the original isolates in 6 mol/L HCl and heating at 110 °C overnight. The DH was determined as the ratio of free amino groups in fermented samples to those in the total acid hydrolyzed samples.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were first extracted (0.1 mol/L tris(hydroxymethyl)aminomethane (Tris), 1% SDS, pH 9.0) at a 1:10 ratio then centrifuged (Eppendorf Centrifuge 5418, Mississauga, ON, Canada) at 15,000×*g* for 10 min at room temperature (21–23 °C) (Khorsandi, Stone, et al., 2024). The diluted (× 4 with water) supernatant was mixed 1:1 (v/v) with buffer (2 × Laemmli Sample Buffer, Bio-Rad, 1610737, Bio-Rad Laboratories, Inc., Hercules, CA, USA; augmented with 1:19 β-mercaptoethanol: buffer) and incubated for 5 min at 95 °C. A 10 μL aliquot of the solution as well as a protein standard (Bio-Rad, 1610377) were loaded onto a precast polyacrylamide gel (Bio-Rad, 4561105) for electrophoresis (30 min at 200 V). The gels were then stained with Coomassie Brilliant Blue R-250 and analyzed with a Gel Doc XR + system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

2.6. Surface charge (zeta potential)

The surface charge was determined using a Zetasizer Nano-ZS90 instrument (Malvern Instruments, Westborough, MA, USA) as described by Can Karaca et al. (2011). Briefly, a 0.05% (w/w) protein solution in deionized water was stirred for 1 h at room temperature (21–23 °C), and then the pH was adjusted to 7.0 using 0.05 mol/L HCl/NaOH. The electrophoretic mobility was measured using a Zetasizer (Malvern Instruments, Westborough, MA, USA). The zeta potential was calculated using the Henry equation:

$$U_E = \frac{2\varepsilon \times \xi \times f(\kappa\alpha)}{3\eta}$$

where ε is permittivity, f ($\kappa\alpha$) is a function related to the Debye length (κ) and the ratio of particle radius (α) and equals 1.5 (by Smoluchowski's

approximation), η is viscosity.

2.7. Protein solubility

A suspension of 0.2 g of protein in 19 mL of water was stirred for 1 h at room temperature (21–23 °C) at pH 7.0 (Stone et al., 2015). The solution was then brought to 20.0 g with water and left for 10 min to allow sedimentation. The upper portion of the solution (approximately 10 g) was transferred to a 15 mL conical tube to centrifuge at 4180×*g* for 10 min at room temperature (21–23 °C). The protein content of the supernatant (5 g) was measured using a micro Kjeldahl unit (Labconco Corporation, Kansas City, MO, USA). The solubility was defined as the ratio between the protein content in the supernatant and the protein content of the starting material (powdered protein isolate).

2.8. Water and oil holding capacity

Water holding and oil holding capacity (WHC and OHC, respectively) were determined using the method described by Stone et al. (2015) with slight modification. For this measurement, 0.5 g (protein basis; initial sample weight) of each sample was mixed with 5 g of water (WHC) or canola oil (OHC) in a 50 mL conical centrifuge tube. The mixtures were vortexed at maximum speed for 10 s every 5 min for 30 min. Then the samples were centrifuged at 3000×*g* for 15 min at room temperature (21–23 °C). After decanting the supernatant, the pellet was weighed. The WHC and OHC were calculated as the pellet weight increase per gram of protein:

$$WHC\ (or\ OHC) = \frac{Wet\ pellet\ wight - initial\ sample\ weight}{initial\ sample\ weight}$$

2.9. Emulsion stability

The emulsion stability (ES) was determined using the method described by Stone and Nickerson (2012). A 1% (w/w) solution based on the protein content was prepared in water, and the pH was adjusted to 7.0 after 1 h of stirring at room temperature (21–23 °C). Five mL of the solution was transferred into a 50 mL centrifuge tube, and 5 g of canola oil was added. The probe of the homogenizer (S-10 N-10G probe, IKA T10 basic ULTRA-TURRAX Homogenizer, IKA Werke GmbH & Co. KG, Staufen im Breisgau, Germany) was placed at the oil/water interface. The sample was homogenized at speed 2 for 3 min. Immediately after homogenization, the emulsion was transferred to a 10 mL graduated cylinder. The emulsion was left to stand for 30 min to allow for separation, after which the volume of the aqueous layer at the cylinder bottom was recorded. The ES was expressed as below:

$$\%ES = (V_B - V_A) / V_B \times 100$$

where V_B is the volume of the aqueous layer before homogenization, and V_A is the volume of the aqueous layer after 30 min of separation.

2.10. Total phenolic content

The total phenolic content (TPC) of the samples was determined using a modified procedure described in Attard (2013). The protein isolates (50 mg) were extracted with 500 μL of aqueous methanol (80% (v/v)), on a shaker for 1 h at room temperature (21–23 °C), followed by centrifugation (17,900×*g*, 10 min, room temperature (21–23 °C)). Per each well, an aliquot (20 μL) of supernatant or of gallic acid standard solution was mixed with 100 μL of Folin-Ciocalteu aqueous solution (reagent: water 1:10, v:v) (cat # 47641, Sigma-Aldrich Co., St Louis, MO, USA) and 80 μL of 1 mol/L sodium bicarbonate aqueous solution. After 15 min incubation, the absorbance was measured at 765 nm using a BioTeK Synergy H1 Hybrid Multi-Mode Reader spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) against the reagent blank (20 μL of 80% (v/v) aqueous methanol). The TPC was calculated as mg of gallic acid equivalents (GAE)/g of sample based on a gallic acid standard curve. For the calibration curve, four independent stock solutions (1 mg/mL) of gallic acid in 80% aqueous methanol (v/v) were prepared (solutions A, B, C and D). Five 2-fold serial dilutions (250, 125, 62.5, 31.25 and 15.63 μg/mL) were prepared from these stocks using 80% aqueous methanol (v/v). Calibration curve solutions and samples were analyzed simultaneously.

2.11. In vitro protein digestibility

The enzymatic pH drop method (Tinus et al., 2012) was applied to measure *in vitro* protein digestibility (IVPD). Samples of 62.5 ± 0.5 mg protein were dissolved in 10 mL water, adjusted to pH 8.0, and kept for 1 h at 37 °C in a water bath. The multi-enzyme solution was prepared by mixing 31 mg of chymotrypsin (bovine pancreas), 16 mg of trypsin (porcine pancreas), and 13 mg of protease (*Streptomyces griseus*) in 10 mL of water and adjusted to pH 8.0. One mL of this enzyme mixture was added to the protein solution and kept at 37 °C for 10 min. The pH was recorded every 1 min. The IVPD was calculated as below:

$$IVPD\ (\%) = 65.66 + 18.10 \times \Delta pH_{10mi}n$$

Where ΔpH_{10min} is the change of pH in 10 min.

2.12. Volatolomics

The volatile compounds in the unfermented and fermented samples were analyzed by HS-SPME-GC/MS (headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry) using an Agilent 6890 GC and 5973 MSD coupled with a PAL RSI 85 autosampler (Agilent Technologies, Santa Clara, CA, USA) (Khorsandi, Shi, et al., 2024). Two grams of each sample were weighed into 20 mL headspace clear glass vials (Chromatographic Specialties, Brockville, ON, Canada) and stored at 4 °C. NaCl of the same weight (2 g) was used as the blank run. According to the conditions established by Azarnia et al. (2011), the volatile compounds from the samples were extracted at 50 °C for 45 min by SPME (Solid Phase Microextraction). The carboxen/polydimethylsiloxane (CAR/PDMS) coating of the SPME fibre assembly (Supelco, Bellefonte, PA, USA) was conditioned at 285 °C for 3 min before extraction, after which it was desorbed at 285 °C for 0.3 min. The GC inlet was set in the pulsed splitless mode and the gradient was initiated at 35 °C using pure helium (flowrate of 1.5 mL/min) on a DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm film thickness, Agilent Technologies). The gradient was held at 35 °C for 3 min, increased to 80 °C at 6 °C/min, then up to 280 °C at 20 °C/min, held for 2 min. The MS was acquired by scan from 35 to 200 m/z at the source 230 °C, quad 150 °C and 70.3eV of fixed electron energy. Volatile compound determination was done using mass spectra library search (National Institute of Standards and Technology (NIST) database (14. L), Agilent Mass Hunter software; Agilent Technologies).

2.13. Statistical analysis

For each inoculated isolate, fermentation was done in duplicate. For each duplicate inoculation, the original isolate, and the controls, all measurements were made in duplicate (n=2). A one-way ANOVA with Tukey's *post hoc* test (SPSS software, IBM SPSS Statistics 28.0, New York, USA) was performed to identify significant differences between samples within a pulse type ($p < 0.05$).

3. Results and discussion

3.1. Microorganism growth

To assess the influence of indigenous microorganisms on the metabolic activities of the test strains, their growth was monitored and

identified through morphological differences and CFU determination (Table 1). For all pulses, the strain *A. niger* was able to outgrow the natural microbiota, while dominant growth of the indigenous microorganisms was observed in all samples inoculated with *L. plantarum*. The growth of *A. oryzae* showed competition to the indigenous microbial communities in chickpea and faba bean samples, whereas for lentil, it was dominated by the natural microbes. Indigenous strains' growth was analyzed by plating on the YPD, MRS and Nutrient Agar. Colony morphology was used to differentiate between indigenous (e.g., bacilli and spore-forming yeasts) organisms and inoculated strains.

3.2. Protein hydrolysis

The protein content (dry basis) of the original, untreated, isolates was 71.5% for chickpea, 81.3% for lentil, and 88.4% for faba bean (Table 2). Fermentation, spontaneous or resulting from inoculation, had little effect on the protein content of the pulse isolates. The protein content of the treated pulses ranged from 70.3% to 73.7% for chickpea, 81.5%–85.1% for lentil, and 86.2%–91.4% for faba bean. An increase in protein content upon fermentation is generally expected due to the secretion of various microbial enzymes to break down sugars which would typically lead to an increase in microbial biomass; however, it was suggested that substrates low in carbohydrates and rich in proteins may transition the typical sugar metabolism towards proteolysis, promoting the utilization of proteins as the primary energy source (Ben-Harb et al., 2019). García Arteaga et al. (2021) also reported little change in the protein content of a pulse (pea) protein isolate after fermentation with lactic acid bacteria, where only one of the six strains (*Leuconostoc mesenteroides* subsp. *cremoris*) decreased the isolate protein content which the authors hypothesized to be due to nitrogen utilization by the bacteria. Chandra-Hioe et al. (2016) found differences in the protein content of fermented flours based on pulse type; inoculated (*L. delbrueckii* subsp. *Bulgaricus* and *Streptococcus thermophillus*) fermented faba bean flour had an enriched protein content whereas for chickpea fermentation (spontaneous/natural or inoculated) a decreased protein content of the flour was observed. As differences in protein/polypeptide composition exist among legumes, differential sensitivity to microbial proteolysis by specific strains may account for such variations among pulse types.

The pulse protein isolates were significantly ($p < 0.05$) hydrolyzed to 9.9%–15.2%, 11.1%–13.4%, and 9.0%–13.1% DH for chickpea, lentil, and faba bean, respectively, upon 48 h fermentation by the test strains (Table 2). Inoculated lentil samples had a narrower range of DH than chickpea and faba bean. All fermented lentil protein isolates, including the 48 h controls, had >10% DH; *A. oryzae* fermentation resulted in the lowest DH (11.1%) which was significantly lower than its respective 30 °C control (13.9%) and the other two fermentation strains. Similarly, *L. plantarum* fermentation resulted in a lower DH than its 37 °C control. In contrast, for chickpea, *L. plantarum* produced a high DH of 15.2%; however, this was not significantly different from the 37 °C control (13.9%). *Aspergillus niger,* but not *A. oryzae,* produced a higher DH in chickpea than the 30 °C control. For faba bean, the fungal strains were significantly less effective at protein hydrolysis (~9% DH) than the indigenous microbial communities (30 °C, 12.2% DH) whereas the bacterium and its control at 37 °C resulted in similar DH (~13%). The fermentative hydrolysis of large molecular weight proteins into smaller molecular weight proteins was also confirmed via SDS-PAGE (Fig. S1). Overall, the fermentation process affected the three pulse protein isolates differently in terms of proteolysis. There are number of possible reasons for this including differences in protein profiles (i.e., globulin/albumin content; legumin-vicilin ratio), carbohydrate and lipid content, and antinutritional factors (i.e. protease inhibitors) (Emkani et al., 2022;

Table 1
Microorganism growth after 48 h fermentation of pulse protein isolates.

Sample	CFU/g of substrate	Remarks
Chickpea		
Original isolate	N/A	N/A
0 h control	Not detected	N/A
48 h control @ 30 °C	10^7	N/A
48 h *A. oryzae*	10^7	Indigenous Mo > Inoculated Mo
48 h *A. niger*	10^6	Indigenous Mo ≪ Inoculated Mo
48 h control @ 37C	10^8	N/A
48 h *L. plantarum*	10^8	Indigenous Mo ≫ Inoculated Mo
Lentil		
Original isolate	N/A	N/A
0 h control	$<10^1$	N/A
48 h control @ 30 °C	10^8	N/A
48 h *A. oryzae*	10^7	Indigenous Mo ≫ Inoculated Mo
48 h *A. niger*	10^7	Indigenous Mo ≪ Inoculated Mo
48 h control @ 37 °C	10^8	N/A
48 h *L. plantarum*	10^8	Indigenous Mo ≫ Inoculated Mo
Faba bean		
Original isolate	N/A	N/A
0 h control	Not detected	N/A
48 h control @ 30 °C	10^7	N/A
48 h *A. oryzae*	10^7	Indigenous Mo > Inoculated Mo
48 h *A. niger*	10^6	Indigenous Mo ≪ Inoculated Mo
48 h control @ 37 °C	10^8	N/A
48 h *L. plantarum*	10^8	Indigenous Mo ≫ Inoculated Mo

Notes: N/A, not applicable; Mo, microorganism.

Table 2
Physicochemical properties of unfermented and fermented (48 h) pulse protein isolates.

Sample	Moisture (%)	Protein (%) (d.b.)	Degree of hydrolysis (%)	Surface charge (mV)
Chickpea				
Original isolate	3.6 ± 0.1^a	71.5 ± 0.4^{ab}	N/A	-32.1 ± 0.4^a
0 h control	2.3 ± 0.3^b	73.1 ± 0.9^{ab}	1.1 ± 0.2^d	-37.3 ± 1.8^b
48 h control @ 30 °C	3.4 ± 0.2^a	71.9 ± 1.7^{ab}	7.6 ± 1.7^c	-35.9 ± 0.8^b
48 h *A. oryzae*	3.8 ± 0.4^a	73.5 ± 0.3^{ab}	9.9 ± 1.0^{bc}	-37.0 ± 1.4^b
48 h *A. niger*	3.8 ± 0.3^a	73.7 ± 2.4^a	13.8 ± 1.4^a	-32.7 ± 0.6^a
48 h control @ 37 °C	4.0 ± 0.7^a	70.3 ± 1.6^b	12.2 ± 2.3^{ab}	-36.0 ± 1.5^b
48 h *L. plantarum*	2.2 ± 0.1^b	73.7 ± 0.9^a	15.2 ± 0.5^a	-42.7 ± 1.4^c
Lentil				
Original isolate	4.4 ± 0.0^a	81.3 ± 0.9^a	N/A	-28.0 ± 1.1^{ab}
0 h control	2.4 ± 0.2^b	81.5 ± 2.8^a	1.1 ± 0.1^e	-31.6 ± 1.6^{bc}
48 h control @ 30 °C	4.7 ± 0.1^a	82.4 ± 0.4^a	13.9 ± 0.3^{ab}	-37.7 ± 1.1^{de}
48 h *A. oryzae*	4.4 ± 0.1^a	82.1 ± 1.3^a	11.1 ± 0.7^d	-35.2 ± 1.9^{cd}
48 h *A. niger*	5.1 ± 0.7^a	84.3 ± 1.5^a	13.4 ± 0.2^{bc}	-24.2 ± 0.4^a
48 h control @ 37 °C	5.2 ± 0.1^a	84.7 ± 0.7^a	14.3 ± 0.1^a	-41.6 ± 1.9^e
48 h *L. plantarum*	2.6 ± 0.3^b	85.1 ± 1.2^a	12.9 ± 0.1^c	-35.0 ± 2.6^{cd}
Faba bean				
Original isolate	4.4 ± 0.1^b	88.4 ± 2.8^{ab}	N/A	-26.8 ± 2.0^a
0 h control	2.7 ± 0.2^d	87.9 ± 2.8^{ab}	0.6 ± 0.0^d	-29.4 ± 0.5^a
48 h control @ 30 °C	5.3 ± 0.1^a	86.2 ± 2.7^b	12.2 ± 0.2^b	-34.9 ± 1.3^b
48 h *A. oryzae*	4.1 ± 0.2^b	87.3 ± 1.2^{ab}	9.0 ± 0.2^c	-32.7 ± 0.3^b
48 h *A. niger*	3.5 ± 0.2^c	91.4 ± 0.6^a	9.1 ± 0.5^c	-34.3 ± 1.1^b
48 h control @ 37 °C	4.3 ± 0.1^b	87.7 ± 1.6^{ab}	13.6 ± 0.1^a	-34.4 ± 2.5^b
48 h *L. plantarum*	2.7 ± 0.2^d	87.3 ± 1.3^{ab}	13.1 ± 0.1^a	-35.6 ± 1.0^b

Notes: d.b., dry basis; N/A, not applicable; Data with the same superscript letters within a column for each pulse type are not significantly different ($p > 0.05$).

Senanayake et al., 2023).

3.3. Surface charge (zeta potential)

The surface charge of the isolates ranged from −32.1 to −42.7 mV for chickpea, from −24.2 to −41.6 mV for lentil, and from −26.8 to −35.6 mV for faba bean (Table 2). Overall fermentation increased (in magnitude) the surface charge of the isolates by ~5–10 mV. However, for both chickpea and lentil, fermentation with *A. niger* had no effect on the protein surface charge. Fermentation by *L. plantarum* resulted in chickpea protein isolate being more charged as compared to fermentation by the indigenous microbes at the same temperature, whereas fermentation by *A. oryzae* resulted in a similar surface charge as its 30 °C spontaneous fermented control. Inoculation with *A. oryzae* in lentil protein isolate also produced a similar surface charge as its control. In contrast, the 37 °C spontaneous fermented lentil control resulted in a more pronounced surface charge (−41.6 mV) than inoculation with *L. plantarum* (−35.0 mV). While all the fermented faba bean isolates had increased (in magnitude) surface charge compared to the original isolate there were no significant differences between the fermented isolates based on inoculation strain or spontaneous fermentation temperature.

Fermentation opens protein structure by hydrolysis, exposing hidden moieties and charges (Emkani et al., 2022). However, some of the zeta potential values did not correspond well with their respective DH (Table 2). For instance, the *A. niger*-inoculated chickpea and lentil samples had high DH values of 13%–14% but similar surface charges to that of their respective original isolates. In such cases, due to variations in pulse protein composition and the metabolic preference of the microbes, protein hydrolysis may have also occurred at sites with high surface hydrophobicity, resulting in unaffected zeta potential measurements or refolding of the protein. Batbayar et al. (2023) reported differences in the zeta potential of fermented pea protein-enriched flours (46% protein, db) inoculated with different microorganisms (*Bacillus subtilis*, *Rhizopus oryzae*, *R. oligosporus*, *L. plantarum*, and *A. oryzae*) despite all having 10% DH.

3.4. Solubility

The solubility at pH 7.0 was low for the original pulse isolates, with values below 20% (10.7%, 9.2%, and 16.5% for chickpea, lentil, and faba bean, respectively) (Table 3), which was not unexpected for commercial protein ingredients. Fermentation hydrolyzed the proteins and broke them down into smaller constituents (amino acids and peptides), resulting in enhanced protein solubility of ~22%–53%, depending on the fermentation treatment and pulse type. For chickpea, fermentation with either *A. niger* or *L. plantarum* increased the isolate solubility to 37.4% and 43.3%, respectively, whereas fermentation with *A. oryzae* had no effect on solubility compared to its 48 h 30 °C control. For lentil, while all inoculations enhanced solubility over the original isolate only *A. niger* fermentation increased the solubility (44.3%) above that of its 48 h fermented control (36.3%). In contrast, both *A. oryzae* and *L. plantarum* lowered the isolate solubility compared to their 48 h controls. Similarly, for faba bean, fermentation at 30 °C with the natural microbiota present in the isolate increased the solubility more than the fungal inoculations. The faba bean protein isolate fermented by *L. plantarum* had a similar solubility to its fermentation control at 37 °C, reaching almost 50%. In short, all test strains increased protein solubility of the original untreated isolate however when compared to the natural fermentation controls the results were dependent on pulse and strain type.

Besides the size reduction of the protein particles, enhanced surface charge (Table 2) via protein hydrolysis may have also promoted protein-water interactions and improved solubility. However, in some samples protein aggregation might have occurred between the newly exposed hydrophobic regions, diminishing the beneficial effect of size reduction on protein solubility (Kumitch, Stone, Nickerson, et al., 2020).

Table 3
Functional properties of unfermented and fermented (48 h) pulse protein isolates.

Sample	Solubility (%)	Water holding capacity (g/g)	Oil holding capacity (g/g)	Emulsion stability (%)
Chickpea				
Original isolate	10.7 ± 0.2^{d}	2.4 ± 0.0^{a}	0.8 ± 0.0^{b}	80.0 ± 0.0^{ab}
0 h control	8.5 ± 0.2^{d}	2.3 ± 0.1^{a}	0.9 ± 0.0^{ab}	67.0 ± 6.0^{bc}
48 h control @ 30 °C	22.4 ± 4.7^{c}	2.2 ± 0.1^{a}	0.9 ± 0.1^{ab}	80.5 ± 7.5^{a}
48 h *A. oryzae*	23.4 ± 1.3^{c}	2.0 ± 0.1^{b}	0.9 ± 0.0^{ab}	65.5 ± 3.8^{c}
48 h *A. niger*	37.4 ± 3.9^{ab}	1.6 ± 0.1^{d}	1.0 ± 0.0^{a}	81.5 ± 5.0^{a}
48 h control @ 37 °C	34.0 ± 7.3^{b}	1.8 ± 0.1^{bc}	0.9 ± 0.0^{ab}	57.5 ± 3.8^{c}
48 h *L. plantarum*	43.3 ± 2.8^{a}	1.7 ± 0.1^{cd}	0.9 ± 0.1^{ab}	67.0 ± 2.6^{bc}
Lentil				
Original isolate	9.2 ± 0.2^{d}	3.0 ± 0.0^{a}	0.9 ± 0.0^{a}	94.0 ± 0.0^{a}
0 h control	6.9 ± 0.5^{d}	2.5 ± 0.1^{b}	0.9 ± 0.0^{a}	85.5 ± 4.1^{a}
48 h control @ 30 °C	36.3 ± 1.4^{b}	2.1 ± 0.1^{d}	0.8 ± 0.0^{a}	82.5 ± 3.0^{ab}
48 h *A. oryzae*	26.0 ± 0.4^{c}	2.3 ± 0.0^{b}	0.9 ± 0.0^{a}	66.5 ± 4.4^{cd}
48 h *A. niger*	44.3 ± 2.7^{a}	1.8 ± 0.1^{e}	0.9 ± 0.0^{a}	87.0 ± 4.8^{a}
48 h control @ 37 °C	42.3 ± 1.1^{a}	1.8 ± 0.0^{e}	0.9 ± 0.1^{a}	75.0 ± 5.0^{bc}
48 h *L. plantarum*	34.0 ± 0.9^{b}	2.2 ± 0.0^{c}	0.9 ± 0.0^{a}	65.0 ± 4.8^{d}
Faba bean				
Original isolate	16.5 ± 0.0^{d}	5.1 ± 0.5^{a}	1.6 ± 0.0^{a}	93.0 ± 1.4^{a}
0 h control	6.8 ± 0.3^{e}	3.7 ± 0.1^{b}	0.9 ± 0.0^{b}	82.0 ± 3.7^{abc}
48 h control @ 30 °C	46.8 ± 4.6^{b}	2.1 ± 0.1^{cd}	0.8 ± 0.1^{bc}	85.5 ± 4.4^{ab}
48 h *A. oryzae*	27.2 ± 1.2^{c}	2.2 ± 0.0^{c}	0.8 ± 0.0^{c}	65.0 ± 3.5^{d}
48 h *A. niger*	25.5 ± 0.9^{c}	2.0 ± 0.1^{cde}	0.9 ± 0.0^{bc}	78.0 ± 6.9^{bc}
48 h control @ 37 °C	53.3 ± 0.8^{a}	1.8 ± 0.0^{e}	0.8 ± 0.0^{c}	73.5 ± 6.8^{cd}
48 h *L. plantarum*	49.1 ± 4.3^{ab}	1.9 ± 0.1^{de}	0.8 ± 0.0^{c}	75.0 ± 3.8^{bcd}

Notes: Data with the same superscript letters within a column for each pulse type are not significantly different ($p > 0.05$).

Nevertheless, natural fermentation (of the controls) was equally effective in improving the solubility of pulse isolates and in some cases even more so than strain inoculation. The differences between the controls and the inoculated samples showed that even though the growth of *L. plantarum* and *A. oryzae* was largely dominated by indigenous microbes (Table 1), their respective inoculation still had a significant impact on the solubility profile of the protein ingredients. Fermentation (natural or inoculated) remains a viable method to enhance the typically low solubility profile of commercial protein isolates. Lampart-Szczapa et al. (2006) attributed increased protein solubility after fermentation of lupin by lactic acid bacteria to protein hydrolysis. In contrast García Arteaga et al. (2021) reported that fermentation decreased the solubility (at pH 7) of a pea protein isolate; however, the isolates were heat treated (30 min at 80 °C and 10 min at 90 °C) during the fermentation procedure which may have denatured the proteins, contributing to protein aggregation and reduced solubility. The authors did, however, find that at pH 5 the solubility of the fermented isolates (10.6%–13.4%) was significantly higher than the unfermented isolate (7.1%).

3.5. Water and oil holding capacity

The WHC was significantly reduced by most fermentation and control treatments for all pulse types, whereas OHC remained largely unaffected by fermentation except for faba bean where OHC was reduced (Table 3). The decrease in WHC was most notable for faba bean samples where the original isolate had a high WHC of 5.1 g/g and fermentation

(controls and inoculated treatments) decreased the WHC by over 50% to 1.8–2.2 g/g. The reductions in WHC for chickpea and lentil were less extreme. For chickpea, fermentation by *A. niger* and *L. plantarum* resulted in the lowest WHC of 1.6–1.7 g/g whereas *A oryzae* had a higher WHC of 2.0 g/g; however, this was still lower than the original isolate (2.4 g/g). The trend was similar for lentil where the *A. oryzae* fermented sample (2.3 g/g) had the highest WHC of the three strains and *A. niger* the lowest (1.8 g/g); this corresponded to a 23% and 40% reduction, respectively, from the original isolate. The indigenous microbiota reduced WHC to a similar extent to that by inoculation, even though *A. niger* outgrew the natural microbial communities (Table 1). Kumitch, Stone, Nickerson, et al. (2020) also reported that fermentation by *A. oryzae* resulted in better WHC than fermentation by *A. niger* for pea protein-enriched flour despite giving similar DH. The results from the current and aforementioned studies indicate that specific modifications to the structure of the proteins and the size and conformation of the peptides as a result of microbial hydrolysis impact the WHC of pulse proteins.

In addition to WHC, the original faba bean sample also had a higher OHC of 1.6 g/g than the other two pulses (0.8–0.9 g/g). This was reduced to 0.8–0.9 g/g after all fermentation treatments. In contrast, for lentil, the OHC remained at 0.9 g/g after fermentation. The OHC for chickpea also remained largely unchanged by fermentation with the exception of inoculation with *A. niger*, which increased the OHC to 1.0 g/g. Various studies have reported fermentation to increase water and oil binding properties of different legumes; however, the substrates in these studies were either flours or protein concentrates and not protein isolates (Chandra-Hioe et al., 2016; Chawla et al., 2017; Emkani et al., 2022; Lampart-Szczapa et al., 2006; Xing et al., 2020). Generally, as fermentation hydrolyzes large protein molecules, the exposure of hidden reactive sites, both polar and non-polar, would help retain water and oil within the protein network and enhance WHC and OHC, respectively. However, in the case of WHC, the hydrolyzed proteins may have difficulty building a strong protein network and forming micro-capillaries between particles to entrap water molecules. Furthermore, highly soluble protein molecules would also show a weakened ability to retain water due to the loss of their native structure within aqueous solutions. WHC has been reported to be negatively correlated with solubility as extremely soluble proteins dissolve when an abundance of water is used in the test procedure, yielding very low WHC after centrifugation and decanting (Singhal, 2015; Stone et al., 2015).

3.6. Emulsion stability

The ES of the original protein isolates was 80.0% for chickpea, 94.0% for lentil, and 93.0% for faba bean (Table 3). Fermentation either decreased or had no impact on the ES, depending on pulse and treatment type. For both chickpea and lentil, the only treatments that did not significantly lower ES were *A. niger* fermentation and 48 h control fermentation at 30 °C. The other treatments (*A. oryzae*, *L. plantarum*, and 37 °C control fermentation) resulted in ES values of 57.5%–67.0% for chickpea and 65.0%–75.0% for lentil. Faba bean showed some similarities to the other two pulses in that the 48 h control fermentation at 30 °C did not influence ES, however, unlike chickpea and lentil, *A. niger* fermentation of faba bean decreased the isolate ES to 78%. The other treatments also decreased the ES of faba bean with *A. oryzae* having the greatest effect. Overall, fermentation by either *A. oryzae* or *L. plantarum* had a negative effect on pulse protein ES. Microbial hydrolysis was detrimental to the ability of protein particles to stabilize emulsions, likely due to the loss of structure for building strong interfacial films, especially for lentil samples which had >10% DH (Table 2) after all treatments. Beneficial functional changes are often observed with limited protein hydrolysis (i.e., with a DH <10%) where protein functional properties (e.g., foaming and emulsification) are improved due to the exposure of reactive sites (Polanco-Lugo et al., 2014). Sadowska et al. (1999) reported reduced emulsion stabilization properties of lentil flour after natural fermentation; however, the authors related to this to the lower solubility of the protein after fermentation. Çabuk, Stone, et al. (2018) reported modest hydrolysis (9.7%) of protein-enriched pea flour after 5 h of fermentation with *L. plantarum* to improve ES from ~37% to ~56%, whereas further fermentation (11 h; 13.5% DH) reduced the ES to ~20%.

3.7. Total phenolic content

The TPC for the samples is given in Table 4. The unfermented isolates had low TPC ranging from 0.312 mg GAE/g to 0.411 mg GAE/g. Fermentation increased the TPC by varying degrees depending on the fermentation treatment and pulse type. For each pulse the fermentation controls produced isolates with higher TPC when incubated at 37 °C compared to 30 °C. Chickpea was the only pulse where not all fermentation conditions increased the TPC as the 30 °C control had a similar value as the original isolate and 0 h control. However, all three strains increased the TPC of chickpea more than their respective fermentation controls, with *L. plantarum* fermentation producing the highest TPC overall of 1.718 mg GAE/g. In contrast, for lentil, only *A. niger* inoculation resulted in a higher TPC than the indigenous fermentation. Despite this, *A. oryzae* and *L. plantarum* fermented isolates had 4 and 5 times higher TPC, respectively, than the original isolate. For faba bean, the fermentation controls had higher TPC than the inoculated samples. Of the three strains, *L. plantarum* fermented faba bean protein isolate had the highest TPC of 2.148 mg GAE/g, followed by *A. oryzae* at 1.057 mg GAE/g, and *A. niger* at 0.904 mg GAE/g. Within each pulse type the TPC content corresponded well with the respective DH data. Others have reported increased phenolics after legume flour fermentation. Fernandez-Orozco et al. (2007) also reported the TPC to be affected by microorganism strain, where 48 h SSF of cracked soybean seeds with *A. oryze* and *R. oryzae* resulted in a small increase of 15%–19%, whereas

Table 4
In vitro protein digestibility and total phenolic content of unfermented and fermented (48 h) pulse protein isolates.

Sample	Total phenolic content (mg GAE/g)	In vitro protein digestibility (%)
Chickpea		
Original isolate	0.407 ± 0.006^{d}	87.5 ± 1.2^{a}
0 h control	0.411 ± 0.018^{d}	86.3 ± 1.4^{ab}
48 h control @ 30 °C	0.572 ± 0.040^{d}	82.9 ± 2.3^{b}
48 h *A. oryzae*	1.110 ± 0.038^{c}	77.8 ± 1.2^{c}
48 h *A. niger*	1.593 ± 0.186^{ab}	77.7 ± 1.8^{c}
48 h control @ 37 °C	1.390 ± 0.256^{b}	77.3 ± 1.6^{c}
48 h *L. plantarum*	1.718 ± 0.095^{a}	76.2 ± 0.1^{c}
Lentil		
Original isolate	0.368 ± 0.021^{e}	92.2 ± 0.4^{a}
0 h control	0.322 ± 0.026^{e}	91.5 ± 0.8^{a}
48 h control @ 30 °C	1.750 ± 0.139^{c}	78.7 ± 0.5^{bc}
48 h *A. oryzae*	1.484 ± 0.060^{d}	80.9 ± 1.2^{b}
48 h *A. niger*	2.207 ± 0.152^{b}	74.3 ± 1.5^{d}
48 h control @ 37 °C	2.535 ± 0.051^{a}	78.2 ± 1.2^{c}
48 h *L. plantarum*	1.910 ± 0.076^{c}	80.0 ± 0.7^{bc}
Faba bean		
Original isolate	0.378 ± 0.014^{f}	90.2 ± 0.1^{a}
0 h control	0.312 ± 0.020^{f}	89.3 ± 1.0^{a}
48 h control @ 30 °C	1.465 ± 0.052^{c}	79.9 ± 0.5^{c}
48 h *A. oryzae*	1.057 ± 0.017^{d}	82.9 ± 0.3^{b}
48 h *A. niger*	0.904 ± 0.047^{e}	80.8 ± 0.8^{c}
48 h control @ 37 °C	2.369 ± 0.143^{a}	77.5 ± 0.9^{d}
48 h *L. plantarum*	2.148 ± 0.023^{b}	76.7 ± 0.5^{d}

Notes: GAE, gallic acid equivalents. Data with the same superscript letters within a column for each pulse type are not significantly different ($p > 0.05$).

B. subtilis increased the phenolic compounds by over 300%. In a chickpea protein-enriched flour the total phenolic compounds more than doubled after 72 h SSF fermentation with different *Pediococcus* strains (Xing et al., 2020). Fermentation increases TPC through microbial enzyme production and the breakdown of larger structures or bound components in the matrix (Emkani et al., 2022). Free amino acids produced by fermentation can interfere with the Folin-Ciocalteu method of analysis for determining the TPC, therefore LC-MS analysis is needed to confirm our results in order to identify and quantify which phenolic compounds are increasing or being liberated via fermentation. For example, Polanowska et al. (2020) showed a 4–9 fold increase in free amino acids after faba bean fermentation depending on *R. oligosporus* strain but an overall similar decrease in TPC by 44%–50% regardless of strain; however specific phenolic compounds, namely quercetin and kaempferol, were in greater abundance in the fermented material.

3.8. In vitro protein digestibility

The IVPD of the original protein isolates was 87.5% for chickpea, 92.2% for lentil, and 90.2% for faba bean (Table 4), and fermentation treatments reduced protein digestibility for all pulses. For chickpea, the IVPD was decreased to ~77% with no significant differences among the three strains. The indigenous microbes at 30 °C were able to maintain a higher protein digestibility of 82.9% than at the fermentation temperature of 37 °C (77.3%). For lentil, the isolate fermented with *A. niger* had the lowest IVPD, at 74.3%, while the other treatments had IVPD values of ~78%–80%. For faba bean, the reduction in IVPD was less for samples inoculated with fungi (~80%–82%) than with the bacterium (~77%). Fermentation was expected to enhance protein digestibility as the hydrolyzing activities of the microbes open the protein structure for more efficient proteolytic attack by the digestive enzymes and concomitantly decrease the antinutritional factors that inhibit protein digestion (Emkani et al., 2022). The reduced IVPD of the pulse samples after fermentation is hypothesized to be due to the corresponding increase in TPC after fermentation. Phenolic compounds can restrict protein digestion through non-covalent bonding with the protein, restricting protease access to the protein substrate, or by directly bonding with the protease and therefore reducing the enzyme activity (Cirkovic Velickovic & Stanic-Vucinic, 2018). In contrast, Çabuk, Nosworthy, et al. (2018) reported a minor increase in IVPD coupled with an increase in phenolic content of pea protein concentrate after 5–11 h of submerged fermentation with *L. plantarum*. This corresponded to a DH of 9.7%–13.5% for the pea protein concentrate (Çabuk, Stone, et al., 2018). The extent of phenolic compounds interfering with protein digestion depends on the specific type of phenolics present and their direct interactions with the protein substrate, the presence of other compounds in the matrix as well as the pH (Cirkovic Velickovic & Stanic-Vucinic, 2018). Similar to the present study, Ranjan et al. (2019) reported *R. oryzae* SSF (72 h) to decrease IVPD, albeit using a much different substrate (de-oiled rice bran) than in the present study. The reduction in IVPD was despite fermentation also decreasing the antinutrient content (i.e., phytate and trypsin inhibitor).

3.9. Volatile compound analysis

Chromatograms relating to the volatile profiles of the unfermented and fermented samples are shown in Fig. 1. The compounds corresponding to the major peaks were identified and are listed in Table 5. The chromatograms from unfermented chickpea samples showed a dominant peak at approximately 5.8 min retention time that was identified as the aldehyde compound, hexanal. This off-flavour compound is commonly present in many pulses and strongly associated with a green grass-like odour profile and is a product of fatty acid oxidation process (Karolkowski et al., 2021). Although hexanal was identified in the unfermented lentil and faba bean samples, it was not as dominant as in the chickpea samples, probably correlating with the higher fatty acid content in chickpea compared to the other two pulses. The fermented samples from all three pulse isolates showed the presence of up to 13 additional prominent peaks and new volatile compounds compared to the unfermented controls. Most of these new compounds belonged to three major compound classes, acids, aldehydes and ketones. Acidic compounds, such as acetic acid, propanoic acid, 2-methyl-propanoic acid, 3-methyl-butanoic acid, 2-methyl-butanoic acid and 2-methyl-pentanoic acid, usually have acidic and pungent flavours that are commonly associated with fermented dairy products like yogurt, kefir and cheeses (Krastanov et al., 2023). It is interesting to note that while fermented lentil and faba bean samples showed the addition of a diversity of new organic acid compounds, they were mostly absent from the fermented chickpea samples. Many aromatic ketones and some aldehydes, such as 3-methyl-butanal, 4-hydroxyl-4-methyl-2-pentanone, 3-methyl-2-pentanone, 2-butanone, 4-methyl-2-hexanone, 3-methyl-, oxime butanal and 2-heptanone, were newly added during fermentation. Many of these ketone compounds are generally associated with pleasant, fruity, or floral odour notes. Production of new flavour compounds during solid state fermentation of pea and navy bean protein isolates by *A. oryzae* has been previously reported (Khorsandi, Shi, et al., 2024).

4. Conclusions

The present research examined the effect of 48 h SSF (*A. oryzae, A. niger*, or *L. plantarum* and spontaneous fermentation) of chickpea, lentil, and faba bean protein isolates on their physicochemical and functional properties, select nutritional properties, and volatolome profiles. Protein hydrolysis reached ~8%–15% DH after fermentation depending upon the inoculation strain and pulse type. Overall, fermentation increased the surface charge (absolute value) and solubility of the protein isolates and reduced their WHC. Trends in OHC and ES were dependent on fermentation strain and pulse type. The largest increases in TPC caused the lowest IVPD in each fermented pulse. Pulse type was an important factor in how the fermentation conditions affected protein modification. Despite *L. plantarum* SSF being dominated by the growth of indigenous microbes, for the lentil protein isolate the *L. plantarum* inoculated sample had different properties than the control sample (spontaneous fermentation). The role of competitive growth between inoculated and indigenous microbiota still needs to be elucidated. The two fungal species used for fermentation gave different results in chickpea and lentil whereas the faba bean samples had similar results between the two species. Several new compounds from three prominent classes of volatile organic compounds were produced in samples that were fermented by either indigenous or inoculated microbes. Compounds belonging to organic acids and ketones classes should impart a favourable odour profile to the fermented products, compared to the basal flavour note of these substrates that was predominated by hexanal. Further studies correlating flavour chemistry analysis with human sensory evaluations are needed to confirm the utility of using fermentation as a tool for the improvement of the overall flavour profile of pulse ingredients. As well, a deeper explanation of the mechanist effect of fermentation in each pulse would be useful. Overall, fermentation can be used to modify the attributes of pulse protein isolates depending on pulse type, fermentation conditions, and the specific ingredient property under evaluation.

CRediT authorship contribution statement

Andrea K. Stone: Writing – review & editing, Writing – original draft, Formal analysis. **Dai Shi:** Writing – original draft, Formal analysis. **Enyu Liu:** Investigation. **Zahra Jafarian:** Investigation. **Caishuang Xu:** Visualization, Investigation. **Aarti Bhagwat:** Visualization, Investigation. **Yuping Lu:** Methodology, Formal analysis. **Leonid Akhov:** Formal analysis. **Jessica Gerein:** Formal analysis. **Xiumei Han:** Formal analysis. **L. Irina Zaharia:** Writing – review & editing, Formal analysis. **Nandhakishore Rajagopalan:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Takuji Tanaka:** Writing –

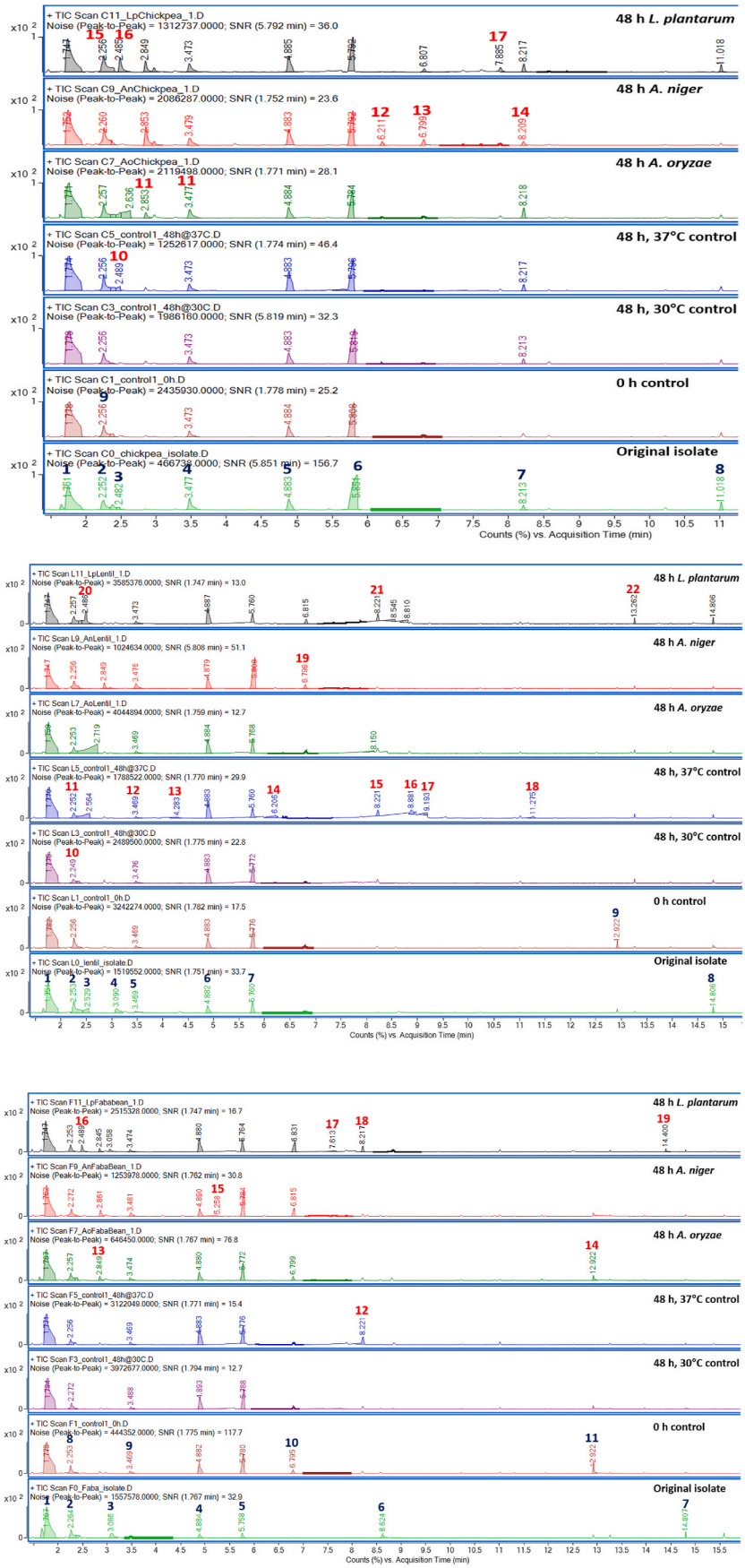

Fig. 1. Volatolome profiles of unfermented and fermented (48 h) pulse protein isolates: Top, chickpea; middle, lentil; bottom, faba bean.

Table 5
Major volatile compounds identified in unfermented and fermented (48 h) pulse protein isolates.

	Peak #	Retention time (min)	Name
Chickpea	1	1.751	Acetonitrile
	2	2.252	2-Butanone
	3	2.482	Ammonium acetate
	4	3.477	Pentanal
	5	4.883	Toluene
	6	5.851	Hexanal
	7	8.213	2-Heptanone
	8	11.018	Furan, 2-pentyl-
	9	2.256	N-Hexane
	10	2.489	Acetic acid
	11	2.853/3.477	Butanal, 3-methyl-
	12	6.211	Silane, dimethyl (2-methoxy ethoxy) butoxy-
	13	6.799	2-Pentanone, 4-hydroxyl-4-methyl
	14	8.209	Beta-Phenylethyl butyrate
	15	2.256	2-Pentanone, 3-methyl-
	16	2.485	Trichloromethane
	17	7.885	Hexanenitrile
Lentil	1	1.751	Acetonitrile
	2	2.253	*n*-Hexane
	3	2.529	Acetic acid
	4	3.090	2-Propanol,1-methoxy
	5	3.469	Pentanal
	6	4.882	Toluene
	7	5.760	Hexanal
	8	14.806	Oxalic acid, 2TMS derivative
	9	12.922	Nonanal
	10	2.249	2-Butanone
	11	2.252	5-Methyloxazolidine
	12	3.469	Butanal,3-methyl-
	13	4.283	Propanoic acid
	14	6.206	Propanoic acid,2-methyl-
	15	8.221	2-Hexanone,4-methyl-
	16	8.881	Butanoic acid,3-methyl-
	17	9.193	Butanoic acid,2-methyl-
	18	11.275	Butanal,3-methyl-,oxime
	19	6.799	2-Pentanone,4-hydroxyl-4-methyl-
	20	2.486	Trichloromethane
	21	8.221	2-Heptanone
	22	13.262	Silane,tetramethyl-
Faba bean	1	1.767	Oxirane,trimethyl-
	2	2.264	*n*-Hexane
	3	3.086	2-Propanol,1-methoxy-
	4	4.884	Toluene
	5	5.758	Hexanal
	6	8.624	Ethanol,2-butoxy-
	7	14.807	Oxalic acid, 2TMS derivative
	8	2.253	Acetonitrile
	9	3.469	Pentanal
	10	6.795	2-Pentanone,4-hydroxyl-4-methyl-
	11	12.922	Hexane,1-(hexyloxy (-2-methyl-
	12	8.221	Pentanoic acid,2-methyl-
	13	2.849	Butanal,3-methyl-
	14	12.922	Ethanol,2-(hexyloxy)-
	15	5.258	Propanoic acid,2-methyl-
	16	2.489	Trichloromethane
	17	7.613	Butanoic acid,3-methyl-
	18	8.217	2-Heptanone
	19	14.400	1-Phenoxypropan-2-ol

For each pulse type, compounds listed below the dashed line are only found in the fermented samples.

review & editing, Supervision, Conceptualization. **Darren R. Korber:** Writing – review & editing, Supervision, Conceptualization. **Michael T. Nickerson:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors gratefully acknowledge support from the Sustainable Protein Production (SPP) program of the National Research Council Canada and by the Aquatic and Crop Resource Development Research Centre as part of its contribution to the SPP program and the Saskatchewan Ministry of Agriculture Strategic Research Chair Program (Protein Quality and Utilization).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2024.104580.

References

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. *Journal of Agricultural and Food Chemistry, 27*(6), 1256–1262. https://doi.org/10.1021/jf60226a042

AOAC. (2003). *Official methods of analysis of AOAC International*. Gaithersburg, MD: AOAC International.

Attard, E. (2013). A rapid microtitre plate Folin-Ciocalteu method for the assessment of polyphenols. *Central European Journal of Biology, 8*(1), 48–53. https://doi.org/10.2478/s11535-012-0107-3

Azarnia, S., Boye, J. I., Warkentin, T., Malcolmson, L., Sabik, H., & Bellido, A. S. (2011). Volatile flavour profile changes in selected field pea cultivars as affected by crop year and processing. *Food Chemistry, 124*, 326–335. https://doi.org/10.1016/j.foodchem.2010.06.041

Batbayar, B., Kryachko, Y., Nickerson, M. T., Korber, D. R., & Tanaka, T. (2023). Solid-state and submerged fermentation effects on functional properties of pea protein-enriched flour. *Cereal Chemistry, 100*(5), 1092–1105. https://doi.org/10.1002/cche.10691

Ben-Harb, S., Saint-Eve, A., Panouillé, M., Souchon, I., Bonnarme, P., Dugat-Bony, E., & Irlinger, F. (2019). Design of microbial consortia for the fermentation of pea-protein-enriched emulsions. *International Journal of Food Microbiology, 293*, 124–136. https://doi.org/10.1016/j.ijfoodmicro.2019.01.012

Byanju, B., Hojilla-Evangelista, M., & Lamsal, B. (2021). Fermentation performance and nutritional assessment of physically processed lentil and green pea flour. *Journal of the Science of Food and Agriculture, 101*(14), 5792–5806. https://doi.org/10.1002/jsfa.11229

Çabuk, B., Nosworthy, M. G., Stone, A. K., Korber, D. R., Tanaka, T., House, J. D., & Nickerson, M. T. (2018). Effect of fermentation on the protein digestibility and levels of non-nutritive compounds of pea protein concentrate. *Food Technology and Biotechnology, 56*(2), 257–264, 10.17113%2Fftb.56.02.18.5450.

Çabuk, B., Stone, A. K., Korber, D. R., Tanaka, T., & Nickerson, M. T. (2018). Effect of *Lactobacillus plantarum* fermentation on the surface and functional properties of pea protein-enriched flour. *Food Technology and Biotechnology, 56*(3), 411–420. https://doi.org/10.17113/ftb.56.03.18.5449

Can Karaca, A., Low, N., & Nickerson, M. T. (2011). Emulsifying properties of chickpea, faba bean, lentil and pea proteins produced by isoelectric precipitation and salt extraction. *Food Research International, 44*(9), 2742–2750. https://doi.org/10.1016/j.foodres.2011.06.012

Chandra-Hioe, M., Wong, C., & Arcot, J. (2016). The potential use of fermented chickpea and faba bean flour as food ingredients. *Plant Foods for Human Nutrition, 71*(1), 90–95. https://doi.org/10.1007/s11130-016-0532-y

Chawla, P., Bhandari, L., Sadh, P. K., & Kaushik, R. (2017). Impact of solid-state fermentation (*Aspergillus oryzae*) on functional properties and mineral bioavailability of black-eyed pea (*Vigna unguiculate*) seed flour. *Cereal Chemistry, 94*(3), 437–442. https://doi.org/10.1094/CCHEM-05-16-0128-R

Christensen, L. F., Garcia-Bejar, B., Bang-Berthelsen, C. H., & Hansen, E. B. (2022). Extracellular microbial proteases with specificity for plant proteins in food fermentation. *International Journal of Food Microbiology, 381*, Article 109889. https://doi.org/10.1016/j.ijfoodmicro.2022.109889

Cirkovic Velickovic, T. D., & Stanic-Vucinic, D. J. (2018). The role of dietary phenolic compounds in protein digestion and processing technologies to improve their antinutritive properties. *Comprehensive Reviews in Food Science and Food Safety, 17*(1), 82–103. https://doi.org/10.1111/1541-4337.12320

Emkani, M., Oliete, B., & Saurel, R. (2022). Effect of lactic acid fermentation on legume protein properties, a review. *Fermentation, 8*(6), 244–286. https://doi.org/10.3390/fermentation8060244

García Arteaga, V., Leffler, S., Muranyi, I., Eisner, P., & Schweiggert-Weisz, U. (2021). Sensory profile, functional properties and molecular weight distribution of fermented pea protein isolate. *Current research in food science, 4*, 1–10. https://doi.org/10.1016/j.crfs.2020.12.001

Granito, M., Frias, J., Doblado, R., Guerra, M., Champ, M., & Vidal-Valverde, C. (2002). Nutritional improvement of beans (*Phaseolus vulgaris*) by natural fermentation. *European Food Research and Technology, 214*(1), 226–231. https://doi.org/10.1007/s00217-001-0450-5

Khorsandi, A., Shi, D., Stone, A. K., Bhagwat, A., Lu, Y., Xu, C., Das, P. P., Polley, B., Akhov, L., Gerein, J., Han, X., Bhowmik, P., Zaharia, L. I., House, J. D., Rajagopalan, N., Tanaka, T., Korber, D. R., & Nickerson, M. T. (2024). Effect of solid-state fermentation on the protein quality and volatile profile of pea and navy bean protein isolates. *Cereal Chemistry, 101*(1), 131–143. https://doi.org/10.1002/cche.10729

Khorsandi, A., Stone, A. K., Shi, D., Xu, C., Das, P. P., Lu, Y., Rajagopalan, N., Tanaka, T., Korber, D. R., & Nickerson, M. T. (2024). Physicochemical and functional properties of fermented pea and navy bean protein isolates. *Cereal Chemistry, 101*(1), 231–247. https://doi.org/10.1002/cche.10740

Krastanov, A., Yeboah, P. J., Wijemanna, N. D., Eddin, A. S., Ayivi, R. D., & Ibrahim, S. A. (2023). Volatile aromatic flavor compounds in yogurt: A review. In S. A. Ibrahim (Ed.), *Current issues and advances in the dairy industry*. London: IntechOpen. https://doi.org/10.5772/intechopen.109034.

Kumitch, H. M., Stone, A. K., Nickerson, M. T., Korber, D. R., & Tanaka, T. (2020). Effect of fermentation time on the physicochemical and functional properties of pea protein-enriched flour fermented by *Aspergillus oryzae* and *Aspergillus niger*. *Cereal Chemistry, 97*(2), 416–428. https://doi.org/10.1002/cche.10257

Kumitch, H. M., Stone, A., Nosworthy, M. G., Nickerson, M. T., House, J. D., Korber, D. R., & Tanaka, T. (2020). Effect of fermentation time on the nutritional properties of pea protein-enriched flour fermented by *Aspergillus oryzae* and *Aspergillus niger*. *Cereal Chemistry, 97*(1), 104–113. https://doi.org/10.1002/cche.10234

Nadeeshani, H., Senevirathne, N., Somaratne, G., & Bandara, N. (2022). Recent trends in the utilization of pulse protein in food and industrial applications. *ACS Food Science & Technology, 2*, 722–737. https://doi.org/10.1021/acsfoodscitech.1c00448

Patel, H., Wang, R., Chandrashekar, O., Pandiella, S., & Webb, C. (2004). Proliferation of *Lactobacillus plantarum* in solid-state fermentation of oats. *Biotechnology Progress, 20*(1), 110–116. https://doi.org/10.1021/bp034176r

Polanco-Lugo, E., Dávila-Ortiz, G., Betancur-Ancona, D., & Chel-Guerrero, L. (2014). Effects of sequential enzymatic hydrolysis on structural, bioactive and functional properties of *Phaseolus lunatus* protein isolate. *Food Science & Technology (Zurich), 34*(3), 441–448. https://doi.org/10.1590/1678-457x.6349

Polanowska, K., Grygier, A., Kuligowski, M., Rudzińska, M., & Nowak, J. (2020). Effect of tempe fermentation by three different strains of *Rhizopus oligosporus* on nutritional characteristics of faba beans. *Lebensmittel-Wissenschaft und -Technologie, 122*, Article 109024. https://doi.org/10.1016/j.lwt.2020.109024

Ranjan, A., Sahu, N. P., Deo, A. D., & Kumar, S. (2019). Solid state fermentation of de-oiled rice bran: Effect on *in vitro* protein digestibility, fatty acid profile and anti-nutritional factors. *Food Research International, 119*, 1–5. https://doi.org/10.1016/j.foodres.2019.01.054

Rivera, J., Siliveru, K., & Li, Y. (2022). A comprehensive review on pulse protein fractionation and extraction: Processes, functionality, and food applications. *Critical Reviews in Food Science and Nutrition, 64*(13), 4179–4201. https://doi.org/10.1080/10408398.2022.2139223

Sadowska, J., Fornal, J., Vidal-Valverde, C., & Frias, J. (1999). Natural fermentation of lentils. Functional properties and potential in breadmaking of fermented lentil flour. *Nahrung, 43*(6), 396–401. https://doi.org/10.1002/(SICI)1521-3803(19991201)43:6%3C396::AID-FOOD396%3E3.0.CO;2-N

Senanayake, D., Torley, P. J., Chandrapala, J., & Terefe, N. S. (2023). Microbial fermentation for improving the sensory, nutritional and functional attributes of legumes. *Fermentation, 9*(7), 635. https://doi.org/10.3390/fermentation9070635

Shi, D., & Nickerson, M. T. (2022). Comparative evaluation of the functionality of faba bean protein isolates with major legume proteins in the market. *Cereal Chemistry, 99*(6), 1246–1260. https://doi.org/10.1002/cche.10589

Sim, S., Srv, A., Chiang, J., & Henry, C. (2021). Plant proteins for future foods: A roadmap. *Foods, 10*(8), 1967. https://doi.org/10.3390/foods10081967

Singhal, A. (2015). *The effect of genotype and the environment on the physicochemical and functional attributes of faba bean protein isolates (Unpublished Master's thesis)*. University of Saskatchewan, Saskatoon, Canada. http://hdl.handle.net/10388/ETD-2015-05-2136.

Stone, A. K., Karalash, A., Tyler, R. T., Warkentin, T. D., & Nickerson, M. T. (2015). Functional attributes of pea protein isolates prepared using different extraction methods and cultivars. *Food Research International, 76*(1), 31–38. https://doi.org/10.1016/j.foodres.2014.11.017

Stone, A. K., & Nickerson, M. T. (2012). Formation and functionality of whey protein isolate (kappa-, iota-, and lambda-type)-carrageenan electrostatic complexes. *Food Hydrocolloids, 27*(2), 271–277. https://doi.org/10.1016/j.foodhyd.2011.08.006

Tinus, T., Damour, M., Van Riel, V., & Sopade, P. (2012). Particle size-starch–protein digestibility relationships in cowpea (*Vigna unguiculata*). *Journal of Food Engineering, 113*(2), 254–264. https://doi.org/10.1016/j.jfoodeng.2012.05.041

Xiao, Y., Xing, G., Rui, X., Li, W., Chen, X., Jiang, M., & Dong, M. (2015). Effect of solid-state fermentation with *Cordyceps militaris* SN-18 on physicochemical and functional properties of chickpea (*Cicer arietinum* L.) flour. *Food Science & Technology (Zurich), 63*(2), 1317–1324. https://doi.org/10.1016/j.lwt.2015.04.046

Xing, Q., Dekker, S., Kyriakopoulou, K., Boom, R. M., Smid, E. J., & Schutyser, M. A. (2020). Enhanced nutritional value of chickpea protein concentrate by dry separation and solid state fermentation. *Innovative Food Science & Emerging Technologies, 59*, Article 102269. https://doi.org/10.1016/j.ifset.2019.102269