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ORIGINAL ARTICLE

Integrated Food Science

Submerged fermentation of lentil protein isolate and its impact on protein functionality, nutrition, and volatile profiles

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Abstract: Fermentation of pulses as a clean processing technique has been reported to have a favorable impact on the functional and nutritional quality of the starting materials. Compared to commonly fermented pulses such as peas and chickpeas, limited information is available on the effect of fermentation on lentils, especially when using a high protein isolate (>80% protein) as compared to seeds or flours. Therefore, in the present work, lentil protein isolate was used as a feedstock for submerged fermentation with *Aspergillus niger*, *Aspergillus oryzae*, or *Lactobacillus plantarum*. After 48 h, the samples showed increased protein content with enhanced solubility and oil-holding capacity. Controlled fermentation, as opposed to spontaneous fermentation, maintained the high foaming capacity; however, all fermented samples had lower foam and emulsion stabilizing properties and reduced water-holding capacity compared to the control. The fermented proteins were also less digestible, possibly due to an increase in phenolics and saponins. New volatile compounds were identified in fermented samples that show promise for improved sensory attributes. Significant differences were observed in specific quality attributes depending on the microbial strain used. Further research is required to better understand the fermentative metabolism of microbial communities when provided high-protein lentil ingredients as growth substrates.

KEYWORDS

Lactobacillus plantarum, *Aspergillus*, pulse protein, digestibility, hydrolysis

Practical Application: Fermented lentil protein isolate has promising flavor profiles that may improve its sensory properties for food application.

1 | INTRODUCTION

Lentils (*Lens culinaris*) are nutrient-dense edible seeds from the legume family. They are good sources of protein (26.5%–31.6%), dietary fiber (16.0%–18.6%), vitamins (e.g., folate and B vitamins), minerals (e.g., iron and potassium), and antioxidants (e.g., polyphenols) (Ganesan & Xu, 2017; Ramdath et al., 2020). The consumption of lentils has been positively linked to the prevention of diabetes, obesity, cancers, and cardiovascular diseases (Ganesan & Xu, 2017). The functional and nutritional quality of lentils promises value in substituting animal proteins in various food applications. However, like most plant-based protein sources, due to their inherent limitations in protein structure (low flexibility and high content of indigestible components), amino acid composition (lacking sulfur-containing amino acids), antinutrients (e.g., enzyme inhibitors), and flavor (beany notes), lentils face challenges in value-added utilization (Nadeeshani et al., 2022; Sim et al., 2021).

Different processing strategies have proven effective in addressing the utilization challenges of plant proteins, including lentils. Fermentation, regarded as a clean process, involves the hydrolysis of large macronutrients, such as protein, starch, and lipids, into smaller molecules through the microbial secretion of various enzymes, modifying substrate composition (Sharma et al., 2020). Typically, during fermentation, antinutritional factors (ANFs) are degraded and protein structure is opened, enhancing the overall protein digestibility and nutrient availability (Emkani et al., 2022). The structural modification of the proteins also affects functional properties due to changes in surface properties and solubility (Emkani et al., 2022). The hydrolytic process generates new compounds while facilitating the removal/reduction of the original flavors, resulting in unique sensory profiles of the products (Sharma et al., 2020).

Literature data is available on fermented legumes, such as soybeans (Razavizadeh et al., 2022; Rui et al., 2017), peas (Batbayar et al., 2023; Çabuk, Nosworthy et al., 2018; Çabuk, Stone et al., 2018; Kumitch et al., 2020), chickpeas and faba beans (Chandra-Hioe et al., 2016; Coda et al., 2015; Xiao et al., 2015), and the effect on their qualities such as functional and nutritional properties and flavor profile. However, a research gap exists in terms of assessing protein functionality modifications of fermented lentil protein isolate (LPI). Most fermentation studies have used whole legume seeds or flours as substrates, of which carbohydrates (starch) remain the major nutrient available for microbial growth. Because substrate composition greatly affects the metabolism of microorganisms (Ben-Harb et al., 2019), ingredients that are high in protein, that is, protein isolates, are expected to exhibit quite varied responses during fermentation processing. Hence, the

present project aims to investigate the above gaps of knowledge by fermenting LPI (>80% protein) with common strains, *Aspergillus niger*, *Aspergillus oryzae*, and *Lactobacillus plantarum*, under submerged fermentation (SmF) conditions and examining changes to substrate composition, protein surface and functional properties, ANF composition, protein digestibility, and volatile profiles. The results from LPI fermentation are hypothesized to differ from those for other legumes and those in less concentrated protein forms.

2 | MATERIALS AND METHODS

2.1 | Materials

LPI was commercially sourced and produced using an alkaline extraction–isoelectric precipitation process. The strains *A. oryzae* NRRL 5590, *A. niger* NRRL 334, and *L. plantarum* NRRL B-4496 were subcultures of the original cultures from the Agricultural Research Service (ARS) Culture Collection (Peoria, IL, USA). The de Man, Rogosa, and Sharpe (MRS) broth and potato dextrose agar (PDA) plates were from Fisher Scientific. All chemicals (ACS grade) were from Fisher Scientific and VWR Canada.

2.2 | Strains and inoculum preparation

Spores of *A. oryzae* and *A. niger* were collected from PDA plates after cultivation at 30°C for 7 days. Spore suspensions were prepared in 20 mL of sterilized and deionized water, the concentration of which was determined by direct microscopy counting using a hemocytometer (BrightLine). Bacterial cells of *L. plantarum* were obtained by culturing in MRS broth at 37°C for 48 h, the colony-forming unit (CFU) of which was determined by serial dilution and spread plating on MRS media using standard methods.

2.3 | Submerged fermentation

Under aseptic conditions, the LPI (25 g) was suspended in an Erlenmeyer flask with deionized water (225 g) and inoculated with prepared spore/cell suspensions at a concentration of 10^7 CFU/g of protein isolate. The flask was then placed on an orbital shaker operated at 300 rpm in an incubator (Fisher Scientific) at 30°C for fungal strains and 37°C for *L. plantarum* for 48 h. Fermentation reactions were done in triplicate. The 0 h control and spontaneous fermented 48 h controls were also prepared in triplicate under the same conditions without strain inoculation. The

samples were then dried in a freeze dryer for 10 days (18-L Labconco FreeZone Freeze Dryer; Labconco Corporation). Dried samples were ground into fine powders using a coffee burr mill (Cuisinart DBM-8C) and stored at 4°C. The moisture and protein contents were measured according to Association of Official Agricultural Chemists (AOAC) (2003) methods 925.10 and 920.87 ($N\% \times 6.25$), respectively.

2.4 | Determination of degree of hydrolysis (DH)

The degree of hydrolysis (DH) was measured according to a modified method of Adler-Nissen (1979) using the reaction of 2,4,6-trinitrobenzenesulfonic acid (TNBS) with free amino acid groups. Briefly, 0.4 g of the processed LPI was suspended in 40 mL of sodium phosphate buffer (pH 7.8) and stirred overnight at 4°C. The pH was readjusted to 7.8 (using 0.5 M NaOH), and the solution was centrifuged at $8230 \times g$ for 30 min at 4°C. The supernatant was measured for absorption at 340 nm (Genesys 20 spectrophotometer, Thermo Fisher Scientific, Inc.). The original isolates were hydrolyzed by dissolving in 6 N HCl and heating at 110°C overnight to complete total acid hydrolysis. The ratio of free amino groups in processed LPI to those in the total acid-hydrolyzed original LPI was calculated as the DH.

2.5 | Surface properties

2.5.1 | Surface charge (zeta potential)

The surface charge (mV) was measured based on the method of Can Karaca et al. (2011). Protein solutions of 0.05% (w/w) were prepared in deionized water and stirred for 1 h at room temperature. The pH was adjusted to 7.0 (using 0.1 M NaOH), then using a Zetasizer Nano-ZS90 instrument (Malvern Instruments), the electrophoretic mobility (U_E) was measured to calculate the zeta potential (ξ) as follows (Equation 1), 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), and η is viscosity:

$$U_E = \frac{2\epsilon \times \xi \times f(\kappa\alpha)}{3\eta} \quad (1)$$

2.5.2 | Surface hydrophobicity

The surface hydrophobicity was measured according to Kato and Nakai (1980) with modifications. Protein solutions of 0.025% (w/w) in water were prepared and stirred

overnight at 4°C. The solutions were brought to room temperature, and the pH was adjusted to 7.0 (using 0.1 M NaOH), followed by preparing dilutions of 0.005%, 0.010%, 0.015%, and 0.020% in water. The fluorescent probe 8-anilino-1-naphthalenesulfonic acid (ANS) was used, and the fluorescence intensity of the ANS reaction with the samples after 5 min in the dark was measured using a FluoroMax-4 spectrophotometer (HORIBA Jobin Yvon Inc.). The physical slit width was 0.2353 mm, and the excitation and emission wavelengths were set at 390 and 470 nm, respectively. A chart of fluorescence intensity versus protein concentration was constructed (linear regression), and the slope was taken as the surface hydrophobicity of the samples, which was expressed in arbitrary units (a.u.).

2.6 | Protein composition

2.6.1 | Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was used for protein composition analysis. Protein isolates were extracted at a 1:10 ratio (w:v) using a buffer containing 100 mM tris(hydroxymethyl)aminomethane (Tris) (pH 9.0) and 1% SDS at room temperature. After 30 min, the protein sample mixture was centrifuged at $15,000 \times g$ for 10 min using the Eppendorf Centrifuge 5418 (Eppendorf Canada) to collect the supernatant, which was then transferred to clean tubes. The protein extract was then diluted with water four times. At a 1:1 ratio, a sample buffer of β -mercaptoethanol (BME) pre-added 2 \times Laemmli sample buffer (Bio-Rad, 1610737, Bio-Rad Laboratories, Inc.) was added to the tubes to mix with the diluted protein extract. The mixture was heated at 95°C for 5 min. After protein denaturation, 10 μ L of the sample was loaded onto an 8%–16% Mini-PROTEAN[®] TGX[™] Precast Protein Gel (Bio-Rad, 4561105) along with a reference protein molecular weight (MW) ladder (Bio-Rad, 1610377). The gel was run for 30 min at 200 V for protein band separation. Coomassie R-250 stain (Bio-Rad, 1610400) was used to stain the gel, which was then visualized with a Gel Doc XR+ system (Bio-Rad Laboratories, Inc.).

2.6.2 | Capillary gel electrophoresis

Capillary gel electrophoresis (CGE) was performed to analyze the size distribution of protein bands. As described in Das et al. (2023), the sample was reduced using BME, followed by denaturation, before loading on to a

bare-fused silica capillary column (50 μm I.D. \times 30 cm) filled with SDS-MW gel separation buffer. Instrument setup for the model P/ACE MDQ plus electrophoresis system with UV detector and 32Karat software (AB Sciex Pte. Ltd) was based on the application guide (Sciex, RUO-IDV-05-6934-A) for the SDS-MW analysis kit (Sciex, 390953). Protein band separation was done at 15 kV for 35 min with 214 nm UV detection. The percent size distribution of MW ranges was calculated from the peak area data from different time periods for each sample.

2.7 | Protein functionality

2.7.1 | Protein solubility

Protein solubility was determined using the method of Stone et al. (2015). Protein solutions (1% w/w) were prepared in water and stirred for 1 h at room temperature and pH 7.0. The solutions were then centrifuged (4180 \times g for 10 min), and the supernatant was measured for protein content according to Association of Official Agricultural Chemists (AOAC) (2003) method 920.87 (N% \times 6.25) using a micro Kjeldahl unit (Labconco Corporation). The ratio of the supernatant protein content to that in the original solution was calculated as the protein solubility.

2.7.2 | Water- and oil-holding capacities

The water- and oil-holding capacities (WHC and OHC) were measured according to a modified method by Stone et al. (2015). Samples were prepared by mixing 0.5 g of protein in 5 mL of water or 5 g of oil in 50 mL centrifuge tubes and vortexing at maximum speed (9) for 10 s every 5 min, for a total of 30 min. The mixtures were then centrifuged at 3000 \times g for 15 min, and the supernatant was decanted. The pellet weight was recorded to calculate WHC or OHC (g/g) as shown in the following equation:

$$\text{WHC (or OHC)} = \frac{\text{wet pellet weight} - \text{dry sample weight}}{\text{dry sample weight}} \quad (2)$$

2.7.3 | Foaming capacity and foam stability

The foaming capacity (FC) and foam stability (FS) were measured according to Stone et al. (2015). Protein solutions of 1% (w/w) were prepared in water and adjusted to pH 7.0 during 1 h of stirring at room temperature. Fifteen milliliters of the solution were foamed using a homogenizer (IKA T10 basic ULTRA-TURRAX Homogenizer, S-10 N-10G probe; IKA Werke GmbH & Co. KG) at speed 2 for

3 min, after which the sample was immediately transferred to a graduated cylinder (100 mL) to measure the foam volume (V_{f0}) generated. The volume of foam was measured again after 30 min of undisturbed storage (V_{f30}). The FC (%) and FS (%) were then calculated as (Equations 3 and 4)

$$\text{FC} = \frac{V_{f0}}{15 \text{ mL}} \times 100 \quad (3)$$

$$\text{FS} = \frac{V_{f30}}{V_{f0}} \times 100 \quad (4)$$

2.7.4 | Emulsion stability

The emulsion stability (ES) was measured using a modified method of Stone et al. (2015) using protein solutions prepared as described for foaming. Five milliliters of the solution was transferred to a 50 mL centrifuge tube, followed by the addition of 5 g of canola oil. The mixture was emulsified using the same homogenizer conditions as described for foaming. The generated emulsion was poured into a 10 mL graduated cylinder and left undisturbed for 30 min. The volume of the aqueous layer (V_A) at the cylinder bottom was recorded after 30 min to calculate ES (Equation 5) as

$$\% \text{ES} = (5 \text{ mL} - V_A) / 5 \text{ mL} \times 100 \quad (5)$$

2.8 | Nutritional properties

2.8.1 | Trypsin inhibitor activity

The trypsin inhibitor activity (TIA) was measured according to the American Oil Chemists' Society (AOCS) Method Ba 12a-2020 (Liu et al., 2021). In brief, 1 g of each sample was extracted using 50 mL of 10 mM NaOH for a duration of 3 h. The resulting extract was then diluted to a concentration that would induce trypsin inhibition falling within the range of 30%–70%. Glass culture tubes (13 mm) were set up in a heat block pre-warmed to 37°C (three tubes per sample), and in each tube, 1 mL of the sample extract dilution was added. Additionally, a set of tubes containing 1 mL of distilled water was prepared to serve as reference samples for spectrophotometer readings. To each tube, 2.5 mL of a substrate solution pre-warmed to 37°C, containing Tris, CaCl_2 , and Na-benzoyl-D, L-arginine 4-nitroanilide hydrochloride (DL-BAPNA), was added, and the contents were mixed by vortexing. Of the three tubes created for each sample, one was designated as the blank and the other two as readings. Then 0.5 mL of 30% (v/v) glacial acetic acid was added to each of the blank tubes and

vortexed. Exactly 10 min later, 1.0 mL of trypsin solution (containing 20 µg/mL trypsin in HCl and CaCl₂ solutions) was added to each blank tube and vortexed. Concurrently, 1.0 mL of trypsin solution was added to each of the reading tubes and vortexed to mix. Exactly 10 min later, 0.5 mL of acetic acid solution was added to each sample and vortexed to terminate the reactions. A 1.2 mL aliquot of each reaction mixture was then centrifuged for 10 min at 10,000 rpm. The supernatant was measured for absorbance at 410 nm to calculate the TIA (TIU/mg sample) as

$$\text{TIU} = \frac{\{(A_{410R} - A_{410RB}) - (A_{410S} - A_{410SB})\} \times 50}{\text{mg sample in the assay}} \quad (6)$$

where TIU is the trypsin inhibitor unit(s), A_{410R} = is the reference reading, A_{410RB} = is the reference blank, A_{410S} = is the sample reading, and A_{410SB} = is the sample blank.

2.8.2 | Total phenolic compounds

Each protein isolate was quantified for total phenolic compounds (TPCs) using the method of Attard (2013) with modifications. Protein isolates of 50 mg were extracted for phenolic compounds using 500 µL of 80% (v/v) aqueous methanol at room temperature (21–23°C) in capped centrifuge tubes. The tubes were placed on a shaker for 60 min then centrifuged (Eppendorf Centrifuge 5417R) at 17,900 × g for 10 min to collect the supernatant. A 1:10 (v/v) Folin–Ciocalteu solution was prepared using the Folin–Ciocalteu reagent (cat# 47641, Sigma-Aldrich Co.) and distilled water. Supernatant of 20 µL was mixed with 100 µL of the diluted Folin–Ciocalteu reagent and 80 µL of 1 M sodium bicarbonate aqueous solution. A reagent blank was prepared as 20 µL 80% (v/v) aqueous methanol. The absorbance of the sample mixture was measured after 15 min at 765 nm (BioTeK Synergy H1 Hybrid Reader, Agilent Technologies). To prepare the calibration curve, serial dilutions of 250, 125, 62.5, 31.25, and 15.63 µg/mL were made from 1 mg/mL of gallic acid in 80% (v/v) aqueous methanol stock solutions. The TPC was calculated as mg of gallic acid equivalent (GAE)/g of isolate.

2.8.3 | Total saponins

Total saponin content was determined using a modified procedure as described by Le et al. (2018). Briefly, 50 mg of protein isolate was extracted with 500 µL of 80% aqueous methanol in a 1.5 mL tube placed on a shaker for 60 min at room temperature and then centrifuged (Eppendorf Centrifuge 5417R) at 17,900 × g for 10 min to collect the supernatant for saponin analysis. The reaction was carried

out in 96-well plate (VWR, Cat #10062-900). In each well of the plate, 30 µL of saponin extract was mixed with 15 µL of 8% vanillin solution in ethanol (w/v) and 170 µL of 50% sulfuric acid. After incubation of the plate for 30 min at 55°C, the absorbance of the mixture was measured at 540 nm using plate reader (BioTeK Synergy H1 Hybrid Reader, Agilent Technologies). The total saponin content was calculated as milligram aescin equivalents (AE/g) of tissue on the basis of a standard curve consisting five concentrations of aescin (15, 7.5, 3.75, 1.87, and 0.94 mg/mL).

2.8.4 | In vitro protein digestibility

The in vitro protein digestibility (IVPD) was measured according to Tinus et al. (2012). Protein solutions were prepared by dissolving 62.5 ± 0.5 mg of protein in 10 mL of water. A multi-enzyme solution consisting of 31 mg of chymotrypsin (bovine pancreas), 16 mg of trypsin (porcine pancreas), and 13 mg of protease (*Streptomyces griseus*) was prepared in 10 mL of water. The solutions (both enzyme and protein) were adjusted to pH 8.0 and stirred for 1 h in a 37°C water bath. The pH-drop method was utilized where, at 37°C, 1 mL of the enzyme solution was added to each protein solution while stirring, and the pH was recorded every 1 min for a total of 10 min. The IVPD was calculated as shown in the following equation, where $\Delta\text{pH}_{10\text{min}}$ is the pH difference between time 0 and 10 min:

$$\text{IVPD} (\%) = 65.66 + 18.10 \times \Delta\text{pH}_{10\text{min}} \quad (7)$$

2.9 | Volatile profiling

HS-SPME–GC/MS (headspace solid-phase microextraction coupled to gas chromatography–mass spectrometry) (Agilent 6890 GC/5973 MSD, PAL RSI 85 autosampler; Agilent Technologies) was used to analyze the volatile profile of each sample. Samples (2 g) were weighed into HS clear glass vials (20 mL; Chromatographic Specialties). A blank was prepared by substituting the sample with the same amount of NaCl (2 g). The vials were stored at 4°C and brought to room temperature (21–23°C) for 1 h before sample extraction. Samples were extracted by SPME fiber in an Agitator at 50°C for 45 min (Azarnia et al., 2011). The SPME carboxen/Polydimethylsiloxane fiber assembly (Supelco) was conditioned at 285°C for 3 min before the extraction. The GC front inlet was set as pulsed splitless mode. The SPME fiber desorption was performed at 285°C for 0.3 min, followed by elution starting 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

TABLE 1 Physicochemical properties of control, spontaneous fermented, and inoculated lentil protein isolates.

Sample	Fermentation temperature (°C)	Degree of hydrolysis (%)	Moisture (%)	Protein (%) (d.b.)	Surface charge (mV)	Surface hydrophobicity (a.u.)
0-h control	-	0.7 ± 0.0 ^d	2.9 ± 0.1 ^c	85.5 ± 0.3 ^c	-32.1 ± 0.4 ^{bc}	64.4 ± 10.5 ^c
48-h control	30	9.2 ± 0.3 ^{bc}	3.8 ± 0.2 ^b	88.7 ± 0.5 ^b	-32.3 ± 1.8 ^c	66.1 ± 5.0 ^c
48-h control	37	10.5 ± 0.7 ^{ab}	3.7 ± 0.1 ^b	88.5 ± 0.7 ^b	-33.1 ± 0.6 ^c	54.4 ± 1.8 ^c
48-h <i>Aspergillus oryzae</i>	30	10.7 ± 0.6 ^a	5.2 ± 0.1 ^a	87.8 ± 1.0 ^b	-26.4 ± 1.1 ^a	132.8 ± 11.8 ^b
48-h <i>Aspergillus niger</i>	30	10.6 ± 0.6 ^a	5.3 ± 0.3 ^a	88.8 ± 0.6 ^b	-26.9 ± 1.0 ^a	167.6 ± 15.1 ^a
48-h <i>Lactobacillus plantarum</i>	37	8.9 ± 0.4 ^c	5.5 ± 0.4 ^a	91.6 ± 0.5 ^a	-29.3 ± 1.0 ^{ab}	137.8 ± 6.3 ^b

Note: Data with the same superscript letters within a column are not significantly different ($p > 0.05$). Abbreviations: a.u., arbitrary units; d.b., dry basis.

3 min, increased to 80°C at 6°C/min, ramped up to 280°C at 20°C/min, and then 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 70.3 eV of fixed electron energy. Volatile compound determination was done using mass spectra library search (National Institute of Standards and Technology [NIST] database [20.L], Agilent Mass Hunter software; Agilent Technologies).

2.10 | Statistical analysis

For each inoculated isolate, fermentation reactions were done in triplicate ($n = 3$), and all measurements were made in duplicate. A one-way ANOVA with Tukey's post hoc test was performed to identify significant differences between samples ($p < 0.05$), and a simple Pearson correlation analysis was used to determine relevant correlations (SPSS software, IBM SPSS Statistics 28.0).

3 | RESULTS AND DISCUSSION

3.1 | Degree of hydrolysis

Lentil protein fermentation by *A. oryzae* and *A. niger* at 30°C achieved ~11% DH after 48 h whereas that by *L. plantarum* at 37°C was significantly ($p < 0.05$) lower at ~9% (Table 1). Spontaneous fermentation at 30°C resulted in a lower DH (~9%) than the *A.* strains; however, at 37°C, protein hydrolysis (~11%) was more intensive than that in the *L. plantarum*-inoculated sample. A higher proteolytic activity of natural microbial flora than that of *L. plantarum*, shown by the greater release of free amino acids at 37°C, was also observed in the study of Torino et al. (2013) for liquid-state fermented (i.e., SmF) lentil flours. The DH after 48 h in the present study was lower than that reported by Çabuk, Stone et al. (2018) for pea protein-enriched flour

(PPEF; approximately 40% protein, dry basis [d.b.]) fermented for 11 h using *L. plantarum* (SmF). In their study, a DH of 13.5% was achieved after 11 h of fermentation, whereas a DH of 9.7% was obtained at only 5 h; however, a higher initial concentration of substrate was used (25% vs. 10%). In another study with a similar experimental design (PPEF, *L. plantarum* SmF), a 10% DH of PPEF was observed at 24 h, and further incubation to 48 h resulted in a DH of >20% (Batbayar et al., 2023). The major factor responsible for this difference could be the starting materials. Protein concentrates with <50% protein (d.b.) were used in both studies, and because they are relatively abundant in starch (readily utilizable for the microorganisms), a stronger and earlier onset of microbial growth could be anticipated compared to using LPI (>85% protein) in the present work (Table 1). Naturally, crop differences should also be accounted for as lentil protein may be more resistant to proteolytic processes than pea. As all fermented samples had DH below or around 10%, a level often categorized as limited hydrolysis (Polanco-Lugo et al., 2014), improvements in protein functionality can be expected due to the exposure of reactive groups without compromising the protein structures.

3.2 | Protein content

The 0 h control had a protein content of 85.5% on a moisture-free basis, which was significantly increased by ~2%–7% after 48 h of fermentation (Table 1). Similarly, the natural fermentation of lentil flours by Tabera et al. (1995) induced slight (~4%) but statistically significant increases in their protein content over a period of 4 days. Comparing among treatments, spontaneous fermentation and fungal inoculation yielded LPI with similar levels of protein at ~88%–89%, whereas the *L. plantarum*-inoculated isolate had the highest protein content of >90%. Microorganisms during fermentation utilize nutrients, of which

carbohydrates are the major energy source, by secreting extracellular enzymes. The increase in microbial biomass is also another factor contributing to the enhanced overall protein level in the LPI. An increase in pea protein content by ~8% was observed by Çabuk, Stone et al. (2018) coupled with a noticeably reduced crude carbohydrate level (from 53% to 37%) when fermenting PPEF using *L. plantarum* for 11 h. SmF, as opposed to solid-state fermentation (SSF), favors the growth of bacteria over fungi due to the high moisture requirement of the former (Subramaniam & Vimala, 2012). As reported by Batbayar et al. (2023), to achieve the same level of DH (10%), fermentation was prolonged by 24 h for *A. oryzae* compared to that for *L. plantarum*. Accordingly, *L. plantarum* may show better ecological adaptability and therefore a higher carbohydrate (starch) utilization/conversion rate under the SmF conditions used in this study than the fungal strains. This explains the greater magnitude of increase in levels of protein in the *L. plantarum*-inoculated isolate, despite the significantly lower DH compared to that of the fungi-inoculated samples (Table 1).

3.3 | Surface properties

3.3.1 | Surface charge

The surface charge of the 0 h control LPI was -32.1 mV, a value that was not changed following 48 h of spontaneous fermentation at either temperature (Table 1). Inoculation significantly ($p < 0.05$) reduced the magnitude of the negative charge (absolute values becoming smaller) to approximately -26 and -29 mV for the fungi and the bacterium, respectively, with no difference between the strains. However, despite the overall decreasing trend after fermentation, the zeta potential of *L. plantarum*-inoculated LPI was not statistically different from that of the 0 h control, illustrating this strain's ability to maintain high electrostatic repulsion in the protein isolate. Fermentation following inoculation likely promoted cleavages at the protein surface, leading to a portion of the charges being buried and the exposure of previously hidden hydrophobic sites, reducing the overall charge density. Because only the inoculated samples showed such changes while the DH of all fermented samples remained quite similar, it can be speculated that the test strains had different metabolic preferences than those native organisms initially present in raw LPI. A similar decrease in charge density after fermentation (11 h, 13.5% DH) was reported in Çabuk, Stone et al. (2018) for PPEF by *L. plantarum*. The PPEF, however, gained greater surface charge (from -15 to -19 mV) in the study of Batbayar et al. (2023) at 10% DH

following SmF by *A. oryzae* and *L. plantarum*, indicating that modifications to surface charge profiles may also be system-dependent.

3.3.2 | Surface hydrophobicity

Surface hydrophobicity followed a similar trend to the surface charge profile in that spontaneous fermentation had no influence on the hydrophobicity values (Table 1). The initial measurement was ~ 64 a.u. for the 0 h control which more than doubled to ~ 135 a.u. upon inoculation with and 48-h fermentation by *A. oryzae* and *L. plantarum*. *A. niger* induced an even larger change in surface hydrophobicity as this sample achieved the highest value out of all the samples (~ 168 a.u.). Contrary to these results, reduced surface hydrophobicity after SmF by *A. oryzae* and *L. plantarum* was reported by Batbayar et al. (2023) and Çabuk, Stone et al. (2018). SSF by *A. oryzae* and *A. niger* in the study of Kumitch et al. (2020) also resulted in lower surface hydrophobicity values for PPEF (6 h), whereas the property was enhanced for fermented (SSF, *A. oryzae*) protein isolates of pea and navy bean (up to 24 and at 72 h, respectively) in the work of Khorsandi (2022). As with surface charge, fungal growth and metabolism during fermentation can be dependent on fermentation parameters. Interchange of fungal hyphal surface properties (hydrophilic and hydrophobic) is possible over time during adaptation and a period of metabolism by the microbes (Chau et al., 2009). Mycelial growth of filamentous fungi in submerged cultures has been shown to vary in response to a number of factors, of which medium composition is a strong determinant (Gibbs et al., 2000). The composition of macronutrients (e.g., availability of sugars), proteins (e.g., ease of/resistance to hydrolysis), and micronutrients (e.g., availability of trace metals) all affect fungal growth and the production of metabolites, which in turn deliver varied functionality of the resulting protein ingredients. The lack of functional modifications of navy bean protein isolate after SSF compared to that of pea was reported by Khorsandi (2022) due to the resistance of phaseolins to protein hydrolysis. Likewise, lentil protein has a high ratio of *7S/11S*, which is markedly higher (~ 3 -fold) compared to that of pea (Scippa et al., 2010). This could significantly affect the metabolism of microorganisms when used as fermenting substrate; however, detailed structure-functional analysis would be required to draw conclusions as protein composition is also closely related to the cultivar used. The increases in surface hydrophobicity correlated well with the decreases in absolute surface charge (Table 1) ($r = -0.882$; $p < 0.001$), supporting the previous hypothesis of protein hydrolysis and surface property modifications.

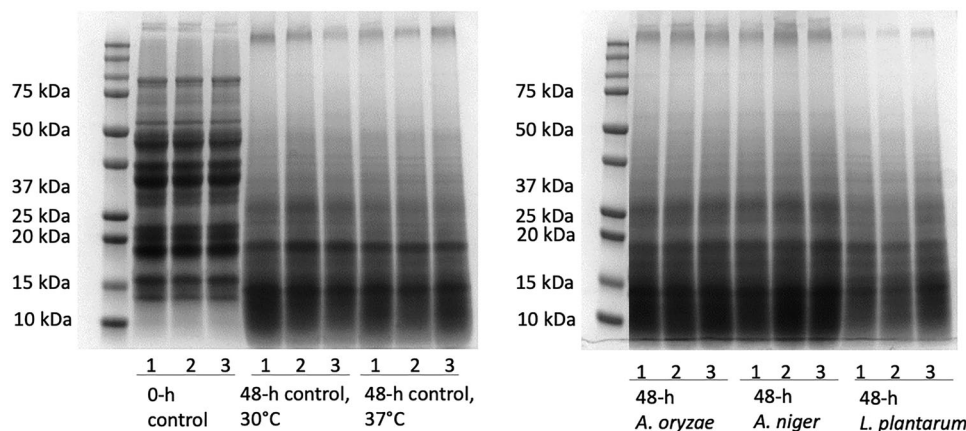


FIGURE 1 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of control, spontaneous fermented (left), and inoculated (right) lentil protein isolates. *Aspergillus oryzae* and *Aspergillus niger* fermentation occurred at 30°C; *Lactobacillus plantarum* fermentation occurred at 37°C.

3.4 | Protein composition

3.4.1 | Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SDS–PAGE analysis of protein composition for the LPIs is presented in Figure 1. Compared to the 0 h control, the 48 h controls (spontaneous fermentation) at both 30 and 37°C showed a reduction in intensity (or complete disappearance) of higher MW bands and a corresponding increase in bands of lower MW. Specifically, bands representing major storage proteins in pulses, that is, legumins at ~40 and ~20 kDa and vicilins at ~50 kDa, were significantly decreased in concentration, leading to the generation of new bands of ~25 and <15 kDa (Boeck et al., 2021). Bands of <20 kDa, which likely represent albumins, were also reduced (Boeck et al., 2021). Proteolytic activities during fermentation are responsible for this breakdown of large protein units into smaller ones. A similar trend was also observed in inoculated samples with the three different microbial strains. Comparing among treatments, protein bands were slightly more intense in samples fermented at 30°C than those at 37°C, especially at ~25 kDa, regardless of the fermentation type (spontaneous fermentation vs. inoculation). No significant visual difference in protein bands was observed among the isolates inoculated with *Aspergillus* strains. Byanju et al. (2021) reported similar findings where *L. plantarum* fermentation of 72 h resulted in notable alterations to the protein composition of lentil flours with reduced band intensity at MW of >36 kDa. Fermented (SSF) PPEF by *Aspergillus* strains in the study of Kumitch et al. (2020) also showed reduced band concentration at ~70 kDa and concurrent increases at ~17–23 kDa as early as 4 h in the process.

3.4.2 | Capillary gel electrophoresis

The CGE analysis of the protein isolates is presented in Figures 2 and 3. Similar to findings from SDS–PAGE, at both temperatures, all the incubated samples (both the uninoculated and spore/bacterial inoculated) showed a left-shift of peaks from the 0 h controls, indicating protein hydrolysis. CGE size distribution profile (Figure 3) confirmed that medium-to-high MW (15–30, 30–45, and >45 kDa) proteins were hydrolyzed, generating smaller protein molecules of >15 kDa. A number of proteins larger than 45 kDa were reduced by one-third, and that for proteins at 30–45 kDa were more profound, from ~20% to ~6%–7%. The decrease in percentage at 15–30 kDa was relatively smaller, from ~30% to ~22%–26%, as hydrolyzed proteins from higher MW compensated for this reduction. The concentration of <15 kDa proteins more than doubled from <20% to ~50%, correlating to the dark background smear of this area on SDS–PAGE gels (Figure 1). No notable difference was observed between treatments (i.e., fermentation type, temperature, and strain). The slight variation among samples shown on SDS–PAGE gels may be specific to the test.

3.5 | Protein functionality

3.5.1 | Solubility

The solubility of lentil proteins in the 0 h control was low at 9.8%, as expected for high-purity commercial plant protein ingredients due to their inherent structural arrangement and composition and heat/chemical denaturation due to processing (Bai et al., 2016; Sá et al., 2022). SmF

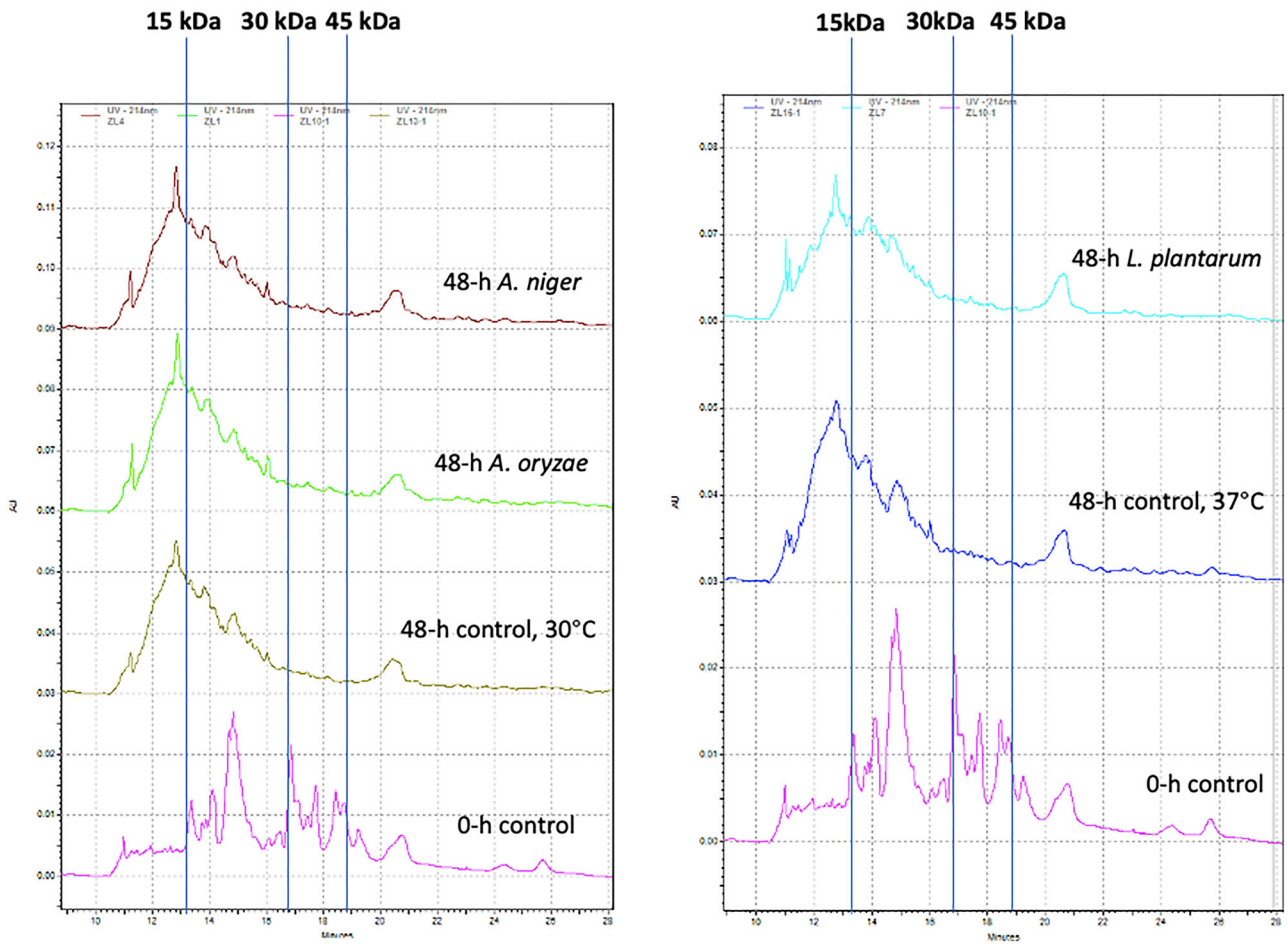


FIGURE 2 Capillary gel electrophoresis (CGE) analysis of control, spontaneous fermented, and inoculated lentil protein isolates processed at 30°C (left) and 37°C (right).

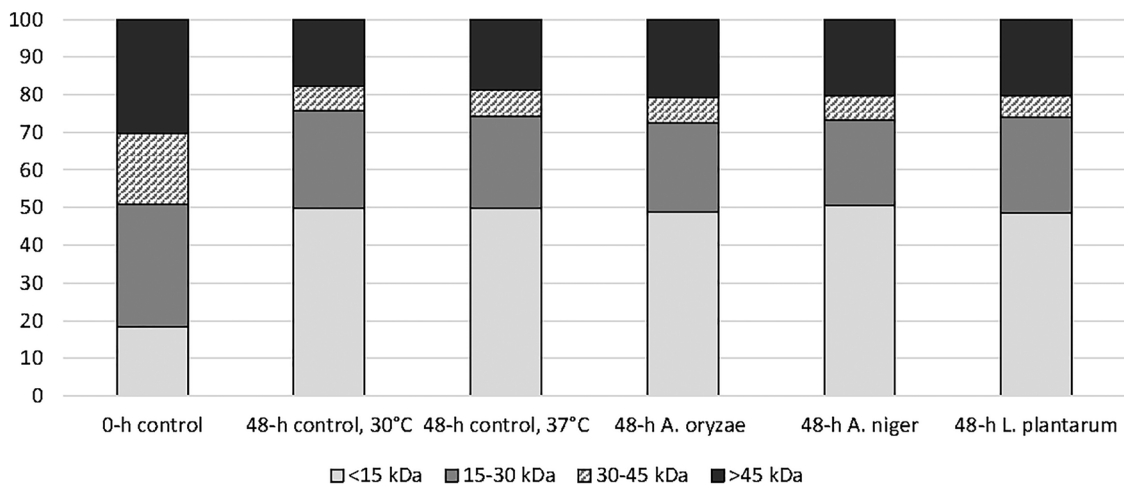


FIGURE 3 Capillary gel electrophoresis (CGE) size distribution profile of control, spontaneous fermented, and inoculated lentil protein isolates.

TABLE 2 Functional properties of control, spontaneous fermented, and inoculated lentil protein isolates.

Sample	Fermentation temperature (°C)	Solubility (%)	Water-holding capacity (g/g)	Oil-holding capacity (g/g)	Foaming capacity (%)	Foam stability (%)	Emulsion stability (%)
0-h control	-	9.8 ± 1.1 ^e	1.8 ± 0.0 ^a	0.7 ± 0.0 ^c	191.1 ± 28.0 ^a	21.3 ± 2.9 ^a	91.7 ± 1.5 ^a
48-h control	30	35.3 ± 2.2 ^{cd}	1.3 ± 0.0 ^b	0.9 ± 0.1 ^b	137.2 ± 17.8 ^b	19.1 ± 4.4 ^a	75.3 ± 2.3 ^b
48-h control	37	38.7 ± 1.6 ^{bc}	1.3 ± 0.1 ^b	0.7 ± 0.0 ^c	135.0 ± 1.7 ^b	13.6 ± 3.3 ^{ab}	68.3 ± 1.2 ^{bc}
48-h <i>Aspergillus oryzae</i>	30	44.8 ± 2.9 ^a	1.1 ± 0.0 ^c	1.0 ± 0.1 ^{ab}	196.7 ± 7.3 ^a	9.4 ± 2.4 ^b	72.8 ± 2.3 ^{bc}
48-h <i>Aspergillus Niger</i>	30	43.5 ± 0.5 ^{ab}	1.1 ± 0.1 ^c	1.1 ± 0.0 ^a	175.0 ± 6.7 ^{ab}	9.8 ± 2.8 ^b	75.3 ± 5.0 ^b
48-h <i>Lactobacillus plantarum</i>	37	32.8 ± 2.1 ^d	1.3 ± 0.0 ^b	0.7 ± 0.0 ^c	156.7 ± 18.8 ^{ab}	9.4 ± 1.6 ^b	67.3 ± 3.2 ^c

Note: Data with the same superscript letters within a column are not significantly different ($p > 0.05$).

significantly ($p < 0.05$) enhanced solubility to $>30\%$ upon all treatments (Table 2). A similar trend to that observed for DH was found for solubility, where *A. oryzae* and *A. niger* induced greater changes than for *L. plantarum*, to approximately 44% and 33%, respectively. Compared to the spontaneous fermented control at 37°C, the *L. plantarum*-inoculated LPI had lower solubility, whereas those with *A. oryzae* and *A. niger* were higher than the spontaneous fermented control at 30°C. As surface charge decreased upon fermentation, it is hypothesized that protein particle size reduction resulting from hydrolysis played a more important role than changes in charge repulsion in determining the aqueous dispersibility of the lentil protein. This can be further supported by SDS-PAGE and CGE data (Figures 1–3), where smaller MW proteins were generated from the breakdown of larger molecules by fermentation, resulting in higher solubility. The lower solubility of *L. plantarum*-inoculated LPI compared to other strains may explain its fainter protein bands on the SDS-PAGE gel (Figure 1). Likely, a smaller number of proteins were solubilized to become available for extraction, leading to low band intensity. Literature regarding the effect of fermentation on the solubility profile of lentil protein has been limited, whereas that for other pulses (mainly pea) is available. SSF (6 h) of PPEF by *Aspergillus* strains in the study of Kumitch et al. (2020) caused a general decreasing trend for protein solubility at pH 3.0, 5.0, and 7.0. A similar PPEF fermented by *L. plantarum* (11 h) in Çabuk, Stone et al. (2018) also showed reduced solubility values at pH 7.0, whereas those at pH 4.0 remained unchanged. The authors attributed the loss of solubility to the aggregation of hydrolyzed proteins due to the newly exposed hydrophobic patches (Kumitch et al., 2020) and the insoluble nature of microbial biomass generated during fermentation (Çabuk, Stone et al., 2018). In the present study, protein size reduction may be a stronger influencing factor, as small MW (<15 kDa) proteins account

for ~50% of total protein after SmF (Figure 3), significantly higher than that reported by Kumitch et al. (2020) (~15%–20%). Prolonged fermentation period (48 h) in the present study likely favored the production of these small MW proteins/peptides, overcoming the effects of reduced charge and increased hydrophobicity (Table 1) and enhancing solubility, despite yielding similar DH values (~10%) compared to those in Kumitch et al. (2020). Naturally, crop differences among pulses can also affect the outcome as lentil protein may react differently to the proteolytic activities of these strains compared to pea. The three- to fivefold improved solubility resulting from the current processing protocols, although not high enough for liquid formulations such as plant-based milks/drinks, suggests great potential of the technique to significantly modify protein functionalities, particularly using *Aspergillus* strains.

3.5.2 | Water- and oil-holding capacities

The WHC was reduced significantly ($p < 0.05$) from 1.8 g/g for the 0 h control to 1.3 g/g upon spontaneous fermentation or with the inoculation of *L. plantarum* (Table 2). An even greater decrease to 1.1 g/g was observed for WHC for the fungal strains. In contrast, the OHC was enhanced after fermenting at 30°C from 0.7 to 0.9 g/g by the indigenous microorganisms and to 1.0–1.1 g/g by the fungal strains. There was no change in OHC for samples fermented at 37°C by either spontaneous fermentation or *L. plantarum* inoculation. Naturally-fermented lentil flours in Sadowska et al. (1999) had higher water hydration and oil-binding capacities than non-fermented flour, irrespective of fermentation temperature and flour-to-water ratios. The authors attributed the change to the swelling of starch and protein molecules in water, leading to more opened flour particles absorbing more water/oil. The

diminished WHC in the present research can be partially associated with the exposed hydrophobic regions, given that only the inoculated samples showed increases in surface hydrophobicity and the two properties were negatively correlated ($r = -0.617$; $p < 0.01$). Enhanced protein solubility after SmF (Table 2) is most likely the primary driver for reduced WHC ($r = -0.961$; $p < 0.001$). With more protein particles being solubilized in water and centrifuged away, fewer remain to bind and retain water molecules via protein–water interactions or the formation of microcapillaries for physical entrapment. As for OHC, the proteins can show improved ability to interact with nonpolar oil droplets due to the enhanced hydrophobicity ($r = 0.637$; $p < 0.01$) and reduction in surface charge ($r = -0.708$; $p < 0.01$). Enhanced WHC after fermentation was observed for flours from pea, chickpea, black eye pea, and African oil bean, whereas decreased values were found for lupin and pigeon pea flours (Adebowale & Maliki, 2011; Akubor & Chukwu, 1999; Chawla et al., 2017; Kumitch et al., 2020; Olukomaiya et al., 2020; Xiao et al., 2015). Similarly, OHC increased for fermented chickpea, faba bean, pea, soy, and lupin and decreased for Mucuna bean (Chandra-Hioe et al., 2016; Kumitch et al., 2020; Olukomaiya et al., 2020; Razavizadeh et al., 2022; Udensi & Okoronkwo, 2006). In short, the protein functionality of pulses is highly specific to pulse type, substrate composition, and processing and test parameters utilized. The current findings indicate that *Aspergillus* strains may be better candidates for improving the mouthfeel (by enhancing OHC) of fermented lentil protein ingredient-based formulations.

3.5.3 | Foaming capacity and foam stability

Varied effects of SmF were observed between FC and FS (Table 2). For FC, spontaneous fermentation, regardless of temperature, significantly ($p < 0.05$) reduced the values from 191% to ~136%, while inoculation did not induce any significant changes to FC. The FS was initially low at only ~21%; however, this was further decreased by fermentation by each of the three strains to ~9%–10%. The fermenting activities of the indigenous LPI microbes did not significantly influence this attribute. The stability of the foam was expected to improve with fermentation as surface charge density decreased and hydrophobicity increased (Table 1), which was hypothesized to result in a more compact protective film surrounding the air bubble. However, because the lentil proteins became three- to five-fold more soluble as compared to the 0 h control, protein films at the air–water interface may lack sufficient rigidity to prevent bubble collapse. The effect of fermentation on the foaming properties of lentil protein has not been reported, whereas that from enzyme hydrolysis is available. FC and FS of

lentil protein concentrate examined in Rezvankhah et al. (2021) had similar changes to the present findings. In their study, FC was maintained at ~80% after 180 min of hydrolysis by 2% Alcalase (20% DH) and Flavourzyme (8.5% DH) separately and was enhanced when treated with 1% Alcalase (15.7% DH) as well as combining both enzymes at 2% (47% DH). FS after 60 min, regardless of treatment, was reduced by ~4%–30%. According to the authors, a high DH could be linked to impaired foaming properties as the result of elevated solubility. Similar decreases in foaming properties were also reported for pea (PPEF) by Kumitch et al. (2020) (*A. oryzae* and *A. niger* SSF, ~10% DH) and Batbayar et al. (2023) (*A. oryzae* and *L. plantarum* SSF and SmF, 10% DH). Loss of high and medium MW proteins after fermentation (as indicated in Figures 1–3) may also compromise protein structure and contribute to reduced foaming.

3.5.4 | Emulsion stability

The ES was notably reduced by SmF by both indigenous and inoculated strains from >90% to ~67%–75% (Table 2). The differences in ES among most fermented samples were statistically insignificant ($p > 0.05$). It was expected that limited hydrolysis of proteins induced by fermentation would enhance ES; however, the lentil proteins did not show any improvement in this attribute. The process of breaking open the protein structure favored solubility and OHC as previously discussed; however, these factors may adversely impact film properties when it comes to foaming and emulsifying, namely, film thickness and viscoelasticity. Although smaller peptides migrate faster to the oil–water interface, their low ability to reduce the interfacial tension and stabilize droplets could be detrimental to emulsifying properties (Zhao et al., 2012). The surface charges being overall less negative may also contribute to reduced ES due to weakened electrostatic repulsion; however, this does not apply to those naturally fermented protein samples with unchanged charge profiles. Naturally fermented lentil flours in Sadowska et al. (1999) also showed reduced emulsifying properties, some of which did not even form an emulsion. Similarly, at pH 7.0, SmF of PPEF by *A. oryzae* and *L. plantarum* to a 10% DH significantly lowered the ES from ~87% to ~39% and ~26%, respectively, as reported by Batbayar et al. (2023). Interestingly, as opposed to SmF, the SSF preparation of the same set of samples was able to maintain high ES with slightly increased values from ~87% to >92%. However, the authors did not address the reason behind this phenomenon. Çabuk, Stone et al. (2018) presented SmF data with initially increasing ES for *L. plantarum*-inoculated PPEF after 5 h followed by reductions till reaching 11 h

TABLE 3 Select antinutrients and protein digestibility of control, spontaneous fermented, and inoculated lentil protein isolates.

Sample	Fermentation temperature (°C)	Trypsin inhibitor activity (TIU/mg)	Total phenolic compounds (mg GAE/g)	Total saponins (mg AE/g)	In vitro protein digestibility (%)
0-h control	-	1.7 ± 0.1 ^a	0.5 ± 0.0 ^c	42.7 ± 5.0 ^c	89.6 ± 0.9 ^a
48-h control	30	1.7 ± 0.2 ^a	1.1 ± 0.1 ^{ab}	48.6 ± 7.5 ^c	80.1 ± 0.6 ^{bc}
48-h control	37	1.5 ± 0.1 ^a	1.0 ± 0.1 ^b	49.6 ± 1.0 ^{bc}	79.3 ± 0.5 ^{bcd}
48-h <i>Aspergillus oryzae</i>	30	1.8 ± 0.1 ^a	1.3 ± 0.1 ^a	65.1 ± 2.5 ^a	78.7 ± 0.8 ^{cd}
48-h <i>Aspergillus niger</i>	30	1.8 ± 0.2 ^a	1.3 ± 0.2 ^a	61.2 ± 5.6 ^{ab}	77.6 ± 0.8 ^d
48-h <i>Lactobacillus plantarum</i>	37	1.6 ± 0.0 ^a	1.1 ± 0.0 ^{ab}	51.8 ± 0.5 ^{bc}	81.3 ± 0.8 ^b

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

Abbreviation: GAE, gallic acid equivalent.

(to a level lower than the control), whereas Kumitch et al. (2020) reported unchanged high ES by SSF using *A. oryzae* and *A. niger*. It can be speculated that the fermentation medium somewhat affected the outcome of fermentation, even with similar substrate materials (sourced from the same company for all three studies).

3.6 | Nutritional properties

3.6.1 | Trypsin inhibitor activity

Protease inhibitors form complexes with enzymes by mimicking the structure of protein substrates and subsequently reducing the overall protein digestibility of pulses. Therefore, reducing protease inhibitor activity through processing is important for enhancing pulse utilization. However, the present SmF processing, regardless of temperature or strain treatment, did not induce significant ($p < 0.05$) changes in TIA in LPI (Table 3) from that of the control (1.7 TIU/mg). All samples had similar TIA (1.5–1.8 TIU/mg). Vidal-Valverde et al. (1993) and Wagh et al. (2021) reported decreased TIA of fermented (natural—4 days and *Lactobacilli* inoculated—3 days, respectively) lentils; however, such an effect was not observed by Byanju et al. (2021) with *L. plantarum* fermentation (3 days). A similar discrepancy among studies was also present for faba bean, where *Lactobacilli* fermentation was effective in lowering TIA in a study by Coda et al. (2015) but not in work by Chandra-Hioe et al. (2016). It is generally believed that the microbial degradation of the inhibitors is responsible for their decreasing contents after fermentation. In contrast, Wang et al. (1972) suggested that the release of trypsin inhibitors from the bound, inactive form, by microbial proteases of *Rhizopus oligosporus* may be responsible for the elevated levels of TIA in fermented

soybeans. Although no data is available on lentils fermented using the present test strains, the insignificant changes in TIA may be attributed to a counter effect resulting from the partial trypsin inhibitor degradation coupled with a concurrent release of those inhibitors that were previously bound due to the proteolytic activities of the microorganisms.

3.6.2 | Total phenolic compounds

Phenolic compounds in pulses possess strong antioxidant properties owing to the presence of hydroxyl (OH) groups in their chemical structure, which serve to neutralize free radicals and prevent oxidative damage (Kiokias et al., 2008). However, from the perspective of protein quality, phenolic compounds have a high binding affinity toward proteins and can form insoluble complexes that reduce protein digestibility (Cirkovic Velickovic & Stanic-Vucinic, 2018). The TPC content (Table 3) was 0.5 mg GAE/g in the 0 h control and doubled ($p < 0.05$) in the 48-h spontaneous fermented controls at both temperatures. As for controlled fermentation, inoculation increased the TPC (1.1–1.3 mg GAE/g), with no differences among the three microorganisms. Natural SmF led to a general increase in TPC content of lentil flour in Bartolomé et al. (1997), whereas Tabera et al. (1995) reported doubled tannin levels, similar to the present finding for the 48 h controls. It is well established that during fermentation, fungi and bacteria produce various active hydrolytic enzymes, for example, carbohydrases and β -glucosidases, releasing bound phenolic compounds from cell wall matrices and further breaking down these compounds into free, more active derivatives (Duenas et al., 2005; Esteban-Torres et al., 2015). Regarding inoculated fermentation, reported modulation of TPC content in lentils has mixed results in

the literature. For example, Dhull et al. (2020) reported TPC of *Aspergillus*-fermented (SSF) lentil to increase by 78.8%–122.8% with the formation of new phenolic compounds (resorcinol and cinnamic acid). On the contrary, total identified phenolics in Bautista-Expósito et al. (2018) decreased from approximately 1.3 to 0.9 mg/g after alkaline pH-controlled *L. plantarum* fermentation (SmF) of lentil flour; the conversion among phenolic compounds was evident from the analysis of individual phenolic fractions. In addition to strain type, variation in the results between the two studies may have arisen from differences in experimental settings where water-soluble phenolics can leach into fermentation medium when a large quantity of water is present (i.e., SmF). According to Vattem et al. (2004), depletion of nutrients (e.g., starch) may induce stress responses in microorganisms, triggering the production of oxidative enzymes to polymerize the released free phenolics.

3.6.3 | Total saponins

Like phenolics, saponins are a class of naturally occurring compounds in plants with anti-inflammatory and antioxidant properties; however, when consumed in large quantity, they can lead to adverse effects (Francis et al., 2002). Saponins precipitate proteins and reduce their bioavailability while also affecting the absorption of certain minerals and vitamins in the human body. The content of total saponins was 42.7 mg AE/g for the 0 h control and was not significantly affected by spontaneous fermentation (~49–50 mg AE/g) or fermentation with *L. plantarum* (51.8 mg AE/g) ($p > 0.05$). *Aspergillus* strain inoculation, however, significantly ($p < 0.05$) increased the total saponins to ~61–65 mg AE/g. There is limited information on the effect of fermentation on the saponin content of lentils, whereas that for other legumes is more available. Lactic acid bacteria (LAB) fermentation of yellow and red lentil flours was effective in reducing saponin content, more so than by germination (De Pasquale et al., 2020). For other legumes, varied effects of fermentation have been reported. Higher saponin content after fermentation was observed for chickpeas (kabuli and desi) and pigeon peas in Toor et al. (2021), whereas that for soybeans was decreased, agreeing with the results for soybeans in Rui et al. (2017). However, Nwanekezi et al. (2017) reported that saponin content decreased after fermentation in pigeon peas. Similar results were reported for mung bean and locust bean in Onwurafor et al. (2014) and Yakubu et al. (2022), respectively. Saponin degradation was a result of microbial β -glucosidase activities, which reduce the water solubility of saponins by cleaving sugar side chains from steroid and triterpenoid saponins (Szakács

& Madas, 1979). Massmann et al. (2022) offered a different view when addressing the elevated saponin content of pea protein concentrates after *Aspergillus* fermentation. Their 3D interaction model indicated that the increase in saponin levels was rather an increase in the extractability of these compounds, as microbes likely facilitated the hydrolysis of cell walls and subsequently released previously bound saponins, resulting in high extraction yields showing increased saponin levels in the fermented materials. Nevertheless, the higher saponin content observed in the *Aspergillus*-inoculated LPI samples may adversely affect nutrient availability.

3.6.4 | In vitro protein digestibility

The IVPD of the 0 h control was high at nearly 90% and dropped to ~78%–81% after fermentation ($p < 0.05$, Table 3). At 37°C, the magnitude of reduction was similar between the *L. plantarum*-inoculated (81.3%) and spontaneous fermented samples (79.3%). The *A. oryzae*-inoculated LPI also had a similar IVPD (78.7%) to that of the 30°C control (80.1%), whereas *A. niger* further lowered the value (77.6%). Comparing among inoculation treatments, *L. plantarum* resulted in higher IVPD than the *Aspergillus* strains. The current findings agreed with those reported by Tabera et al. (1995) and Olukomaiya et al. (2020), where fermentation of lentil (natural fermentation) and lupin (*Aspergillus* inoculation) flours, respectively, led to a reduction in IVPD. However, contradictory results were obtained by Alrosan et al. (2021), Asensio-Grau et al. (2020), and Shekib (1994). The increase in IVPD after fermentation was attributed to the reduction of ANF and the opening of protein structures, enhancing protein solubility and enzymatic attack efficiency. Although the TIA was unaffected under the present experimental conditions, the quantity of phenolics and saponins was significantly increased (Table 3), which may have interfered with protein digestion. IVPD was negatively correlated with both TPC ($r = -0.943$; $p < 0.001$) and total saponins ($r = -0.678$; $p < 0.01$). On the other hand, a bacterial pellet of *L. plantarum* cells prepared by Çabuk, Nosworthy et al. (2018) had significantly lower IVPD (69.6%) compared to the control and fermented pea protein concentrates (80.0%–83.2%). As microorganisms metabolize and utilize substrate proteins, the production of less digestible microbial proteins may contribute adversely to the overall protein digestion in fermented samples. Olukomaiya et al. (2020) also suggested that fermentation induced changes in the substrate composition, locking proteins within fiber matrices and decreasing their availability for the hydrolytic activities of the digestive enzymes.

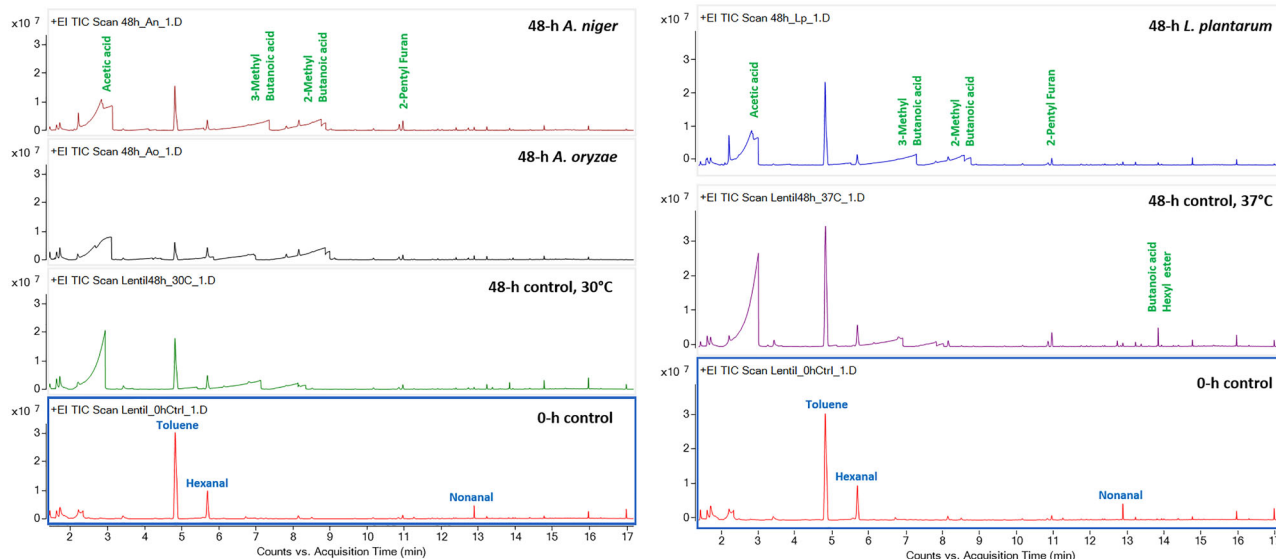


FIGURE 4 Volatile profiles of control, spontaneous fermented, and inoculated lentil protein isolates processed at 30°C (left) and 37°C (right). The names of the compounds identified from the most prominent peaks are shown. The prominent compounds present in the unfermented 0-h control samples are shown in blue and the new compounds identified in the spontaneous and inoculated fermentation samples are shown in green.

3.7 | Volatile profiling

During fermentation, macronutrients are broken down into smaller molecules, such as amino and fatty acids, which serve as precursors for flavor compound formation. Volatiles thus generated typically include alcohols, aldehydes, acids, ketones, esters, and others (Wieczorek & Drabińska, 2022). The volatile profile of control and 48 h fermented LPI is presented in Figure 4. The predominant peak at ~4.8 min in the 0 h control was toluene, which is associated with a sweet, pungent, benzene-like odor (World Health Organization [WHO], 2004). The next abundant peak at ~5.6 min was hexanal, followed by nonanal at ~12.8 min. Hexanal provides a green, fatty, leafy, vegetative, fruity, and clean flavor with a woody nuance (Mockus et al., 2023), and nonanal has a strong fruity or floral odor (Kim et al., 2019). Although toluene and hexanal were still present in all samples, their peak area was reduced after fermentation (Supporting Information section). Experimental data regarding the volatile profiles of fermented lentils has been limited. Sanchez-García et al. (2024) reported a decreased level of hexanal in fermented pardina lentils, whereas that for castellana lentils increased; variation between lentil cultivars was also significant when comparing unfermented samples, with hexanal and 1-hexanol being the most abundant in each cultivar, respectively. All the spontaneous fermented and inoculated samples had a rich diversity of new compounds. The abnormal peak shape of most of these new

compounds is a phenomenon that occurs due to their interaction with the fixed phase of the GC column, as they are mostly acids. Some of these newly formed compounds were identified as acetic acid, propanoic acid, 2-methyl propanoic acid, butanoic acid, 2-methyl butanoic acid, 3-methyl butanoic acid, hexanoic acid, butanoic acid pentyl ester, and butanoic acid hexyl ester. New volatile compound formation in LAB-fermented red and green lentils was reported to include acetic acid, hexanoic acid, pantolactone, (E)-non-2-enal, (E)-2-nonen-1-ol, (2E,4E)-nona-2,4-dienal, and 2-undecenal in Mockus et al. (2023); the complexity and intensity of the volatile profiles differed significantly among lentil cultivars. The present finding indicates that SmF was able to modify the volatile profile of LPI by introducing new compounds.

4 | CONCLUSIONS

LPI was subjected to fermentation for 48 h under different conditions: at 30°C using *A. oryzae* and *A. niger*, at 37°C using *L. plantarum*, and spontaneous fermentation (uninoculated) carried out at both temperatures. Fermented lentil proteins showed lower surface charge coupled with elevated surface hydrophobicity. Positive changes brought on by fermentation with any of the three strains studied included enriched protein content and three- to fivefold higher solubility, whereas negative impacts included reduced WHC, FS, ES, and IVPD. OHC

was increased by *Aspergillus* inoculation but not by *L. plantarum*, and the former also increased solubility to a greater extent, possibly due to a higher DH. For most properties studied, there were no differences between the two *Aspergillus* strains, which gave similar DH. The combination of higher quantities of phenolics and saponins after fermentation was hypothesized to account for the reduced IVPD. The volatile profiles of the fermented samples were enriched with new compounds, warranting further investigation into their sensory profiles for their use as food ingredients.

AUTHOR CONTRIBUTIONS

Dai Shi: Writing—original draft; Formal analysis; Visualization. **Andrea K. Stone:** Writing—original draft; Writing—review and editing; Formal analysis; Visualization. **Zahra Jafarian:** Investigation; Formal analysis. **Enyu Liu:** Investigation; Formal analysis. **Caishuang Xu:** Investigation; Visualization; Formal analysis. **Aarti Bhagwat:** Investigation; Visualization; Formal analysis. **Yuping Lu:** Validation; Writing—review and editing; Formal analysis. **Peng Gao:** Investigation; Visualization; Formal analysis. **Brittany Polley:** Investigation; Visualization; Formal analysis. **Pankaj Bhowmik:** Funding acquisition; Writing—review and editing; Supervision. **Nandhakishore Rajagopalan:** Funding acquisition; Conceptualization; Supervision; Writing—review and editing; Visualization. **Takuji Tanaka:** Conceptualization; Writing—review and editing; Supervision. **Darren R. Korber:** Conceptualization; Supervision; Writing—review and editing. **Michael T. Nickerson:** Conceptualization; Funding acquisition; Writing—review and editing; Supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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