Author/s:
Feng, X;Ng, K;Ajlouni, S;Zhang, P;Liang, Z;Fang, Z

Title:
Enhancement of Protein Hydrolysis and Bioactivity in Hempseed Cake via Solid-State Fermentation Using Aspergillus niger, Bacillus subtilis, and Lactobacillus rhamnosus

Date:
2025-10-01

Citation:
Feng, X., Ng, K., Ajlouni, S., Zhang, P., Liang, Z. & Fang, Z. (2025). Enhancement of Protein Hydrolysis and Bioactivity in Hempseed Cake via Solid-State Fermentation Using Aspergillus niger, Bacillus subtilis, and Lactobacillus rhamnosus. Food and Bioprocess Technology, 18 (10), pp.8633-8651. https://doi.org/10.1007/s11947-025-03940-4.

Persistent Link:
https://hdl.handle.net/11343/362218

Food and Bioprocess Technology (2025) 18:8633–8651
https://doi.org/10.1007/s11947-025-03940-4

RESEARCH

Enhancement of Protein Hydrolysis and Bioactivity in Hempseed Cake via Solid-State Fermentation Using *Aspergillus niger*, *Bacillus subtilis*, and *Lactobacillus rhamnosus*

Xiaoyu Feng[1] · Ken Ng[1] · Said Ajlouni[1] · Pangzhen Zhang[1] · Zijian Liang[1] · Zhongxiang Fang[1]

Received: 27 April 2025 / Accepted: 19 June 2025 / Published online: 4 July 2025

Abstract
Hempseed cake, a by-product of hempseed oil extraction, contains proteins and other compounds whose bioactivity can be enhanced through solid-state fermentation (SSF). Thus, this study investigated the effects of SSF using *Aspergillus niger*, *Bacillus subtilis*, and *Lactobacillus rhamnosus* on the degree of protein hydrolysis (DH), protein composition, and bioactive properties of the fermented hempseed cake. An orthogonal design was employed to determine the optimal fermentation conditions of each microorganism. Fermentation significantly ($P < 0.05$) increased DH and soluble protein and free amino acid (FAA) contents, with *B. subtilis* showing the highest increase (DH, 2.77% → 48.72%; soluble protein, 28.12 mg/g dw → 208.21 mg/g dw; FAA, 4.09 mg/g dw → 52.81 mg/g dw). SDS-PAGE confirmed the hydrolysis of macromolecular proteins into peptides. SSF also enhanced the antioxidant capacity of fermented hempseed cake as measured by ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity, as well as increased inhibition against ACE (angiotensin-converting enzyme), AChE (acetylcholinesterase), and intestinal α-glucosidase activity. The highest ABTS radical scavenging activity was observed with *L. rhamnosus* fermentation (4.98 mg TE/g), while *B. subtilis* fermentation yielded the strongest ACE (88.22%) and AChE (84.39%) inhibitory activities. *A. niger* exhibited the highest α-glucosidase inhibitory activity (26.04%). Pearson correlation analyses supported that these enhancements were linked to protein hydrolysis. Among the tested strains, *B. subtilis* generally exhibited superior performance in protein hydrolysis. These findings highlight SSF as a promising approach to enhance the nutritional and bioactive properties of hempseed cake, offering potential applications in functional food and nutraceutical development.

Keywords Orthogonal design · Hempseed proteins · Solid-state fermentation · Antioxidant · ACE inhibition · AChE inhibition

Introduction

Hemp (*Cannabis sativa*) is the same species as marijuana, so the wide utilization of hemp has been hindered due to public misconceptions. However, the low levels of the psychoactive compound tetrahydrocannabinol (THC; < 0.3%, w/w) in hemp have led to its legalization for cultivation in Australia and New Zealand in 2016 (Leonard et al., 2020). Originally grown for its stem fiber, the high oil and protein content in hempseed attracts increasing interest for its food applications. Hempseed cake, a by-product of hemp after its oil extraction, is particularly rich in nutritional protein (up to 50% w/w) (Leonard et al., 2020). Notably, hempseed proteins exhibit superior digestibility and unique amino acid profiles compared to soy protein (Wang et al., 2008). Although native hempseed proteins are commonly utilized, they generally possess less bioactivity than their derived peptides, which can be produced via enzymatic hydrolysis during digestion or food processing (Cruz-Casas et al., 2021). Hempseed peptides produced through enzymatic hydrolysis have demonstrated antioxidant, antidiabetic, antihypertensive, and neuroprotective activities (Malomo & Aluko, 2016; Ren et al., 2016; Tang et al., 2009; Teh et al., 2016). Microbial fermentation offers a promising approach for enhancing the nutritional value of hempseed cake proteins, as it provides diverse proteases with higher hydrolysis

✉ Zhongxiang Fang
zhongxiang.fang@unimelb.edu.au

[1] School of Agriculture, Food, and Ecosystem Sciences, Faculty of Sciences, University of Melbourne, Parkville, VIC 3010, Australia

efficiency and lower production costs for generating bioactive peptides (Cruz-Casas et al., 2021).

Fermentation is a traditional industrial process widely used to improve the shelf life, nutritional value, and organoleptic properties of food (Kaprasob et al., 2017). Solid-state fermentation (SSF) is a process that involves cultivating microorganisms on solid substrates, which provide both nutrients and physical support, with minimal or no free-flowing water (Kumar et al., 2021). This method offers several advantages over conventional liquid fermentation systems, such as lower water and energy requirements, higher hydrolysed protein production, and less stringent requirements for sterile conditions (Dey et al., 2016). Peptides released from proteins are key targets for enhancing functional health benefits through microbial biotransformation (Kaprasob et al., 2017). During fermentation, microorganisms secrete proteases and cellulases, which break down the substrate cell wall structure and depolymerize large proteins into smaller peptides and some to its amino acids (Feng et al., 2023; Leonard et al., 2021a, 2021b). The resulting bioactive protein metabolites contribute to diverse health functional properties such as antioxidant, antidiabetic, and antihypertensive activities (Magro et al., 2019).

Microbial strains such as *Bacillus* spp., *Lactobacillus* spp., and non-pathogenic fungal species are commonly used as starter cultures in SSF systems (Feng et al., 2023). These microorganisms are classified as "Generally Recognized as Safe" (GRAS) by the US Food and Drug Administration and are known for their producing active and complex enzyme systems (de Castro et al., 2014). Previous studies have demonstrated that SSF can lead to significant improvements in the health benefits of legumes and cereals (Feng et al., 2023). However, there remains a research gap in applying SSF to enhance the nutritional quality of hempseed cake. Literature comparing the effects of different starter cultures on hempseed cake is scarce. Therefore, this study investigated the effects of *Aspergillus niger* (a filamentous fungus), *Lactobacillus rhamnosus* (a lactic acid bacteria), and *Bacillus subtilis* (an amylases and proteases producing bacteria) on the SSF of hempseed cake. To the best of our knowledge, this is the first study to compare the impact of various microbial species on the nutritional value and bioactivities of hempseed cake using SSF. The findings of this research will provide a practical method for the utilization of hempseed cake as a valuable resource in the food and nutraceutical industries.

Materials and Methods

Material and Reagent

Hempseed cake was supplied by Australian Primary Hemp (Geelong, VIC, Australia). *B. subtilis* BSL1 (ATCC 23857), *A. niger* van Tieghem (ATCC 16888), and *L. rhamnosus* GG (ATCC 53103) were procured from American Type Culture Collection (ATCC, Manassas, VA, USA). All remaining reagents, unless otherwise specified, were of analytical grade acquired from Sigma-Aldrich (Castle Hill, NSW, Australia).

Starter Culture Preparation

Culture preparation was conducted following the method described by Chen et al., (2023), with minor modifications. The inoculum for *A. niger* was prepared by incubating *A. niger* van Tieghem on potato dextrose agar (Oxoid, Thermo Fisher Scientific, Waltham, MA, USA) at 26 °C for 48 h, followed by harvesting spores into sterile 0.9% saline. *B. subtilis* was cultured in nutrient broth (Merck, Darmstadt, Germany) and incubated at 26 °C for 24 h. For *L. rhamnosus*, the inoculum was prepared by growing the culture in de Man, Rogosa, and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 37 °C for 24 h. The temperature was set referring to the ATCC website handling information. The cells were harvested by centrifugation at 4000 g for 10 min, washed twice with sterile 0.9% saline, and resuspended in 0.9% saline for subsequent use.

Orthogonal Experimental Design Optimization of SSF Conditions for the Hempseed Cake

Orthogonal design, a fractional factorial design approach commonly used to develop experiments involving multiple-level factors, was employed to select representative combinations and optimize fermentation conditions for the degree of protein hydrolysis (DH) (Table 1) (Oles, 1993). An L_9 (3^4) table was applied, enabling the evaluation of four factors, each with three levels. Fermentation time, added amount of water, and inoculum size were selected as factors. The blank column was set to estimate random error according to the orthogonal design theory. Based on preliminary single-factor experimental findings, three levels were defined for each factor. For *A. niger*, the levels were fermentation time (5, 7, and 9 days), water addition amount (0.8, 1, and 1.2 mL/g dry weight dw), and inoculum size (1×10^5, 1×10^6, and 1×10^7 spores/g dw), resulting in samples labelled "AN1- AN9." For *B. subtilis*, the levels included fermentation time (7, 9, and 11 days), amount of water (0.8, 1, and 1.2 mL/g dw), and inoculum size (1×10^6, 1×10^7, and 1×10^8 CFU/g dw), generating samples labelled "BS1–BS9." For *L. rhamnosus*, the levels were fermentation time (5, 7, and 9 days), amount of water (0.8, 1, and 1.2 mL/g dw), and inoculum size (1×10^6, 1×10^7, and 1×10^8 CFU/g dw), producing samples labelled "LR1–LR9." The influence of each factor on DH was determined by variance analysis, and the comparative importance of each factor was determined by range analysis.

Table 1 Orthogonal design of solid-state fermentation of hempseed cake

Treatments	Fermentation time (A)	Amount of added water (B)	Inoculum size (C)	Blank (D)
A. niger (temperature: 26 ℃)				
AN1	1 (5 days)	1 (0.8 ml/g dw)	1 (1×10^5 spores/g dw)	1
AN2	1	2 (1 ml/g dw)	3 (1×10^7 spores/g dw)	2
AN3	1	3 (1.2 ml/g dw)	2 (1×10^6 spores/g dw)	3
AN4	2 (7 days)	1	3	3
AN5	2	2	2	1
AN6	2	3	1	2
AN7	3 (9 days)	1	2	2
AN8	3	2	1	3
AN9	3	3	3	1
B. subtills (temperature: 26 ℃)				
BS1	1 (7 days)	1 (0.6 ml/g dw)	1 (1×10^6 CFU/g dw)	1
BS2	1	2 (0.8 ml/g dw)	3 (1×10^8 CFU/g dw)	2
BS3	1	3 (1 ml/g dw)	2 (1×10^7 CFU/g dw)	3
BS4	2 (9 days)	1	3	3
BS5	2	2	2	1
BS6	2	3	1	2
BS7	3 (11 days)	1	2	2
BS8	3	2	1	3
BS9	3	3	3	1
L. rhamnoses (temperature: 37 ℃)				
LR1	1 (5 days)	1 (0.8 ml/g dw)	1 (1×10^6 CFU/g dw)	1
LR2	1	2 (1 ml/g dw)	3 (1×10^8 CFU/g dw)	2
LR3	1	3 (1.2 ml/g dw)	2 (1×10^7 CFU/g dw)	3
LR4	2 (7 days)	1	3	3
LR5	2	2	2	1
LR6	2	3	1	2
LR7	3 (9 days)	1	2	2
LR8	3	2	1	3
LR9	3	3	3	1

SSF Process of Hempseed Cake

The fermentation process is illustrated in Fig. 1. Briefly, hempseed cake was autoclaved at 121 °C for 20 min. After cooling, 10 g of hempseed cake was aseptically transferred into 125-mL sterile plastic containers inside a biosafety cabinet, inoculated with the previously prepared inoculum, and mixed with sterile Milli-Q water (Merck, Bayswater, VIC, Australia). The mixture was thoroughly blended under aseptic conditions using sterile glass rods. The samples were incubated in a laboratory incubator (Thermoline I100G-300-D, Wetherill Park, NSW, Australia) with sterilized cotton plugs without agitation. The fermentation temperature was set at 26 °C for *A. niger* and *B. subtilis*, and 37 °C for *L. rhamnosus*, for durations specific to the experimental design (Table 1). Following fermentation, the samples were dried in a hot air oven (Qualtex, Melbourne, VIC, Australia) at 50 °C for 24 h and subsequently ground into powder using a commercial coffee grinder (Sunbeam EM0405 Multigrinder II, Melbourne, VIC, Australia) to achieve a particle size of less than 0.5 mm. The powdered samples were stored in 125-mL plastic containers at − 20 °C for further analysis. Autoclaved, unfermented hempseed cake was used as the control (CON) to account for the impact of the autoclaving process on the substrate.

Degree of Protein Hydrolysis (DH)

DH is defined as the ratio of free amino acid groups to fully hydrolysed amino acid groups and was determined using the 2,4,6-trinitrobenzene sulfonic acid (TNBS) assay, based on the method described by Yu et al., (2023), with minor modifications. Fermented hempseed cake samples were mixed with 0.1 M PBS buffer (pH 7.4, 1:15 w/v) and then shaken at 140 rpm for 1 h at room temperature. The mixture was then centrifuged at 8000 g with Beckman Coulter Allegra V-15R centrifuge (Beckman Coulter, Brea, CA, USA) for 10 min, and the supernatant was collected. For the assay, 25 µL

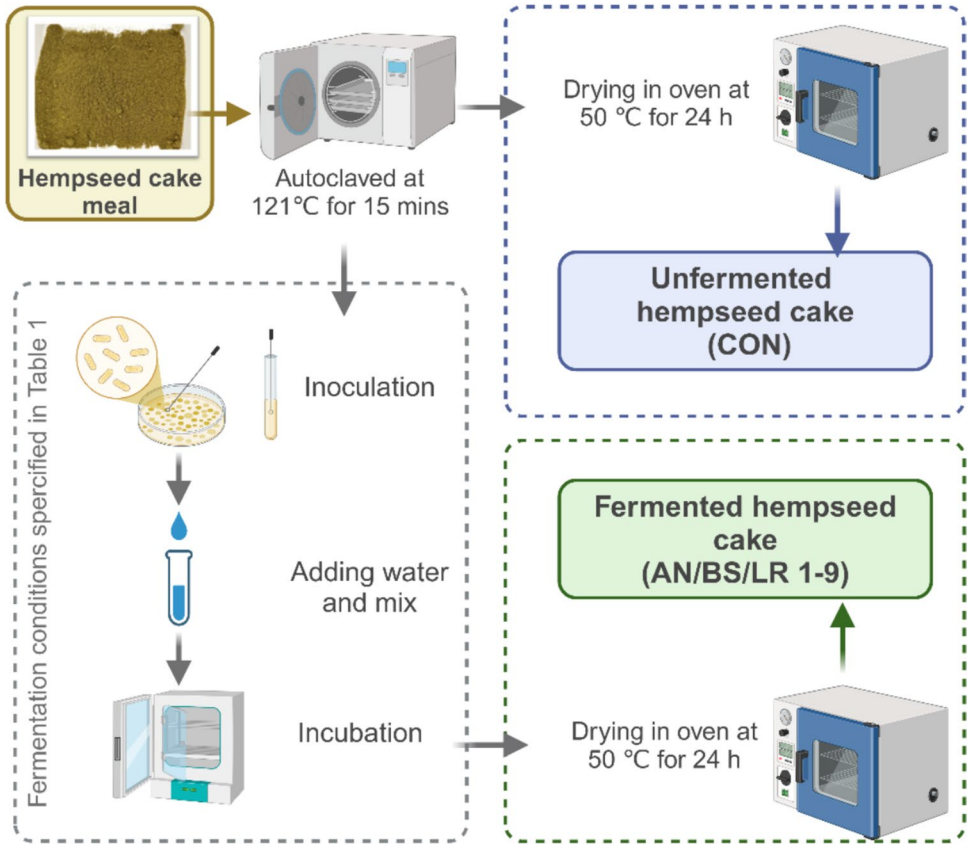


Fig. 1 Flowchart of SSF of hempseed cake

of the supernatant was mixed with 200 µL pH 8.2 phosphate buffer and 200 µL 0.1% TNBS in capped tubes. The reaction mixture was incubated in a 50 °C water bath for 1 h. The reaction was terminated by adding 400 µL of 0.1 M HCl, and the absorbance was measured at 340 nm using a Thermo Scientific Varioskan LUX Multimode Microplate Reader (Thermo Fisher Scientific, Scoresby, VIC, Australia).

The total amino groups were determined using unfermented hempseed samples completely hydrolyzed with 6 M HCl at 120 °C for 24 h. The DH (%) was calculated using the equation:

$$\mathrm{DH}(\%) = \frac{A_{\mathrm{Sample}} - A_{\mathrm{Blank}}}{A_{\mathrm{Total}} - A_{\mathrm{Blank}}} \times 100$$

where A_{Sample} represents the absorbance of the unfermented or fermented sample at 340 nm, A_{Blank} is the absorbance measured with deionized water in place of the sample, and A_{Total} is the absorbance of the completely hydrolysed sample.

Soluble Protein Content, Crude Protein Content, and Total Carbon Content

The extract described in "Degree of Protein Hydrolysis (DH)" was also used to determine the soluble protein content, which was measured using the Pierce BCA Protein Assay Kit (23,225, Pierce™ BCA Protein Assay Kit, Thermofisher Scientific, San Jose, CA, USA) following the manufacturer's protocol, with bovine serum albumin (BSA) serving as the protein standard. Crude protein and carbon contents were analyzed as total nitrogen and carbon contents using the Dumas combustion method with a Leco TruMac CNS Analyzer (LECO Corporation, St. Joseph, MI, USA) with a conversion factor of $N \times 6.25$.

Free Amino Acid Profile by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC–DAD)

To extract free amino acids, fermented hempseed cake was mixed with 0.1 M HCl (1:15, w/v), shaken at 140 rpm for 1 h, and centrifuged at 8000 g for 10 min, and the supernatant was filtered through a 0.45-µm filter. An Agilent HPLC system (1260 Infinity II, Agilent Technologies Pty Ltd., Santa Clara, CA, USA) coupled with a diode array detector (DAD) was applied to quantify the amino acids, using the method of Yao et al., (2023), with minor modification. The separation was achieved on a Phenomenex Aeris™ PEPTIDE XB-C18 column (250×4.6 mm, 3.6 µm, Phenomenex, Torrance, CA, USA). The mobile phase consisted of (A) 10 mM Na_2HPO_4, 10 mM Na_2B4O_7, and 5 mM NaN_3 (pH 8.2) and (B) HPLC-grade acetonitrile, HPLC-grade methanol, and deionized water (45:45:10, v/v/v). The gradient program

was set as follows: 0–0.53 min, 2% B; 0.53–20.10 min, 57% B; 20.10–20.30 min, 100% B; 20.30–24.75 min, 100% B; 24.75–25.50 min, 2% B; 25.50–27.75 min, 2% B. Absorbance was recorded at 334 nm. The column was maintained at 40 °C with a flow rate of 1 mL/min. Online derivatization of amino acids was performed by sequentially mixing 1 µL of the sample with 2.5 µL of borate buffer (Agilent, 5061–3339, Santa Clara, CA, USA), 0.5 µL of o-phthalaldehyde (OPA) (Agilent, 5061–3335), and 0.4 µL of 9-fluorenylmethylchloroformate (FMOC) (Agilent, 5061–3337). Then, 32 µL of injection diluent (prepared by adding 0.4 mL of H_3PO_4 to 100 mL of mobile phase A) was added and mixed. A 2 µL aliquot of this mixture was injected for analysis. The concentration of each amino acid was calculated based on the calibration curves of amino acid mix standards (A9781, Sigma-Aldrich, Castle Hill, NSW, Australia) and expressed as mg/g.

SDS–Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight distribution of fermented hempseed cake proteins was determined using SDS-PAGE as described by Laemmli, (1970). Two sets of samples were prepared: one to analyze the total protein profile and the other to analyze the soluble protein profile. To extract the total protein, 1 mL of 1X Laemmli buffer (Bio-Rad Laboratories, Hercules, CA, USA) was added to 15 mg fermented sample and mixed for 1 h. For the soluble protein profile, supernatants of fermented sample extracted with PBS buffer (pH 7.4, 1:10 ratio) were freeze-dried, redissolved in deionized water at 10 mg/mL, and then mixed with 2X Laemmli buffer (1:1 ratio). All samples were heated in a 100 °C water bath for 10 min and centrifuged at 8000 g for 5 min, and 10 µL of the resulting supernatant was loaded into the wells of precast 4–20% polyacrylamide gels (Mini-PROTEAN TGX Precast Gels, Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was conducted for 40 min at 200 V (60 mA and 100 W) at room temperature in a vertical electrophoresis cell (Bio-Rad Laboratories, Hercules, CA, USA). Precision Plus Protein Unstained Standards (Bio-Rad Laboratories, Hercules, CA, USA), with a molecular weight range of 10–250 kDa, were included as markers. Gels were stained for 30 min using a staining solution containing 50% methanol (v/v), 10% acetic acid (v/v), and 0.15% Coomassie Brilliant Blue R-250 (w/v), followed by overnight destaining with a solution of 20% methanol (v/v) and 10% acetic acid (v/v).

Bioactivities of Fermented Hempseed Cake Peptide Fraction

To evaluate the bioactivities of the peptide fraction from unfermented or fermented hempseed cake, the samples were washed with methanol (1:15 w:v, shaken at 140 rpm for 1 h) three times to remove phenolic compounds and residual oil. The washed samples were then thoroughly dried and extracted with pH 7.4 PBS (1:10 w/v) to obtain an extract of the hempseed cake peptide.

ABTS Radical Scavenging Activity

The antioxidant activity of the hempseed cake peptide extracts was assessed by ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) radical scavenging assay in a microplate format following Leonard et al., (2021a, 2021b) with minor modifications. An ABTS stock solution was prepared by mixing 7.4 mM ABTS with 2.6 mM potassium persulfate (1:1) and allowing the mixture to stand in the dark for 12 h. The stock solution was diluted with methanol to prepare an ABTS working solution with an absorbance of 1.1 ± 0.02 at 734 nm. An aliquot of 30 µL of peptide extract was mixed with 270 µL of ABTS working solution and shaken for 30 s. Subsequently, the mixture was incubated for 15 min in the dark at room temperature. The absorbance was then measured at 734 nm using the Thermo Scientific Varioskan LUX Multimode Microplate Reader. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (0–0.1 mg) was used to generate the standard curve, and the results were expressed as mg Trolox equivalent (TE)/g dw.

Alpha-Glucosidase Inhibitory Activity

A mammalian α-glucosidase inhibitory assay was used to assess the antidiabetic potential of the fermented hempseed cake following the method of Rosa et al., (2023). Intestinal α-glucosidases were extracted from 10 mg/mL rat intestinal acetone powder (I1630, Sigma Aldrich, Castle Hill, NSW, Australia) by ultrasonic treatment (10 min) in an ice bath. The mixture was centrifuged at 8000 g for 10 min, and the supernatant containing the enzyme was collected. For the assay, an aliquot of 50 µL of 0.1 M phosphate buffer, 20 µL peptide extract, and 10 µL of α-glucosidase solution were mixed in 96-well microplates. The mixture was incubated at 37 °C for 10 min. After incubation, 20 µL of 5 mM p-nitrophenyl-a-d-glucopyranoside (pNPG; pH 6.8) was added and the mixture was incubated at 37 °C for 50 min. The absorbances at 0 and 50 min were recorded at 405 nm using the Thermo Scientific Varioskan LUX Multimode Microplate Reader. The α-glucosidase inhibitory activity was calculated using the formula:

$$\alpha - glucosidase\ inhibition\,(\%) = \frac{\Delta A_{405}^{Control} - \Delta A_{405}^{Sample}}{\Delta A_{405}^{Control}} \times 100$$

ACE Inhibitory Activity

Angiotensin-converting enzyme (ACE) inhibitory activity was assessed following the method of Sonklin et al.,

(2020), with minor modifications. Briefly, 0.1 g of rabbit lung acetone powder (L0756, Sigma-Aldrich, Castle Hill, NSW, Australia) was mixed with 10 mL of 100 mM sodium borate buffer (pH 8.3) containing 5% (v/v) glycerol. The mixture was shaken at 140 rpm for 2 h and then incubated at 4 °C overnight to extract the ACE. For the assay, 150 µL of 0.5 mM N-[3-(2-furyl)acryloyl]-L-phenylalanylglycine (FAPGG; dissolved in 50 mM Tris–HCl buffer containing 0.3 M NaCl, pH 8.2) was mixed with 20 µL of ACE solution (20 mU) and 20 µL of the peptide extract. The reduction in absorbance at 340 nm was recorded at 0 and 30 min at 37 °C using a Thermo Scientific Varioskan LUX Multimode Microplate Reader. pH 7.4 PBS buffer was used as a blank. The ACE inhibitory activity was calculated as follows:

$$\text{ACE inhibition}\,(\%) = (1 - \frac{\text{Slope of the sample curve}}{\text{Slope of the blank curve}}) \times 100$$

AChE Inhibitory Activity

The acetylcholinesterase inhibition assay was performed following the method of Su et al., (2024), with minor modifications. Acetylthiocholine iodide (ATCI) was used as the substrate, and *Electrophorus electricus* acetylcholinesterase (AChE) was used as the enzyme. The reaction mixture consisted of 25 µL of 15 mM ATCI, 125 µL of 3 mM 5,5′-dithiobis-(2-nitrobenzoic acid) dissolved in 0.1 M PBS (pH adjusted to 8.0), 50 µL of pH 7.4 PBS containing 0.1% BSA, and 50 µL of fermented hempseed cake PBS extract. After incubation at 25 °C for 20 min, 25 µL of AChE solution (100 mU) was added to initiate the reaction. The absorbance at 405 nm was recorded every 30 s for 20 min at 25 °C using a Thermo Scientific Varioskan LUX Multimode Microplate Reader. The AChE inhibitory activity was calculated using the following formula:

$$\text{AChE inhibition}\,(\%) = (1 - \frac{\text{Average rate of sample}}{\text{Average rate of blank}}) \times 100$$

Data Analysis

Except otherwise stated, each experiment was conducted in triplicates, and the means ± standard deviations were reported. The significant difference of means among samples in each experiment was determined by one-way ANOVA with Fisher grouping at a 95% confidence level using Minitab (Minitab 21.1.0, Sydney, Australia). Data visualization was performed using Origin 2024b (OriginLab Corporation, Northampton, MA, USA).

Results and Discussion

Analysis of Orthogonal Design Experiment for Degree of Hydrolysis of Fermented Hempseed Cake

Table 2 presents the results of the L_9 (3^4) orthogonal design, which analyzed the effects of three tested factors—fermentation time (A), water addition amount (B), and inoculum size (C). Variance analysis revealed that all three factors significantly ($P < 0.05$) affected DH for *A. niger* fermented samples (AN). Conversely, for *B. subtilis* (BS) and *L. rhamnoses* (LR) fermented samples, only fermentation time (A) exhibited a significant effect ($P < 0.05$). Range analysis was conducted to assess the relative importance of each factor based on their range (R) values, with a higher R-value indicating a greater effect. For AN, water addition amount was the most influential factor, followed by inoculum size and fermentation time. For BS and LR, fermentation time had the greatest influence, followed by inoculum size and water addition amount. These findings highlight the need for microorganism-specific optimization of fermentation conditions. Based on the k values, the optimum fermentation conditions for DH were identified as $A_2B_1C_3$ for AN, $A_3B_2C_3$ for BS, and $A_3B_2C_1$ for LR. Specifically, these conditions correspond to 7 days of fermentation time, 0.8 mL/g water addition, and 1×10^6 spores/g inoculation for AN; 11 days of fermentation time, 0.8 mL/g water addition, and 1×10^8 CFU/g inoculation for BS; and 9 days of fermentation time, 1 mL/g water addition, and 1×10^6 CFU/g inoculation for LR.

Figure 2 illustrates the results of DH. Fermentation significantly ($P < 0.05$) increased the DH of hempseed cake in all tested conditions for the three microorganisms. Among the tested microbial cultures, AN increased DH from 2.77% to a maximum of 33.61% in AN4. Higher DH values were observed in BS and LR, reaching 48.72% in BS8 and 45.63% in LR8, respectively. These results indicated that AN's ability to hydrolyze hempseed protein was lower compared to BS and LR. These differences can be attributed to differences in the amount and activity of proteases produced by each microorganism, as well as different affinities of these enzymes or substrates (Banerjee & Ray, 2017). Previous studies comparing the impact of different starter cultures on the DH of soybean meal reported similar results, with *B. subtilis* outperforming *Lactobacillus* and *Aspergillus* spp. (Yang et al., 2020). It is worth noting that a higher DH does not necessarily translate to enhanced bioactivity, as the functional properties of protein hydrolysates also depend on the nature and sequence of the produced peptides.

Table 2 Variance and range analysis of orthogonal design for degree of hydrolysis (DH) in solid-state fermented hempseed cake

Analysis of Variances														
Factors	AN				BS				LR					
	A	B	C	D (error)	A	B	C	D (error)	A	B	C	D (error)		
SS	18.21	232.43	24.48	0.15	118.36	48.08	28.10	2.75	80.31	12.78	30.80	3.49		
DF	2	2	2	2	2	2	2	2	2	2	2	2		
F value	119.79	1529.18	161.04	1.0	43.03	17.48	10.22	1	23.00	3.66	8.82	1.0		
P value	0.008	0.001	0.006	0.5	0.02	0.05	0.09	0.5	0.04	0.21	0.10	0.5		
Significance	*	*	*		*				*					
Range analysis														
Factors	AN				BS				LR					
	A	B	C	D (error)	A	B	C	D (error)	A	B	C	D (error)		
k_1	23.85	29.77	23.06	23.95	38.84	44.01	42.04	44.16	35.71	37.56	41.16	40.05		
k_2	25.58	24.41	22.33	23.92	45.37	46.59	43.25	43.07	39.01	40.03	39.83	38.53		
k_3	22.10	17.36	26.14	23.66	47.32	40.94	46.24	44.30	43.01	40.14	36.74	39.16		
R	3.48	12.41	3.81	0.29	8.48	5.65	4.20	1.24	7.31	2.58	4.42	1.52		
Best level	A_2	B_1	C_3		A_3	B_2	C_3		A_3	B_2	C_1			

SS Sum of squared deviations, *DF* Degrees of freedom

Chemical Composition of the Fermented Hempseed Cake

Table 3 presents the chemical composition of solid-state fermented hempseed cake. For crude protein and total carbon content, fermentation with BS and LR revealed insignificant ($P > 0.05$) changes across all tested conditions. However, AN fermentation significantly ($P < 0.05$) increased the crude protein content, rising from 50.41% in the control to a maximum of 57.29% in AN7. Additionally, total carbon content notably decreased from 48.18% in the control to a minimum of 39.09% in AN5. The observed increase in crude protein content post-fermentation could be attributed to the consumption of carbon sources by microorganisms in growth, which leads to nitrogen enrichment and subsequently enhances crude protein levels (Feng et al., 2023). A similar increase in the crude protein of hempseed after fermentation with the non-filamentous fungus, *Pleurotus ostreatus,* has been reported with a crude protein increase from 15.89 to 24.32% after 11 days of SSF of hempseed (Eliopoulos et al., 2022). Among the tested microorganisms, AN demonstrated a superior ability to utilize the carbon sources in hempseed cake compared to BS and LR. Similarly, Lin et al., (2022) showed that crude protein content in broad bean koji fermented with fungi was higher than in samples fermented with bacteria.

Table 3 also indicates that all three tested microbial cultures significantly ($P < 0.05$) increased the soluble protein content of hempseed cake after fermentation. Similar to the trends observed in DH, BS and LR generally outperformed AN, with soluble protein content increasing from 28.12 mg/g in the control to a maximum of 115.4 mg/g in AN5, 208.21 mg/g in BS9, and 206.64 mg/g in LR8. In BS-fermented samples, BS3, BS6, and BS9 exhibited elevated soluble protein content, which could be attributed to the shared fermentation condition of the highest water addition amount (1 mL/g dw). For LR-fermented groups, higher inoculum sizes, such as those used in LR2, LR5, and LR8, were generally associated with increased soluble protein content. The increase in soluble protein content can be attributed to the enzymatic degradation of macromolecular proteins into soluble peptides and amino acids by bacterial enzymes, as well as the formation of new microbial soluble proteins during fermentation (Wang et al., 2021). These soluble proteins generated post-fermentation are essential for enhancing nutritional quality, digestibility, and functional properties (Gao et al., 2024). A previous study employing SSF of hempseed cake with *Bacillus licheniformis* for 72 h also reported an increase in soluble protein content from 15.87 ± 0.61 to 69.40 ± 1.06 mg/g (Rambu et al., 2024).

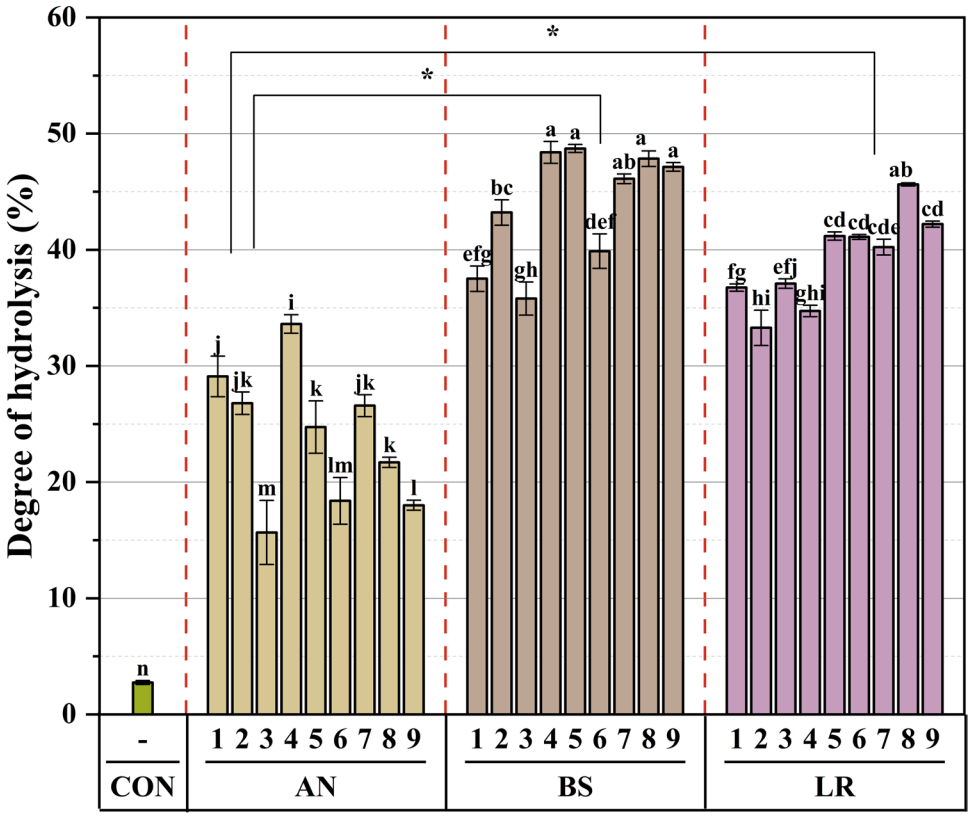


Fig. 2 Degree of hydrolysis (DH) of solid-state fermented hempseed cake. CON, control (unfermented sample); AN/BS/LR 1–9 represent solid-state fermented samples corresponding to fermentation conditions listed in Table 1

Molecular Weight Distribution of Fermented Hempseed Protein

SDS-PAGE is a simple yet effective tool for monitoring the protein hydrolysis during fermentation. The molecular weight distribution of fermented hempseed proteins is shown in Fig. 3. The whole protein (Fig. 3 a, b, c) and freeze-dried soluble protein (Fig. 3 d, e, f) profiles are presented. In general, despite the use of different starter cultures, the SDS-PAGE profiles of fermented hempseed cake exhibited similar patterns. Notably, a persistent band around 50 kDa (Fig. 3a) present in the unfermented protein profile was observed in AN-fermented samples but was absent in BS- and LR-fermented samples, correlating with the lower degree of hydrolysis (DH) observed in AN. Within the same starter culture group, no significant differences were detected in both protein profiles, indicating that fermentation conditions had a minimal impact on the molecular weight distribution of the hempseed proteins.

From the whole protein profile (Fig. 3 a,b,c), the most intense bands at 50 kDa and above 100 kDa in the control (CON) sample disappeared after fermentation, confirming that these large proteins were effectively hydrolyzed during the fermentation process. The band around 50 kDa in the CON sample corresponds to the edestin monomer (54 kDa), a major component of hempseed protein, which makes up 60–80% of the total protein content (Docimo et al., 2014). In contrast, for the AN/BS/LR 1–9 samples, the most intense bands appeared around 37 kDa and 10 kDa. Band I and II may correspond to the acidic (~ 34 kDa) and basic chains (18–20 kDa) of edestin, as reported by previous studies (Docimo et al., 2014; House et al., 2010). The band around 10 kDa (band III) in the CON has been identified as albumin, which constitutes a significant portion of the remaining hempseed protein fraction (Malomo & Aluko, 2015). However, this 10 kDa band may also contain newly formed low molecular weight peptides produced during fermentation as indicated by the increased intensity of this band in fermented samples compared to CON. Peptides with molecular weights below 10 kDa are known for their rapid digestion, absorption rate, and high bioactive properties (Dai et al., 2017).

The appearance of Band I and increased intensity of Band III of the freeze-dried soluble protein (Fig. 3 d,e,f) profiles indicates that, after fermentation, the insoluble macromolecular weight proteins were hydrolyzed into smaller soluble proteins, which aligns with the observed increase in soluble protein content post-fermentation. However, these findings contrast with those reported for enzymatically hydrolyzed hempseed, where 12 h of treatment with five different proteases—alcalase, bromelain, flavourzyme, neutrase, and papain—resulted in the complete degradation of proteins above 20 kDa into fragments of 20 kDa or smaller (Yoon et al., 2023). This would suggest that microbial fermentation may lead to limited breakdown of hempseed proteins compared to enzyme-based hydrolysis.

Table 3 Chemical composition of fermented hempseed cake

		CON	1	2	3	4	5	6	7	8	9	Mean
Crude protein (%)	AN	50.41 ± 0.3^{d}	55.59 ± 0.9^{abc}	53.86 ± 3.5^{abc}	52.19 ± 3.0^{bcd}	55.72 ± 1.4^{abc}	52.64 ± 3.4^{bcd}	50.04 ± 0.5^{bcd}	57.29 ± 0.9^{a}	55.84 ± 3.7^{ab}	51.14 ± 0.4^{cd}	53.81
	BS	50.41 ± 0.3^{a}	52.91 ± 0.9^{a}	52.35 ± 3.4^{a}	51.49 ± 2.9^{a}	52.68 ± 1.3^{a}	51.43 ± 3.4^{a}	50.16 ± 0.4^{a}	52.83 ± 0.9^{a}	51.7 ± 3.4^{a}	51.91 ± 0.4^{a}	51.79
	LR	50.41 ± 0.3^{a}	50.29 ± 0.8^{a}	50.68 ± 3.3^{a}	50.08 ± 2.9^{a}	51.03 ± 1.3^{a}	50.05 ± 3.3^{a}	50.45 ± 0.4^{a}	50.38 ± 0.8^{a}	49.4 ± 3.2^{a}	49.8 ± 0.4^{a}	50.24
Total carbon (%)	AN	48.18 ± 0.1^{a}	40.48 ± 0.7^{cd}	39.45 ± 2.6^{cd}	44.91 ± 2.6^{ab}	38.39 ± 0.9^{d}	38.09 ± 2.5^{d}	40.87 ± 0.3^{cd}	39.62 ± 0.7^{cd}	39.41 ± 2.6^{cd}	42.2 ± 0.3^{bc}	40.38
	BS	48.18 ± 0.1^{a}	48.01 ± 0.8^{a}	48.06 ± 3.1^{a}	47.4 ± 2.7^{a}	47.9 ± 1.2^{a}	47.25 ± 3.1^{a}	45.83 ± 0.4^{a}	48.15 ± 0.8^{a}	48.06 ± 3.1^{a}	47.59 ± 0.4^{a}	47.64
	LR	48.18 ± 0.1^{a}	46.64 ± 0.8^{a}	46.72 ± 3.1^{a}	46.61 ± 2.7^{a}	46.96 ± 1.2^{a}	46.45 ± 3.0^{a}	45.41 ± 0.4^{a}	46.81 ± 0.8^{a}	46.01 ± 3.0^{a}	46.61 ± 0.4^{a}	46.47
Soluble protein (mg/g)	AN	28.12 ± 0.7^{f}	110.57 ± 9^{ab}	99.88 ± 6.6^{bc}	77.54 ± 9.3^{de}	92.1 ± 6.3^{c}	115.4 ± 1.8^{a}	70.62 ± 8.7^{e}	87.76 ± 4.6^{cd}	99.87 ± 11.6^{bc}	76.65 ± 10.5^{de}	92.27
	BS	28.12 ± 0.7^{g}	149.69 ± 7.7^{f}	170.51 ± 7.4^{de}	192.04 ± 9.9^{abc}	180.41 ± 7.8^{cd}	182.41 ± 4.8^{bcd}	200.11 ± 7.3^{ab}	159.5 ± 8^{ef}	178.71 ± 6.1^{cd}	208.21 ± 1.7^{a}	180.18
	LR	28.12 ± 0.7^{e}	201.76 ± 12.4^{ab}	199.38 ± 13.1^{ab}	174.27 ± 7.5^{d}	179.58 ± 12^{cd}	199.97 ± 8.1^{ab}	198.68 ± 2.6^{ab}	182.73 ± 7.9^{cd}	206.64 ± 9.9^{a}	190.08 ± 10.5^{bc}	195.27

Values represent the mean ± standard deviation of three replicates. Different letters in the same row indicate significant differences ($P < 0.05$). *CON*, control (unfermented sample); AN/BS/LR 1–9, solid-state fermented samples corresponding to the fermentation conditions listed in Table 1; mean, average of nine groups within same stater culture

Free Amino Acid Profile of Fermented Hempseed Cake

Free amino acids (FAAs) contribute significantly to both the nutritional value and flavor of food products (Tian et al., 2024). The FAA contents of the hempseed cake samples are presented in Fig. 4. In general, all fermented samples exhibited higher FAA contents compared to the control (CON), which had an FAA content of 4.09 mg/g dw. The choice of starter culture had a significant effect on the FAA content. Among the tested microbes, *B. subtilis* (BS) groups produced significantly ($P < 0.05$) more FAAs (Fig. 4b) than *A. niger* (AN) (Fig. 4a) and *L. rhamnosus* (LR) (Fig. 4c). Specifically, the BS groups achieved a maximum FAA content of 55.46 mg/g in BS9, compared to a peak of 7.1 mg/g in AN3 and 14.52 mg/g in LR1. Interestingly, a previous study by Łopusiewicz et al., (2022) indicated that fermented hempseed cake with commercial kefir and yogurt starter cultures showed a decrease in total FAA after fermentation, indicating the limitation of microbial strains used in that study.

Fermentation conditions significantly influence the FAA profile in fermented hempseed cake, with fermentation time and water addition amount having distinct effects on total and individual FAA contents. Prolonged fermentation generally led to a reduction in the total FAA content in AN and LR fermented groups, as seen in AN/LR 7, 8, and 9 compared to AN/LR 1, 2, and 3. However, this trend was reversed in BS fermented groups, where longer fermentation times resulted in higher total FAA levels (BS 7, 8, 9). The amount of added water was particularly impactful in LR groups. Lower water addition levels (LR1, 4, 7) resulted in higher total FAA content compared to high-moisture groups (LR3, 6, 9). The effects on individual amino acids further highlighted the role of fermentation parameters. In the AN group, prolonged fermentation reduced glutamic acid levels and led to the disappearance of threonine and aspartic acid (AN 7, 8, 9). In contrast, extended fermentation in BS samples increased glutamic acid, isoleucine, arginine, and glycine levels. Serine content was notably higher in low-moisture BS samples (BS 1, 4, 7). A similar trend was observed in LR samples, where lower water content (LR1, 4, 7) resulted in elevated serine, arginine, glutamic acid, and aspartic acid levels but reduced isoleucine content compared to high-moisture groups (LR 3, 6, 9). These results underscore the importance of optimizing fermentation conditions to meet specific nutritional and functional objectives in the amino acid composition of fermented hempseed cake.

Glutamic acid was the most abundant amino acid in both unfermented and fermented hempseed cake. This amino acid plays a critical role in energy metabolism, contributing to the regulation of oxidative stress and the enhancement of intestinal barrier function (Chen et al.,

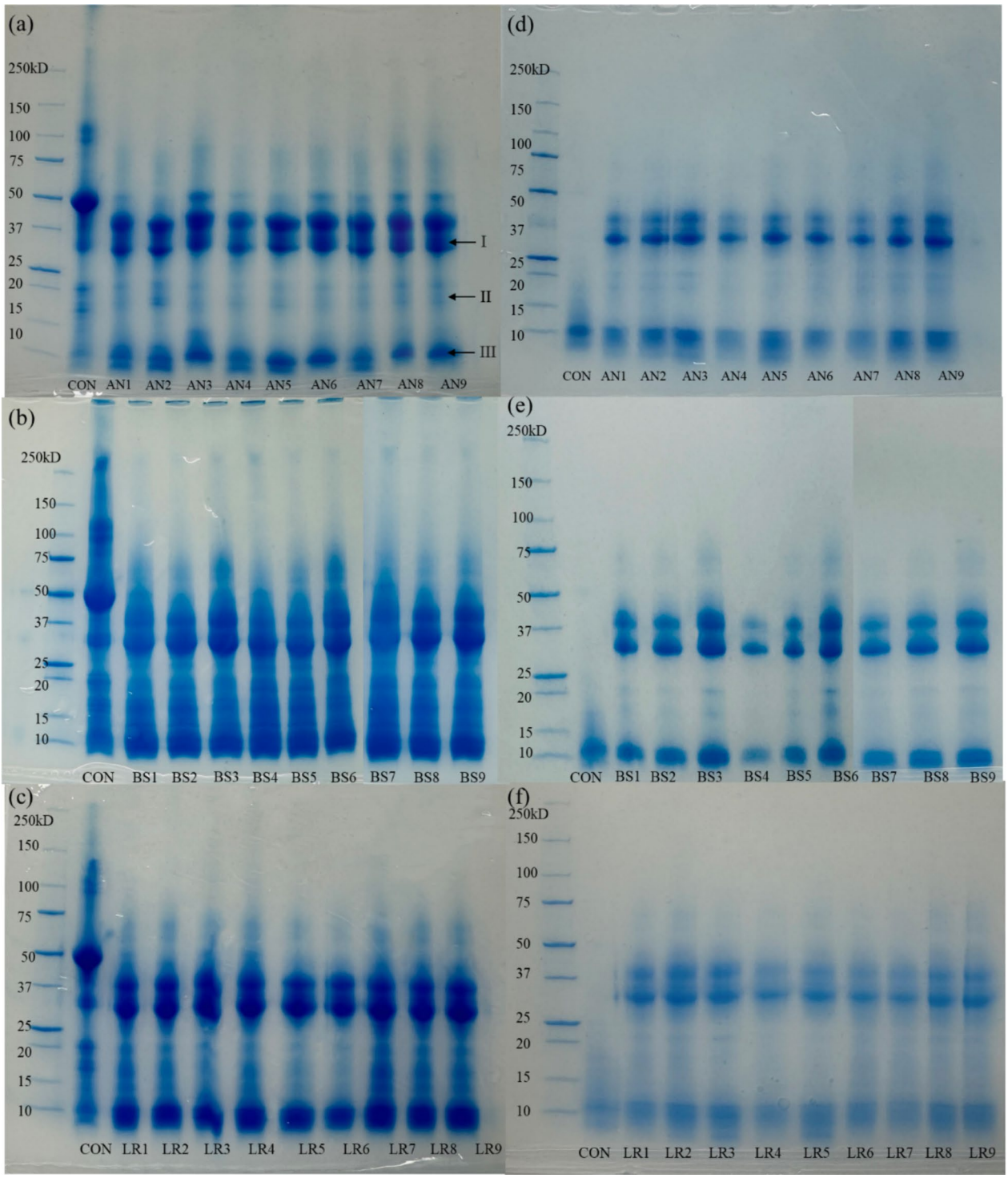


Fig. 3 SDS-PAGE profiles of fermented hempseed cake. Left: whole protein profiles. Right: freeze-dried soluble protein fractions. **a**, **d** *Aspergillus niger* fermented group; **b**, **e** *Bacillus subtilis* fermented group; **c**, **f** *Lactobacillus rhamnosus* fermented group. CON, unfermented sample; AN/BS/LR 1–9, solid-state fermented samples corresponding to the fermentation conditions listed in Table 1

2021). Importantly, it is a component of glutathione, an essential endogenous cellular antioxidant found in all cells, where it helps protect against oxidative damage (Hussin et al., 2022). Fermentation also resulted in a significant increase in valine and leucine levels across all fermented groups. These branched-chain amino acids are known for their antioxidant properties, which are attributed to their ability to scavenge free radicals and chelate

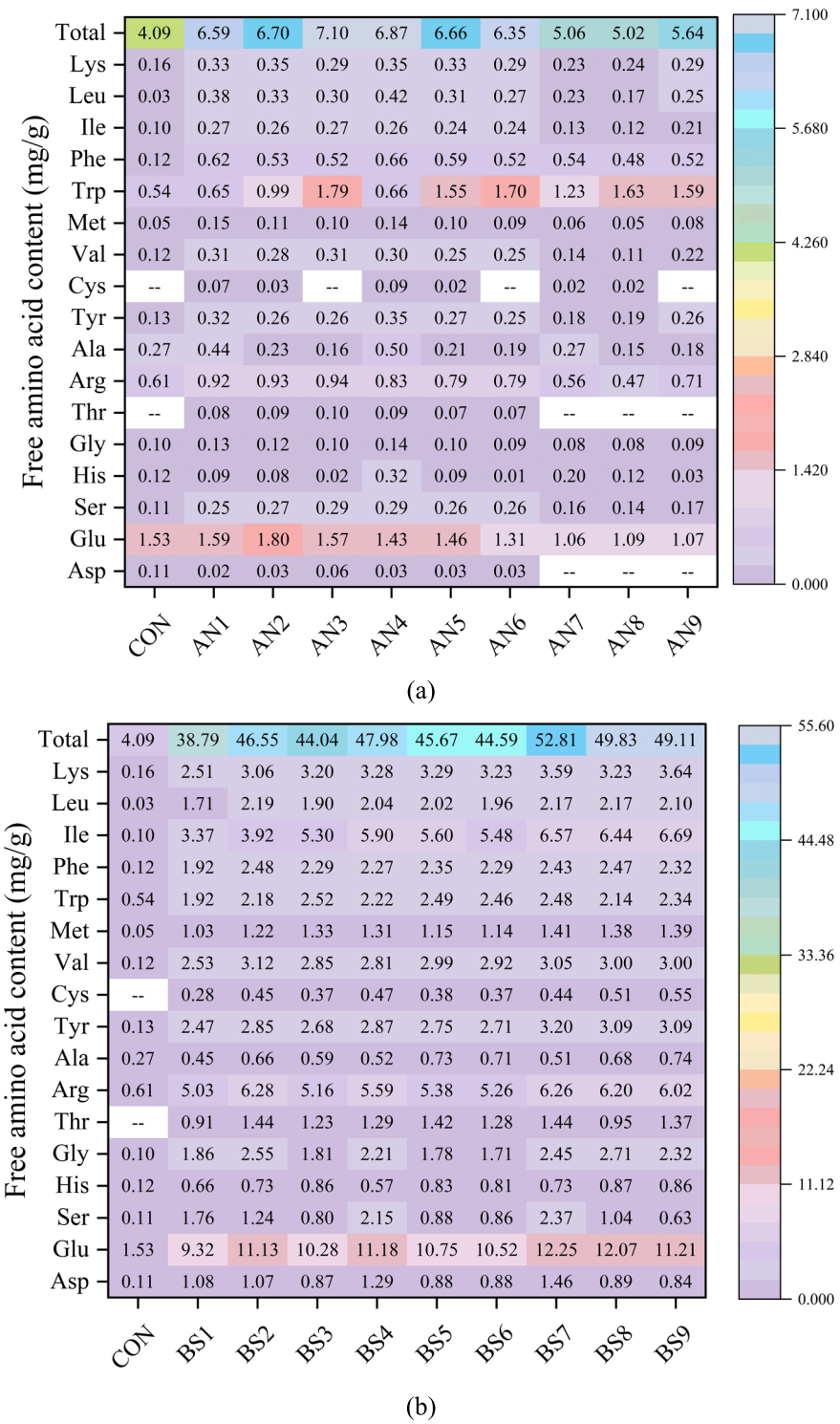


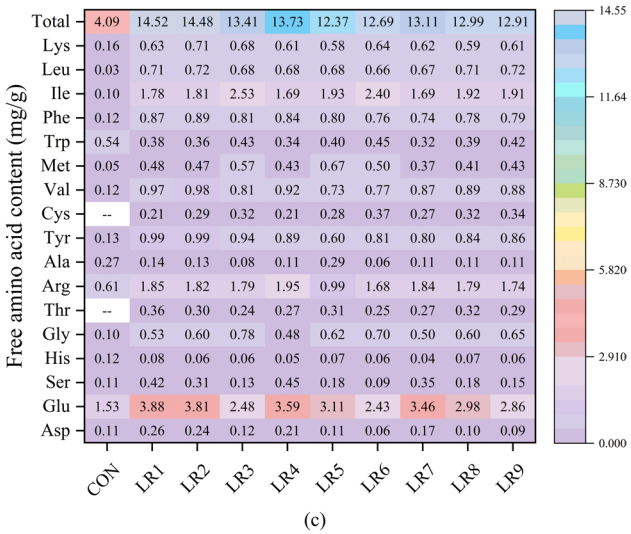
Fig. 4 Free amino acid content of fermented hempseed cake. **a** *Aspergillus niger* fermented hempseed cake; **b** *Bacillus subtilis* fermented hempseed cak; **c** *Lactobacillus rhamnoses* fermented hempseed cake. CON, control (unfermented sample); AN/BS/LR 1–9, solid-state fermented samples corresponding to the fermentation conditions listed in Table 1. The symbol "–" indicates that the amino acid content was not detected. Abbreviations: Asp, aspartic acid; Glu, glutamic acid; Ser, serine; His, histidine; Gly, glycine; Thr, threonine; Arg, arginine; Ala, alanine; Tyr, tyrosine; Cys, cysteine; Val, valine; Met, methionine; Trp, tryptophan; Phe, phenylalanine; Ile, isoleucine; Leu, leucine; Lys, lysine

metal cations (McGarrah & White, 2023). Furthermore, lysine content was significantly enhanced after fermentation. As lysine is an essential amino acid, improving its content in hempseed cake would enhance the nutritional value of the fermented hempseed product (Leonard et al., 2020).

Antioxidant Property of Fermented Hempseed Cake

Figure 5a illustrates the antioxidant property of fermented hempseed cake, as measured by ABTS radical scavenging antioxidant activity. Fermentation significantly ($P < 0.05$) enhanced radical scavenging in most samples, except for

Fig. 4 (continued)



(c)

AN3, AN6, and AN9, which showed insignificant change ($P > 0.05$). The BS- and LR-fermented groups exhibited similar activity, whereas the AN-fermented groups generally displayed lower levels. The highest activity was observed in LR8 and BS5, increasing from 1.78 mg TE/g in CON to 4.98 mg TE/g and 4.88 mg TE/g, respectively. In contrast, the AN group showed a comparatively low activity with 4.52 mg TE/g in AN4. Within each starter culture, fermentation conditions had a significant impact on the radical scavenging activity of the final products. For example, in the AN group, prolonged fermentation time (AN7, AN8, AN9) and higher water addition (AN3, AN6, AN9) resulted in decreased activity. The results align with Eliopoulos et al., (2022), who reported a decline in antioxidant activity with prolonged fermentation of hempseed cake with *Pleurotus ostreatus* for 9, 13, and 17 days.

The increase in radical scavenging activity after fermentation is consistent with previous studies. SSF of hemp protein isolates with *B. subtilis* has been shown to enhance free radical scavenging activity (Karabulut et al., 2023). Similarly, the fermentation of hempseed cake using commercial kefir and yogurt starter cultures, primarily composed of lactic acid bacteria, also led to increased free radical scavenging activity, aligning with the trends observed in LR-fermented samples (Łopusiewicz et al., 2022). However, comparative studies on the effects of different starter cultures remain limited. The enhanced antioxidant activity observed in fermented hempseed cake may be attributed to the proteolytic activity occurring during fermentation. This is supported by a strong positive correlation between ABTS scavenging activity and protein-related attributes, including soluble protein content, DH, and free amino acid content (Fig. 6). Hemp protein hydrolysates and peptides have been shown to exhibit antioxidant properties. Tang et al., (2009) reported increased antioxidant activity following enzymatic hydrolysis of hempseed proteins using six different proteases, which was attributed to the release of antioxidant peptides. Furthermore, certain amino acids such as methionine and cysteine are recognized for their antioxidant properties (Kim et al., 2020). As shown in Fig. 4, the levels of these amino acids increased after fermentation, which may have further contributed to the antioxidant activity of the fermented hempseed cake.

Alpha-Glucosidase Inhibitory Activity

The antidiabetic potential of fermented hempseed cake was assessed based on its ability to inhibit rat intestinal α-glucosidase. Intestinal α-glucosidases are membrane-bound enzymes that catalyze the release of glucose from maltose and other oligosaccharides derived from starch (Kumar et al., 2011). Inhibiting α-glucosidase can help regulate postprandial hyperglycemia, making it a promising strategy for diabetes management (Hossain et al., 2020). Due to the side effects of synthetic α-glucosidase inhibitors (e.g., acarbose), such as diarrhea, bloating, and nausea, there is growing interest in identifying natural and more patient-tolerable α-glucosidase inhibitors (Kumar et al., 2011).

As shown in Fig. 5b, fermented hempseed extract (1:10 w/v) exhibited significantly ($P > 0.05$) higher α-glucosidase inhibitory activity than the unfermented sample, though inhibition remained relatively low, not exceeding 30% in any sample. Despite lower soluble protein content and DH, the AN group exhibited relatively higher inhibition than the other two groups, with AN7 showing the greatest increase from 8.57% in the control (CON) to 26.05%. In the BS and LR groups, BS9 and LR9 displayed the highest activities at 25.13% and 22.01%, respectively. This result suggests that AN fermentation may produce more α-glucosidase

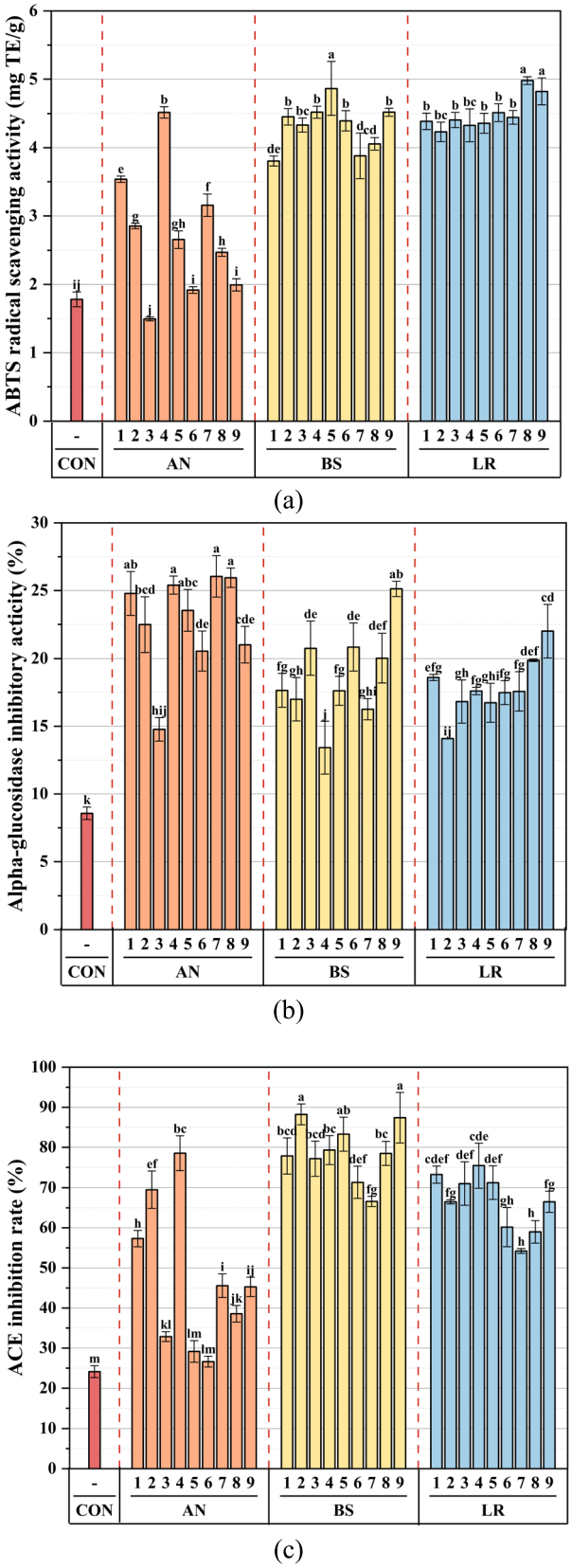


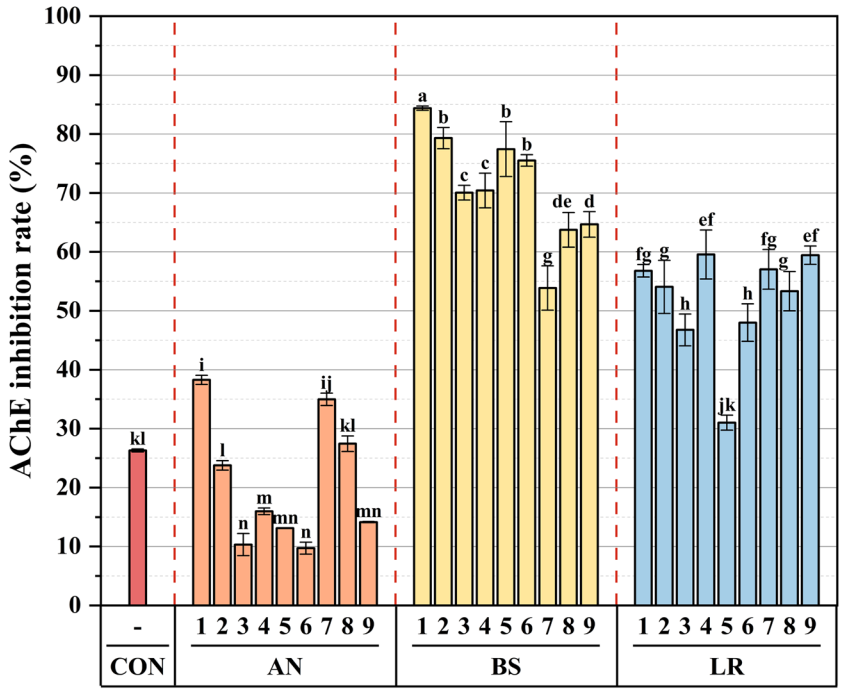
Fig. 5 Bioactivities of the protein fraction from fermented hempseed cake. **a** ABTS radical scavenging activity; **b** α-glucosidase inhibition activity; **c** ACE inhibition activity; **d** AChE inhibition activity. CON, control (unfermented sample); AN/BS/LR 1–9, solid-state fermented samples corresponding to the fermentation conditions listed in Table 1. Different letters indicate significant differences ($P < 0.05$)

inhibitory peptides with specific amino acid sequences compared to the other two starter cultures.

Fermentation-induced improvements in α-glucosidase inhibition have been linked to the production of medium- and short-chain peptides during fermentation in lupin, quinoa, and wheat fermented with *Lactobacillus* spp. (Ayyash et al., 2019). Previous research has also shown that protease-hydrolyzed hempseed protein exhibits significantly higher α-glucosidase inhibitory activity than the intact protein (Ren et al., 2016). Hemp protein hydrolysates prepared using the protease alcalase demonstrated strong α-glucosidase inhibition (58.26%) at a DH of 27.24% (Ren et al., 2016). This discrepancy suggests potential differences between microbial hydrolysis and enzymatic hydrolysis. As revealed by SDS-PAGE analysis, microbial hydrolysis retains macromolecular protein fractions in the soluble protein extract, which may interfere with α-glucosidase inhibition. Nevertheless, as shown in Fig. 5b, selected samples from AN and BS groups—namely AN1, AN4, AN7, AN8, and BS9—exhibited higher inhibitory activity. Peptide analysis suggests that the most effective α-glucosidase inhibitors are tri- to hexapeptides containing hydroxylated amino acids (e.g., serine, threonine, and tyrosine) at the N-terminal (Ibrahim et al., 2018). Both *A. niger* and *B. subtilis* are known producers of serine proteases, which may facilitate the generation of such bioactive peptides (Anand et al., 2025; Dong et al., 2021).

Angiotensin-Converting Enzyme (ACE) Inhibitory Activity

The antihypertensive potential of fermented hempseed cake was assessed by the inhibition of ACE activity. ACE inhibitors reduce the production of angiotensin II, leading to decreased vasoconstriction and lower blood pressure (Xue et al., 2021). Figure 5c presents the ACE inhibitory results. Fermentation significantly ($P < 0.05$) enhanced ACE inhibition in hempseed PBS extract (1:10 w/v) for all fermented samples, except for AN5 and AN6. Among the groups, BS-fermented samples exhibited the highest activity, with BS2 achieving an inhibition rate of 88.22%, compared to 24.13% in the unfermented control (CON). The AN group followed, with AN4 reaching a peak inhibition of 78.54%, while LR4 exhibited 75.45%. Similar high ACE inhibitory activities



(d)

Fig. 5 (continued)

(> 80%) have been reported in camel milk fermented with lactic acid bacteria (Ayyash et al., 2018). Fermentation conditions had a substantial impact on ACE inhibitory activity, prolonged fermentation time resulted in decreased ACE inhibitory activity in AN and LR groups (AN/LR7, 8, 9). This reduction may be attributed to the degradation of ACE inhibitory peptides within the fermented matrix over time.

Pearson correlation matrix shows a strong positive correlation between ACE inhibitory activity and protein attributes (Fig. 6), indicating that proteolysis during fermentation may contribute to enhanced ACE inhibition. Previous research has demonstrated that enzymatic hydrolysis of hempseed proteins using various proteases (e.g., AFP, HT, ProG, actinidin, and zingibain) could enhance ACE inhibition activity (Teh et al., 2016). Several ACE inhibitory peptides derived from hempseed protein have been identified. For instance, GVLY, LGV, and RVR have shown low IC_{50} values, highlighting their potential as antihypertensive agents (Orio et al., 2017). Similarly, Girgih et al., (2014) identified peptides including WVYY, WYT, SVYT, and IPAGV, with WVYY exhibiting the strongest ACE inhibition. Overall, hemp protein hydrolysates show strong potential as antihypertensive agents, and SSF presents a promising alternative to enzymatic hydrolysis for producing bioactive ACE inhibitory peptides.

Acetylcholinesterase (AChE) Inhibitory Activity

AChE is a critical enzyme responsible for breaking down the neurotransmitter acetylcholine in synaptic clefts, thus playing a vital role in nerve function (Sang et al., 2022). Inhibition of AChE is a significant therapeutic target for treating Alzheimer's disease and other neurodegenerative conditions (Sang et al., 2022). Figure 5d presents the AChE inhibitory activity of fermented hempseed PBS extract (1:10 w/v). In general, fermentation with BS and LR significantly ($P < 0.05$) enhanced inhibitory activity. The most notable increase was observed in BS1, where inhibition rose from 26.30% in the control to 84.39%. The LR group followed, with LR4 exhibiting the highest activity at 59.56%. In contrast, the AN group showed mixed results, with most samples displaying reduced AChE inhibitory activity after fermentation, except for AN1, which demonstrated a significant increase to 38.28%. Similarly, a previous study reported increased AChE inhibition in *Artemisia capillaris* fermented by the probiotic *Leuconostoc mesenteroides* (Choi et al., 2022).

In the BS and LR groups, the Pearson correlation matrix also indicates a positive correlation between AChE inhibitory activity and protein attributes, suggesting that the enhancement of AChE inhibitory activity in fermented

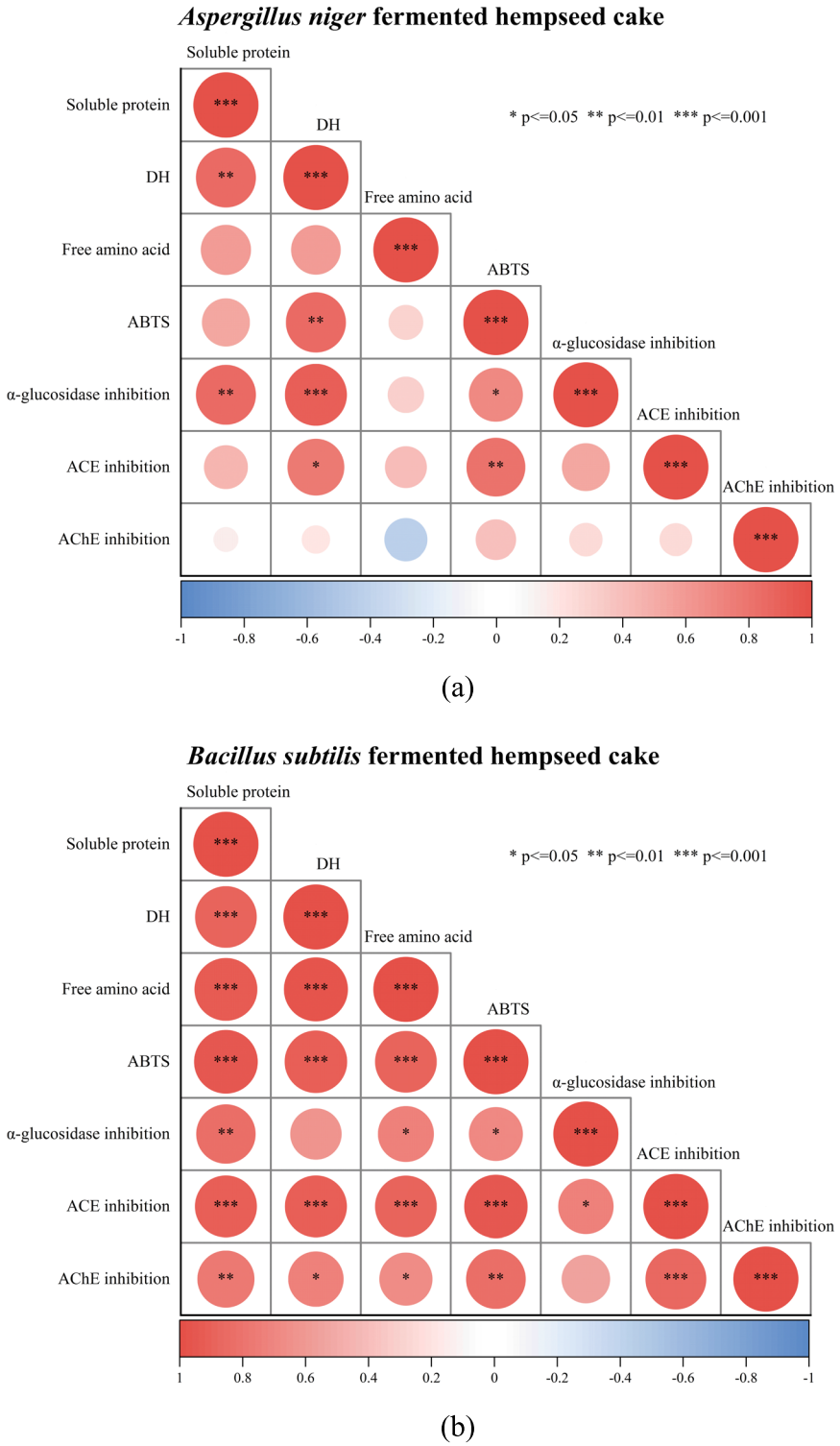
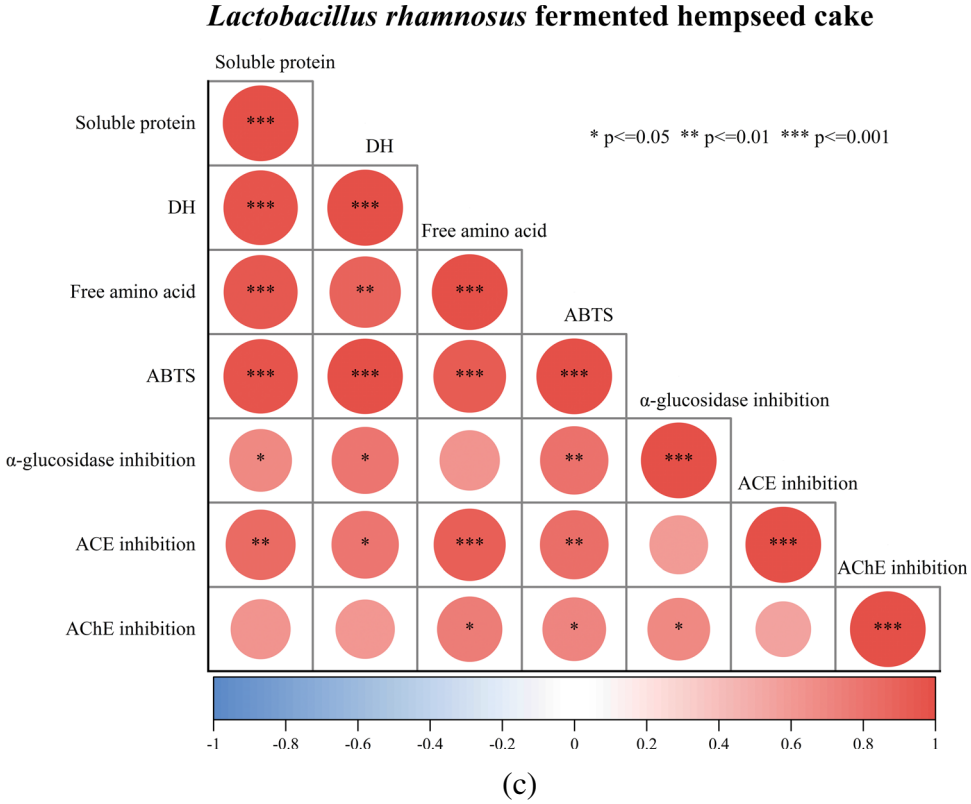


Fig. 6 Pearson correlation matrix of various attributes of solid-state fermented hempseed cake. **a** *Aspergillus niger*–fermented group, **b** *Bacillus subtilis*–fermented group, and **c** *Lactobacillus rhamnosus*–fermented group. DH, degree of hydrolysis

hempseed cake is driven by protease hydrolysis during fermentation (Fig. 6). However, the choice of starter culture and fermentation conditions is critical. In the AN group, despite the production of new peptides, no improvement in AChE inhibitory activity was observed. AChE inhibition depends largely on the type and sequence of amino acids in the peptide chain (Malomo & Aluko, 2016). Similar patterns have been observed in enzymatic hydrolysis of hempseed protein. For instance, 1% pepsin hydrolysis increased AChE inhibitory activity to 53.7%, whereas no inhibitory activity

Fig. 6 (continued)



was detected with 4% pepsin or 1% alcalase (Malomo & Aluko, 2016). Furthermore, previous studies have found that basic amino acids such as arginine, cystine, tyrosine, and lysine show stronger binding to AChE compared to other amino acids (Moreira et al., 2022). The increased content of these amino acids after fermentation (Fig. 4) may also contribute to the enhanced AChE inhibitory activity observed in this study.

Conclusion

This study demonstrated the potential of SSF as an efficient approach to improve the nutritional and bioactive properties of hempseed cake, an underutilized by-product. Fermentation significantly ($P < 0.05$) increased DH, soluble protein content, and free amino acid contents in all fermented samples. *B. subtilis* (BS) showed the most pronounced effects, with DH rising from 2.77 to 48.72%, soluble protein from 28.12 to 208.21 mg/g, and FAA from 4.09 to 52.81 mg/g. SDS-PAGE confirmed the breakdown of macromolecular proteins into smaller peptides. Bioactivity assessment revealed that *L. rhamnosus* (LR) enhanced antioxidant activity most effectively (ABTS increased to 4.98 mg TE/g in LR8), while BS achieved the highest ACE (88.22%) and AChE (84.39%) inhibition. *A. niger* (AN7) exhibited the strongest α-glucosidase inhibition (26.04%). These improvements were attributed to protein hydrolysis during fermentation, as supported by Pearson correlation analysis. Overall, *B. subtilis* outperformed the other two strains, and fermentation conditions within each culture significantly impacted the outcomes, emphasizing the need for process optimization.

Acknowledgements This research was made possible through the financial support of AgriFutures Australia as part of the Emerging Industries Australian Hemp Industry Program of Research – Theme 3, Project 3.1, to improve the health and nutritional value of hemp oil cake protein by fermentation. The authors would like to thank Southern Cross University for their role in managing the Australian Industrial Hemp Program of research and Charles Sturt University for their role in project management. We extend our gratitude to the Melbourne research scholarship of the University of Melbourne Scholarship.

Author Contribution X.F. and Z.F. conceptualized the study. X.F. planned and conducted the experiments, performed formal analysis, data curation, software analysis, and visualization, and wrote the original draft. Z.L. contributed to the development of methodology. K.N., S.A., and P.Z. supervised the project and contributed to reviewing and editing the manuscript. Z.F. provided supervision, resources, project administration, and secured funding. All authors provided critical feedback and helped shape the research and the final manuscript.

Funding Open. Access funding enabled and organized by CAUL and its Member Institutions. This study was funded by AgriFutures Australia.

Data Availability The authors declare that the data supporting the findings of this study are available within the paper. Data used to support the findings of this study are available from the corresponding authors upon request. No datasets were generated or analysed during the current study.

Declarations

Conflict of interest The authors declare no competing interests.

References

Anand, S. S., Nair, B. G., SadasivanNair, S., & GopalakrishnaPai, J. (2025). Proteases from marine endophyte, *Bacillus subtilis* ULB16: Unlocking the industrial potential of a marine-derived enzyme source. *Biocatalysis and Agricultural Biotechnology*, *64*, 103503.

Ayyash, M., Al-Nuaimi, A. K., Al-Mahadin, S., & Liu, S.-Q. (2018). In vitro investigation of anticancer and ACE-inhibiting activity, α-amylase and α-glucosidase inhibition, and antioxidant activity of camel milk fermented with camel milk probiotic: A comparative study with fermented bovine milk. *Food Chemistry, 239*, 588–597. https://doi.org/10.1016/j.foodchem.2017.06.149

Ayyash, M., Johnson, S. K., Liu, S.-Q., Mesmari, N., Dahmani, S., Al Dhaheri, A. S., & Kizhakkayil, J. (2019). In vitro investigation of bioactivities of solid-state fermented lupin, quinoa and wheat using *Lactobacillus* spp. *Food Chemistry, 275*, 50–58. https://doi.org/10.1016/j.foodchem.2018.09.031

Banerjee, G., & Ray, A. K. (2017). Impact of microbial proteases on biotechnological industries. *Biotechnology and Genetic Engineering Reviews, 33*(2), 119–143. https://doi.org/10.1080/02648725.2017.1408256

Chen, S., Wu, X., Duan, J., Huang, P., Li, T., Yin, Y., & Yin, J. (2021). Low-protein diets supplemented with glutamic acid or aspartic acid ameliorate intestinal damage in weaned piglets challenged with hydrogen peroxide. *Animal Nutrition, 7*(2), 356–364. https://doi.org/10.1016/j.aninu.2020.12.005

Chen, Q., Liu, B., Liu, G., Shi, H., & Wang, J. (2023). Effect of *Bacillus subtilis* and *Lactobacillus plantarum* on solid-state fermentation of soybean meal. *Journal of the Science of Food and Agriculture, 103*(12), 6070–6079. https://doi.org/10.1002/jsfa.12683

Choi, J., Yoon, J., & Kim, M. (2022). Optimization of fermentation conditions of *Artemisia capillaris* for enhanced acetylcholinesterase and butyrylcholinesterase. *Foods, 11*(15), 2268. https://doi.org/10.3390/foods11152268

Cruz-Casas, D. E., Aguilar, C. N., Ascacio-Valdés, J. A., Rodríguez-Herrera, R., Chávez-González, M. L., & Flores-Gallegos, A. C. (2021). Enzymatic hydrolysis and microbial fermentation: The most favorable biotechnological methods for the release of bioactive peptides. *Food Chemistry: Molecular Sciences, 3*, 100047. https://doi.org/10.1016/j.fochms.2021.100047

Dai, C., Ma, H., He, R., Huang, L., Zhu, S., Ding, Q., & Luo, L. (2017). Improvement of nutritional value and bioactivity of soybean meal by solid-state fermentation with *Bacillus subtilis*. *Lwt, 86*, 1–7. https://doi.org/10.1016/j.lwt.2017.07.041

de Castro, R. J. S., Nishide, T. G., & Sato, H. H. (2014). Production and biochemical properties of proteases secreted by *Aspergillus niger* under solid state fermentation in response to different agroindustrial substrates. *Biocatalysis and Agricultural Biotechnology, 3*(4), 236–245. https://doi.org/10.1016/j.bcab.2014.06.001

Dey, T. B., Chakraborty, S., Jain, K. K., Sharma, A., & Kuhad, R. C. (2016). Antioxidant phenolics and their microbial production by submerged and solid state fermentation process: A review. *Trends in Food Science & Technology, 53*, 60–74. https://doi.org/10.1016/j.tifs.2016.04.007

Docimo, T., Caruso, I., Ponzoni, E., Mattana, M., & Galasso, I. (2014). Molecular characterization of edestin gene family in *Cannabis sativa L. Plant Physiology and Biochemistry, 84*, 142–148. https://doi.org/10.1016/j.plaphy.2014.09.011

Dong, Z., Yang, S., & Lee, B. H. (2021). Bioinformatic mapping of a more precise Aspergillus niger degradome. *Scientific Reports, 11*(1), 693. https://doi.org/10.1038/s41598-020-80028-3

Eliopoulos, C., Markou, G., Chorianopoulos, N., Haroutounian, S. A., & Arapoglou, D. (2022). Preliminary research concerning the enrichment of industrial hemp extract residues via solid state fermentation with *Pleurotus ostreatus*. *Applied Sciences, 12*(5), 2376. https://doi.org/10.3390/app12052376

Feng, X., Ng, K., Ajlouni, S., Zhang, P., & Fang, Z. (2023). Effect of solid-state fermentation on plant-sourced proteins: A review. *Food Reviews International*, 1–38. https://doi.org/10.1080/87559129.2023.2274490

Gao, Y., Hu, M., Meng, W., Wen, W., Zhang, P., Fan, B., Wang, F., & Li, S. (2024). Study on the quality of soybean proteins fermented by Bacillus subtilis BSNK-5: Insights into nutritional, functional, safety, and flavor properties. *Food Chemistry, 443*, 138523. https://doi.org/10.1016/j.foodchem.2024.138523

Girgih, A. T., He, R., & Aluko, R. E. (2014). Kinetics and molecular docking studies of the inhibitions of angiotensin converting enzyme and renin activities by hemp seed (*Cannabis sativa L.*) peptides. *Journal of Agricultural and Food Chemistry*, *62*(18), 4135–4144. https://doi.org/10.1021/jf5002606

Hossain, U., Das, A. K., Ghosh, S., & Sil, P. C. (2020). An overview on the role of bioactive α-glucosidase inhibitors in ameliorating diabetic complications. *Food and Chemical Toxicology, 145*, 111738. https://doi.org/10.1016/j.fct.2020.111738

House, J. D., Neufeld, J., & Leson, G. (2010). Evaluating the quality of protein from hemp seed (Cannabis sativa L) products through the use of the protein digestibility-corrected amino acid score method. *Journal of agricultural and food chemistry, 58*(22), 11801–11807. https://doi.org/10.1021/jf102636b

Hussin, H., Hanafi, N. S., Lee, A. C., Salleh, M. M., Sam, S. C., & Abd-Aziz, S. (2022). Amino acids from oil producing plants. *Biorefinery of Oil Producing Plants for Value-Added Products, 2*, 653–671. https://doi.org/10.1002/9783527830756.ch32

Ibrahim, M. A., Bester, M. J., Neitz, A. W., & Gaspar, A. R. (2018). Structural properties of bioactive peptides with α-glucosidase inhibitory activity. *Chemical Biology & Drug Design, 91*(2), 370–379. https://doi.org/10.1111/cbdd.13105

Kaprasob, R., Kerdchoechuen, O., Laohakunjit, N., Sarkar, D., & Shetty, K. (2017). Fermentation-based biotransformation of bioactive phenolics and volatile compounds from cashew apple juice by select lactic acid bacteria. *Process Biochemistry, 59*, 141–149. https://doi.org/10.1016/j.procbio.2017.05.019

Karabulut, G., Kapoor, R., & Feng, H. (2023). Biotransformation approaches using solid-state fermentation and germination with

high-intensity ultrasound to produce added-value hemp protein nanoaggregates. *Food Bioscience, 56*, 103400. https://doi.org/10.1016/j.fbio.2023.103400

Kim, J.-H., Jang, H.-J., Cho, W.-Y., Yeon, S.-J., & Lee, C.-H. (2020). In vitro antioxidant actions of sulfur-containing amino acids. *Arabian Journal of Chemistry, 13*(1), 1678–1684.

Kumar, S., Narwal, S., Kumar, V., & Prakash, O. (2011). α-glucosidase inhibitors from plants: A natural approach to treat diabetes. *Pharmacognosy Reviews, 5*(9), 19. https://doi.org/10.4103/0973-7847.79096

Kumar, V., Ahluwalia, V., Saran, S., Kumar, J., Patel, A. K., & Singhania, R. R. (2021). Recent developments on solid-state fermentation for production of microbial secondary metabolites: Challenges and solutions. *Bioresource Technology, 323*, 124566. https://doi.org/10.1016/j.biortech.2020.124566

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227*(5259), 680–685.

Leonard, W., Zhang, P., Ying, D., & Fang, Z. (2020). Hempseed in food industry: Nutritional value, health benefits, and industrial applications. *Comprehensive Reviews in Food Science and Food Safety, 19*(1), 282–308. https://doi.org/10.1111/1541-4337.12517

Leonard, W., Zhang, P., Ying, D., Adhikari, B., & Fang, Z. (2021a). Fermentation transforms the phenolic profiles and bioactivities of plant-based foods. *Biotechnology Advances, 49*, 107763. https://doi.org/10.1016/j.biotechadv.2021.107763

Leonard, W., Zhang, P., Ying, D., Xiong, Y., & Fang, Z. (2021). Effect of extrusion technology on hempseed (Cannabis sativa L) oil cake: Polyphenol profile and biological activities. *Journal of Food Science, 86*(7), 3159–3175. https://doi.org/10.1111/1750-3841.15813

Lin, H., Zhou, B., Zhao, J., Liao, S., Han, J., Fang, J., Liu, P., Ding, W., Che, Z., & Xu, M. (2022). Insight into the protein degradation during the broad bean fermentation process. *Food Science & Nutrition, 10*(8), 2760–2772. https://doi.org/10.1002/fsn3.2879

Łopusiewicz, Ł, Waszkowiak, K., Polanowska, K., Mikołajczak, B., Śmietana, N., Hrebień-Filisińska, A., Sadowska, J., Mazurkiewicz-Zapałowicz, K., & Drozłowska, E. (2022). The effect of Yogurt and Kefir starter cultures on bioactivity of fermented industrial by-product from Cannabis sativa production—Hemp press cake. *Fermentation, 8*(10), 490. https://doi.org/10.3390/fermentation8100490

Magro, A. E. A., Silva, L. C., Rasera, G. B., & De Castro, R. J. S. (2019). Solid-state fermentation as an efficient strategy for the biotransformation of lentils: Enhancing their antioxidant and antidiabetic potentials. *Bioresources and Bioprocessing, 6*(1), 1–9. https://doi.org/10.1186/s40643-019-0273-5

Malomo, S. A., & Aluko, R. E. (2015). A comparative study of the structural and functional properties of isolated hemp seed (*Cannabis sativa L.*) albumin and globulin fractions. *Food Hydrocolloids, 43*, 743–752. https://doi.org/10.1016/j.foodhyd.2014.08.001

Malomo, S. A., & Aluko, R. E. (2016). In vitro acetylcholinesterase-inhibitory properties of enzymatic hemp seed protein hydrolysates. *Journal of the American Oil Chemists' Society, 93*, 411–420. https://doi.org/10.1007/s11746-015-2779-0

McGarrah, R. W., & White, P. J. (2023). Branched-chain amino acids in cardiovascular disease. *Nature Reviews Cardiology, 20*(2), 77–89. https://doi.org/10.1038/s41569-022-00760-3

Moreira, T. F. M., Pessoa, L. G. A., Seixas, F. A. V., Ineu, R. P., Gonçalves, O. H., Leimann, F. V., & Ribeiro, R. P. (2022). Chemometric evaluation of enzymatic hydrolysis in the production of fish protein hydrolysates with acetylcholinesterase inhibitory activity. *Food Chemistry, 367*, 130728.

Oles, P. J. (1993). Fractional factorial design approach for optimizing analytical methods. *Journal of AOAC International, 76*(3), 615–620. https://doi.org/10.1093/jaoac/76.3.615

Orio, L. P., Boschin, G., Recca, T., Morelli, C. F., Ragona, L., Francescato, P., Arnoldi, A., & Speranza, G. (2017). New ACE-inhibitory peptides from hemp seed (Cannabis sativa L) proteins. *Journal of agricultural and food chemistry, 65*(48), 10482–10488. https://doi.org/10.1021/acs.jafc.7b04522

Rambu, D., Dumitru, M., Ciurescu, G., & Vamanu, E. (2024). Solid-state fermentation using Bacillus licheniformis-driven changes in composition, Viability and In Vitro Protein Digestibility of Oilseed Cakes. *Agriculture, 14*(4), 639. https://doi.org/10.3390/agriculture14040639

Ren, Y., Liang, K., Jin, Y., Zhang, M., Chen, Y., Wu, H., & Lai, F. (2016). Identification and characterization of two novel α-glucosidase inhibitory oligopeptides from hemp (*Cannabis sativa L.*) seed protein. *Journal of Functional Foods, 26*, 439–450. https://doi.org/10.1016/j.jff.2016.07.024

Rosa, L. S., Santos, M. L., Abreu, J. P., Rocha, R. S., Esmerino, E. A., Freitas, M. Q., Mársico, E. T., Campelo, P. H., Pimentel, T. C., & Silva, M. C. (2023). Probiotic fermented whey-milk beverages: Effect of different probiotic strains on the physicochemical characteristics, biological activity, and bioactive peptides. *Food Research International, 164*, 112396. https://doi.org/10.1016/j.foodres.2022.112396

Sang, Z., Wang, K., Dong, J., & Tang, L. (2022). Alzheimer's disease: Updated multi-targets therapeutics are in clinical and in progress. *European Journal of Medicinal Chemistry, 238*, 114464. https://doi.org/10.1016/j.ejmech.2022.114464

Sonklin, C., Alashi, M. A., Laohakunjit, N., Kerdchoechuen, O., & Aluko, R. E. (2020). Identification of antihypertensive peptides from mung bean protein hydrolysate and their effects in spontaneously hypertensive rats. *Journal of Functional Foods, 64*, 103635. https://doi.org/10.1016/j.jff.2019.103635

Su, G., Chen, J., Huang, L., Zhao, M., Huang, Q., Zhang, J., Zeng, X., Zhang, Y., Deng, L., & Zhao, T. (2024). Effects of walnut seed coat polyphenols on walnut protein hydrolysates: Structural alterations, hydrolysis efficiency, and acetylcholinesterase inhibitory capacity. *Food Chemistry, 437*, 137905. https://doi.org/10.1016/j.foodchem.2023.137905

Tang, C.-H., Wang, X.-S., & Yang, X.-Q. (2009). Enzymatic hydrolysis of hemp (*Cannabis sativa L.*) protein isolate by various proteases and antioxidant properties of the resulting hydrolysates. *Food Chemistry*, *114*(4), 1484–1490. https://doi.org/10.1016/j.foodchem.2008.11.049

Teh, S.-S., Bekhit, A.E.-D.A., Carne, A., & Birch, J. (2016). Antioxidant and ACE-inhibitory activities of hemp (*Cannabis sativa L.*) protein hydrolysates produced by the proteases AFP, HT, Pro-G, actinidin and zingibain. *Food Chemistry, 203*, 199–206. https://doi.org/10.1016/j.foodchem.2016.02.057

Tian, Y., Zhang, R., Li, G., Zeng, T., Chen, L., Xu, W., Gu, T., Tao, Z., Du, X., & Lu, L. (2024). Microbial fermented feed affects flavor amino acids and yolk trimethylamine of duck eggs via cecal microbiota–yolk metabolites crosstalk. *Food Chemistry, 430*, 137008. https://doi.org/10.1016/j.foodchem.2023.137008

Wang, X.-S., Tang, C.-H., Yang, X.-Q., & Gao, W.-R. (2008). Characterization, amino acid composition and in vitro digestibility of hemp (Cannabis sativa L) proteins. *Food Chemistry, 107*(1), 11–18. https://doi.org/10.1016/j.foodchem.2007.06.064

Wang, R., Dong, P., Zhu, Y., Yan, M., Liu, W., Zhao, Y., Huang, L., Zhang, D., & Guo, H. (2021). Bacterial community dynamics reveal its key bacterium, Bacillus amyloliquefaciens ZB, involved in soybean meal fermentation for efficient water-soluble protein

production. *Lwt, 135*, 110068. https://doi.org/10.1016/j.lwt.2020.110068

Xue, L., Yin, R., Howell, K., & Zhang, P. (2021). Activity and bioavailability of food protein-derived angiotensin-I-converting enzyme–inhibitory peptides. *Comprehensive Reviews in Food Science and Food Safety, 20*(2), 1150–1187. https://doi.org/10.1111/1541-4337.12711

Yang, H., Qu, Y., Li, J., Liu, X., Wu, R., & Wu, J. (2020). Improvement of the protein quality and degradation of allergens in soybean meal by combination fermentation and enzymatic hydrolysis. *Lwt, 128*, 109442. https://doi.org/10.1016/j.lwt.2020.109442

Yao, S., Li, W., Wu, Y., Martin, G. J., & Ashokkumar, M. (2023). The impact of high-intensity ultrasound-assisted extraction on the structural and functional properties of hempseed protein isolate (HPI). *Foods, 12*(2), 348. https://doi.org/10.3390/foods12020348

Yoon, H.-J., Park, G.-H., Lee, Y.-R., Lee, J.-M., Ahn, H.-L., & Lee, S.-O. (2023). Enzymatic preparation and antioxidant activities of protein hydrolysates from hemp (*Cannabis sativa L*) seeds. *Food Science and Preservation*, *30*(3), 434–445. https://doi.org/10.11002/kjfp.2023.30.3.434

Yu, X., Chen, Y., Qi, Z., Chen, Q., Cao, Y., & Kong, Q. (2023). Preparation and identification of a novel peptide with high antioxidant activity from corn gluten meal. *Food Chemistry, 424*, 136389. https://doi.org/10.1016/j.foodchem.2023.136389