

Article

Microbial Bioprocessing of Sunflower Protein Extracts: Nutritional, Techno-Functional and Bioactive Outcomes of *Lactobacillus helveticus* and *Bacillus subtilis* Fermentation Products

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Abstract

Fermentation has significant potential for valorizing de-oiled sunflower meal (DSM) protein for human consumption. Here, DSM extracts were fermented for 48 h with *Lactobacillus helveticus* B-4526 and *Bacillus subtilis* B-3387, and their nutritional, bioactive, and techno-functional properties were evaluated. DSM extract supported the growth of both organisms, with cell numbers increasing by >1.5 log CFU/mL for *B. subtilis* and approximately 1 log CFU/mL for *L. helveticus*. During fermentation, *B. subtilis* degraded protein bands in the 23–46 kDa range, whereas *L. helveticus* promoted protein aggregation due to acidification, which may have reduced protein solubility. Antioxidant activities (DPPH, ABTS, and CUPRAC assays) and ACE-inhibitory activity increased in both fermentations, with *B. subtilis* showing the strongest effect. FTIR analysis indicated fermentation-induced alterations in the protein secondary structure. The total amino acid content decreased after *B. subtilis* fermentation but remained similar after *L. helveticus* fermentation. Phytic acid present in the meal decreased as a result of extraction/fermentation workflow. *B. subtilis* fermentation improved solubility at pH 4.0 but reduced foaming and emulsifying properties. Both fermentations decreased the oil-holding and water-holding capacities. Overall, this study underscores the need to match the fermenting microorganism to the desired outcome, as enhancements in one property often involve trade-offs with others.

Keywords: upcycling by-product; fermentation; antioxidant activity; techno-functional properties; amino acid composition



Academic Editor: Yuthana
Phimolsiripol

Received: 12 June 2026

Revised: 10 July 2026

Accepted: 12 July 2026

Published: 30 July 2026

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1. Introduction

The global food system accounts for approximately 30% of anthropogenic greenhouse gas emissions, making it a primary driver of climate change. Most of these emissions (60%) come from animal protein production; livestock farming uses 77% of agricultural land but provides only 18% of the global calories and 37% the of protein [1]. With the population expected to reach 10 billion by 2050 and protein demand expected to double, the current production model is unsustainable [2]. This necessitates the transformation of the food system based on high resource efficiency and a circular bioeconomy.

Oilseed meals, agro-industrial by-products of oil extraction, represent a valuable and largely underutilized source of protein for food diversification strategies. Sunflower (*Helianthus annuus* L.) is one of the most widely produced oil crops globally, ranking after soybean and rapeseed, and generating substantial quantities of de-oiled sunflower meal (DSM) as a co-product [3]. DSM typically contains crude protein ranging from 30 to 50%, depending on agricultural, genotypic, and processing conditions, and presents a relatively balanced amino acid profile, with lysine as the main limiting amino acid [4,5].

Despite its nutritional potential, its use remains largely confined to animal feeds. A major limiting factor is the high content of phenolic compounds, particularly chlorogenic acid, which binds to DSM proteins. Under alkaline extraction conditions, this interaction intensifies, resulting in green-colored protein isolates that are poorly accepted for food applications [3,4,6]. In addition, the phytic acid present in DSM may further limit the nutritional and functional quality of the proteins by chelating minerals and interacting with proteins, thereby reducing digestibility and potentially affecting solubility and other techno-functional properties [7,8].

Techno-functional properties are also critical determinants of food applications, as they define the suitable use of protein ingredients in different food systems. Properties such as solubility, water- and oil-holding capacity, emulsification, and foaming govern protein behavior during processing and influence the texture, stability, and mouthfeel of the final product [9–11]. Therefore, it is critical to investigate the techno-functional properties of DSM proteins to determine their potential applications.

Microbial fermentation has emerged as a promising bioprocessing strategy for modifying plant proteins [12]. In particular, lactic acid bacteria (LAB) and *Bacillus* species have gained attention for their strong proteolytic activity and capacity to enhance bioactivity and functional properties. *Lactobacillus helveticus* is recognized for its efficient proteolytic system and ability to generate bioactive peptides during fermentation, especially in dairy systems [13]. *Bacillus subtilis*, on the other hand, is known for its extensive extracellular enzyme secretion and broad substrate range, widely used in solid-state fermentation of plant materials [14,15].

The effects of fermentation on the nutritional composition, phenolic content, bioactive properties, molecular structure, and techno-functional properties of DSM protein extracts remain poorly understood, particularly when *Lactobacillus helveticus* and *Bacillus subtilis* are used as single fermentation agents. Therefore, this study aimed to evaluate the impact of fermentation with *L. helveticus* and *B. subtilis* on the amino acid composition, total phenolic content, bioactive properties, phytic acid content and structural characteristics (assessed by FTIR spectroscopy) of DSM protein extracts, as well as their techno-functional properties.

2. Materials and Methods

2.1. Materials

Trakya Birlik (Edirne, Türkiye) provided de-oiled sunflower meal. The meal was ground into a fine powder using a coffee grinder (Kiwi KSPG 4812, İstanbul, Türkiye) and sieved through a 50-mesh screen. The proximate composition of the de-oiled sunflower meal (DSM) was $27.7 \pm 0.5\%$ protein, $0.6 \pm 0.01\%$ lipid, $10.2 \pm 0.4\%$ moisture, 5.8% ash, and 55.7% carbohydrate, as measured in our previous work [5]. All chemicals used in this study were of analytical grade, unless otherwise indicated.

2.2. Preparation of Extraction for Fermentation

An alkaline extraction procedure was employed to extract proteins from the DSM. Briefly, DSM was dispersed in deionized water (8% *w/v*) and the pH was adjusted to 9.0. The solution was homogenized with an ultra-turrax (Wiggins D500, Beijing, China) at

14,500 rpm for 40 s at room temperature and stirred at 130 rpm and 35 °C for 45 min for extraction to take place. The extract was centrifuged at $5300\times g$ for 20 min at 4 °C. The supernatant was collected for fermentation and autoclaved at 121 °C for 15 min.

2.3. Fermentations

Bacillus subtilis (B-3387) and *Lactobacillus helveticus* (B-4526) were cultivated in TGY and MRS broth, respectively, for 16–20 h prior to fermentation. Active cultures were separately inoculated into the DSM extracts at 5% (*v/v*). Fermentations with *B. subtilis* were conducted at 28 °C and 130 rpm, whereas *L. helveticus* fermentations were carried out at 37 °C and 100 rpm. Each fermentation was conducted in three biological replicates. Samples were collected at 0, 24, and 48 h to monitor microbial growth, pH, changes in soluble protein content, and protein profile via electrophoresis.

At the end of fermentation, the pH of each sample was adjusted to 8.0 to maximize protein solubility. The samples were then centrifuged at $5300\times g$ for 30 min at 4 °C to remove microbial cells and insoluble components. The resulting supernatants were concentrated using a rotary evaporator (Wiggins, STRIKE 280, Beijing, China) and lyophilized using a freeze-dryer (Teknosem, TRS 4-4V, İstanbul, Türkiye).

The resulting powders were analyzed for their nutritional, functional, and bioactive properties. Unfermented counterparts were prepared using the same procedure, but without microbial inoculation. Since the unfermented control for *B. subtilis* fermentations was maintained at 28 °C and that for *L. helveticus* at 37 °C, a preliminary statistical analysis (One-Way ANOVA) was performed to evaluate the impact of these incubation temperatures on the control extracts. No statistically significant differences ($p > 0.05$) were observed between the two thermal conditions across the measured parameters, with the sole exception of a minor variance in phytic acid content. Therefore, to enhance readability and avoid redundancy, the data from both conditions were pooled and reported as a single baseline “Unfermented DSM extract”.

2.4. Monitoring Microbial Growth and pH

Microbial growth was monitored using the pour plate technique. Briefly, 1 mL of each sample was serially diluted in sterile 0.85% (*w/v*) NaCl solution and plated on TGY agar for *B. subtilis* and MRS agar for *L. helveticus* quantification. The plates were incubated at 28 °C for 24 h (*B. subtilis*) and 37 °C for 48 h (*L. helveticus*). Colonies were counted at the end of the incubation period, and the results were expressed as log colony-forming units per milliliter (log CFU/mL). The pH of the fermentation samples was measured at each time point using a calibrated pH meter (Mettler-Toledo, Milan, Italy).

2.5. Total Soluble Protein Concentration

Total soluble protein concentration was measured according to Lowry et al. [16] using bovine serum albumin as a standard and measured using a spectrophotometer (Shimadzu, UV-1280, Kyoto, Japan).

2.6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The molecular weight distribution of the samples was determined by SDS-PAGE analysis using the mPAGE[®] Lux Casting System (Merck, Darmstadt, Germany). A 13.5% Bis-Tris polyacrylamide gel (1.0 mm) was prepared according to the manufacturer's protocol using the mPAGE[®] Lux reagent kit. The protein concentration of the samples was determined using the method described by Lowry et al. [16] to ensure equal protein loading into the wells. Samples (70 µg protein/well for *B. subtilis* fermentation; 50 µg/well for *L. helveticus* fermentation) were mixed (1:1) with a sample buffer containing 4 mg bromophenol blue, 0.6 mL 1 M Tris-HCl (pH 6.8), 2.5 mL glycerol, and 3 mL 10% SDS, brought to 10 mL

with deionized water, and heated for 10 min at 70 °C in a heating block. Electrophoresis was performed using a MOPS-SDS running buffer at 80 V for 15 min, followed by 130 V for 60 min. After electrophoresis, the gels were stained with Coomassie Brilliant Blue G-250 (Fisher Scientific, BP100-25, Waltham, MA, USA), and excess stain was removed using a destaining solution until clear band visualization was obtained. The mPAGE™ Color Protein Standard (10–203 kDa) (Merck, Darmstadt, Germany) was used as the molecular weight marker.

2.7. Total Amino Acid Content

The experimental procedures for the amino acid analysis of the unfermented DSM extract or fermented samples (cell-free extracts) were identical to those of the protein concentrates described previously [5]. Briefly, 10 mg of unfermented DSM or fermented samples was hydrolyzed under nitrogen in 5 mL of 6 M HCl at 110 °C for 24 h. Chromatography was performed using an Agilent 1260 Infinity HPLC System (Agilent Technologies Inc., Folsom, CA, USA) consisting of an autosampler, a UV detector set at 338 nm (for OPA-amino acids) and 262 nm (for FMOc-amino acids), and a ZORBAX Eclipse-AAA column (4.6 × 150 mm, with 3.5 µm particle size) maintained at 25 °C. The two eluents were solvent A (40 mM sodium phosphate buffer, pH 7.8) and solvent B (a mixture of acetonitrile, methanol, and water in a 45:45:10 ratio). Finally, the amino acid composition was expressed as g amino acids/100 g protein. Additionally, the tryptophan content was not determined because of its degradation under acid hydrolysis conditions.

2.8. Determination of Functional Groups by Fourier-Transform Infrared (FTIR) Spectroscopy

The FTIR spectra of the unfermented DSM extract or fermented samples were recorded using an FTIR spectrometer (Perkin Elmer 100, Waltham, MA, USA) over a wavenumber range of 4000 cm^{−1} to 550 cm^{−1} at a resolution of 4 cm^{−1} with four scans per sample. Prior to analysis, the samples were prepared as attenuated total reflectance (ATR) pellets for analysis. The obtained spectra were subsequently baseline-corrected, normalized, and visualized using OriginPro 2018 software (OriginLab Corp., Northampton, MA, USA).

2.9. Analysis of Total Phenolic Content and Bioactive Properties

The total phenolic content was determined spectrophotometrically using the Folin–Ciocalteu's phenol reagent, as previously reported [17], and is presented as mg gallic acid per gram of sample.

Antioxidant activity was evaluated using different assays: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) inhibition assay, 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay, and cupric ion reducing antioxidant capacity (CUPRAC).

DPPH radical inhibition activity was evaluated according to the procedure described by Silveira Coelho et al. [18] with slight modifications. Briefly, 500 µL of the sample was mixed with 500 µL of 0.2 mM DPPH solution and kept in the dark for 45 min. The absorbance was measured using a UV–Vis spectrophotometer (Shimadzu UV-1280, Kyoto, Japan) at 515 nm. The results were expressed as mg Trolox per gram of sample.

The ABTS radical scavenging assay was performed as described by Re et al. [19]. Briefly, a 7 mM ABTS solution was kept at room temperature in the dark for 12–16 h. The absorbance of the radical solution was adjusted to 0.70 ± 0.02 at 734 nm with phosphate-buffered saline before analysis. Absorbance values were recorded every minute for a total of six minutes using a spectrophotometer. The results were expressed as mg Trolox per gram of sample.

The CUPRAC assay was performed as described by Apak et al. [20]. A reaction mixture was prepared by combining 1 mL each of 0.01 M CuCl₂, ethanolic 7.5 × 10^{−3} N

neocuproine, and 1.0 M ammonium acetate buffer (pH 7.0). The sample (125 µL) and distilled water (975 µL) were added, and the mixture was incubated in the dark for 30 min. The absorbance was measured at 450 nm (Shimadzu UV-1280, Kyoto, Japan), and the results were expressed as mg Trolox per g of sample.

ACE-inhibitory activity was measured using a method based on the release of hippuric acid (HA) from the reaction of hippuryl-His-Leu (HHL) with ACE enzyme. The peaks for HA and HHL were monitored at a wavelength of 228 nm, and the results were expressed as a percentage of inhibition [21].

2.10. Determination of Techno-Functional Properties

The techno-functional properties of the samples were investigated in terms of their solubility, foaming capacity and stability, emulsifying properties, and oil- and water-holding capacities.

The solubility was evaluated using the method described by Dong et al. [22]. Briefly, the pH of 1% (*w/v*) sample dispersions was adjusted to 4, 7, and 9 using 1 M HCl or 1 M NaOH. The solutions were shaken at ambient temperature for 30 min and then centrifuged at $5300 \times g$ for 30 min. Supernatants were collected, and the soluble protein concentration was analyzed using the method described by Lowry et al. [16]. Solubility was expressed as the percentage of soluble protein in the dispersion.

The foaming capacity and stability were measured according to the protocol described by Abd Rahim et al. [23]. A sample solution (1%, *w/v*) was prepared, and the pH of the solution was adjusted to 4, 7 and 9, using either 0.1 M HCl or 0.5 M NaOH. The mixture was homogenized for 2 min at 10,000 rpm using a mechanical homogenizer (Ultra-turrax, Wiggins D500, Beijing, China). The height of the solution before and after homogenization was measured using a ruler and used to calculate the foaming capacity. The foam height was recorded after keeping the sample at room temperature for 15 and 30 min to calculate foaming stability.

The emulsifying properties of the products were evaluated for emulsifying activity index (EAI) and emulsifying stability index (ESI) at pH 7 by using the method described by Abd Rahim et al. [23] with minor modifications. A total of 9.0 mL of 0.1% protein solution was dispersed in 3.0 mL of sunflower oil. The mixtures were homogenized for 2 min at 19,000 rpm (Ultra-turrax, Wiggins D500, Beijing, China). Fifty microliters of the emulsion were pipetted from the bottom of the beaker at 0 and 10 min and mixed with 3.95 mL of 0.1% SDS. The absorbance of the emulsions was measured at a wavelength of 500 nm (Shimadzu UV-1280). EAI and ESI were calculated using the following equations:

$$EAI \left(\frac{\text{m}^2}{\text{g}} \right) = \frac{2 \times 2.303 \times A_0 \times N}{C \times \phi \times 10000} \quad (1)$$

$$ESI \text{ (min)} = \frac{A_0}{A_0 - A_{10}} \times t \quad (2)$$

where N is the dilution factor (80), C is the protein concentration (0.001), and ϕ is the oil fraction (0.25). A_0 and A_{10} represent the absorbances of the mixture at 0 and 10 min, respectively.

The oil- and water-holding capacities were measured according to the method described by Lawal [24]. Briefly, 0.5 g of the sample and 5 mL of deionized water or sunflower oil were mixed in a pre-weighed tube. The suspension was vortexed for 1 min at room temperature. The mixture was allowed to stand for 30 min at 30 °C in an incubator. The suspension was centrifuged at $5300 \times g$ for 30 min. The supernatant was discarded, and the tubes were inverted for 10 min. The tubes containing the sediments were weighed. The

water-holding capacity (WHC) or oil-holding capacity (OHC) was calculated as grams of water or oil per gram of sample.

2.11. Measurement of Phytic Acid Content

The phytic acid content of all samples was determined according to the colorimetric method of Haug and Lantzsch [25] with minor modifications. Briefly, 0.1–0.2 g of each sample was extracted with 5 mL of Na₂SO₄ solution prepared in 0.4 M HCl by shaking at 250 rpm overnight, followed by centrifugation at 1655× *g* for 20 min. An aliquot (1 mL) of the supernatant was transferred into two parallel screw-cap test tubes, mixed with 2 mL of ferric reagent (ammonium iron (III) sulfate dodecahydrate dissolved in 2 N HCl), vortexed, and incubated in a boiling water bath at 95 °C for 30 min. After cooling in an ice bath for 15 min and equilibrating to room temperature, the samples were centrifuged again at 1655× *g* for 10 min. Subsequently, 1 mL of the clarified supernatant was mixed with 3 mL of 1% (*w/v*) 2,2'-bipyridine solution containing thioglycolic acid, and the absorbance was measured at 519 nm using a UV-Vis spectrophotometer. Sodium phytate (C₆H₆O₂₄P₆Na₁₂) was used as the reference standard, and the phytic acid content was calculated and expressed as µg per 1 g of sample.

2.12. Statistical Analysis

All experiments were conducted in triplicate, and results are represented as means (*n* = 3) ± standard deviation. Statistical analyses were performed using Minitab 17 (Minitab Inc., State College, PA, USA). One-way analysis of variance (ANOVA) was followed by Tukey's HSD test for multiple comparisons with a confidence level of 95% (differences were considered significant at *p* < 0.05).

3. Results and Discussion

3.1. Growth of *Bacillus subtilis* and *Lactobacillus helveticus* in Sunflower Meal Extract and Associated pH Changes During Fermentation

Both *B. subtilis* and *L. helveticus* exhibited growth during the fermentation period (Figure 1). *B. subtilis* reached a maximum increase of more than 1.5 log CFU/mL in total viable cell count within 24 h, whereas *L. helveticus* showed an approximately 1 log CFU/mL increase over the same period, indicating that the DSM extract served as a suitable growth medium for both organisms. Beyond 24 h, the cell counts remained stable, indicating entry into the stationary phase. The peak in viable cell numbers at 24 h was consistent with that reported by Karakurt et al. [26], who reported a similar growth pattern during the solid-state fermentation of DSM with *B. subtilis*. However, that study reported a markedly higher increase of approximately 3 log CFU/g, which may be attributed to the use of a different *B. subtilis* strain and controlled bioreactor conditions, including aeration, pH regulation, and mechanical agitation, which were absent in the present flask-scale fermentations.

Regarding pH changes, the medium had an initial pH of approximately 7.5 after autoclave sterilization. Upon inoculation, the pH decreased slightly to 7.2 in the *B. subtilis* culture, whereas in the *L. helveticus* culture, it dropped markedly to 5.6, likely due to the acidic nature of the activated inoculum. After 24 h of fermentation, both cultures exhibited further acidification, reaching pH values 6.7 for *B. subtilis* and 4.4 for *L. helveticus*. This initial acidification in the *B. subtilis* culture is consistent with the preferential utilization of readily available soluble carbohydrates via glycolysis, transiently generating organic acids before the onset of extensive proteolysis [26,27]. Beyond this point, divergent trends were observed. The pH of the *B. subtilis* fermentation increased to 7.5, consistent with extracellular protein degradation and subsequent ammonia release from amino acid deamination, a well-documented alkalization mechanism in *Bacillus*-fermented protein-rich

substrates [28]. On the other hand, the *L. helveticus* culture stabilized at 4.7. To standardize the samples prior to further analysis and to maximize protein solubility, the pH of the fermented products was then adjusted to 8.0.

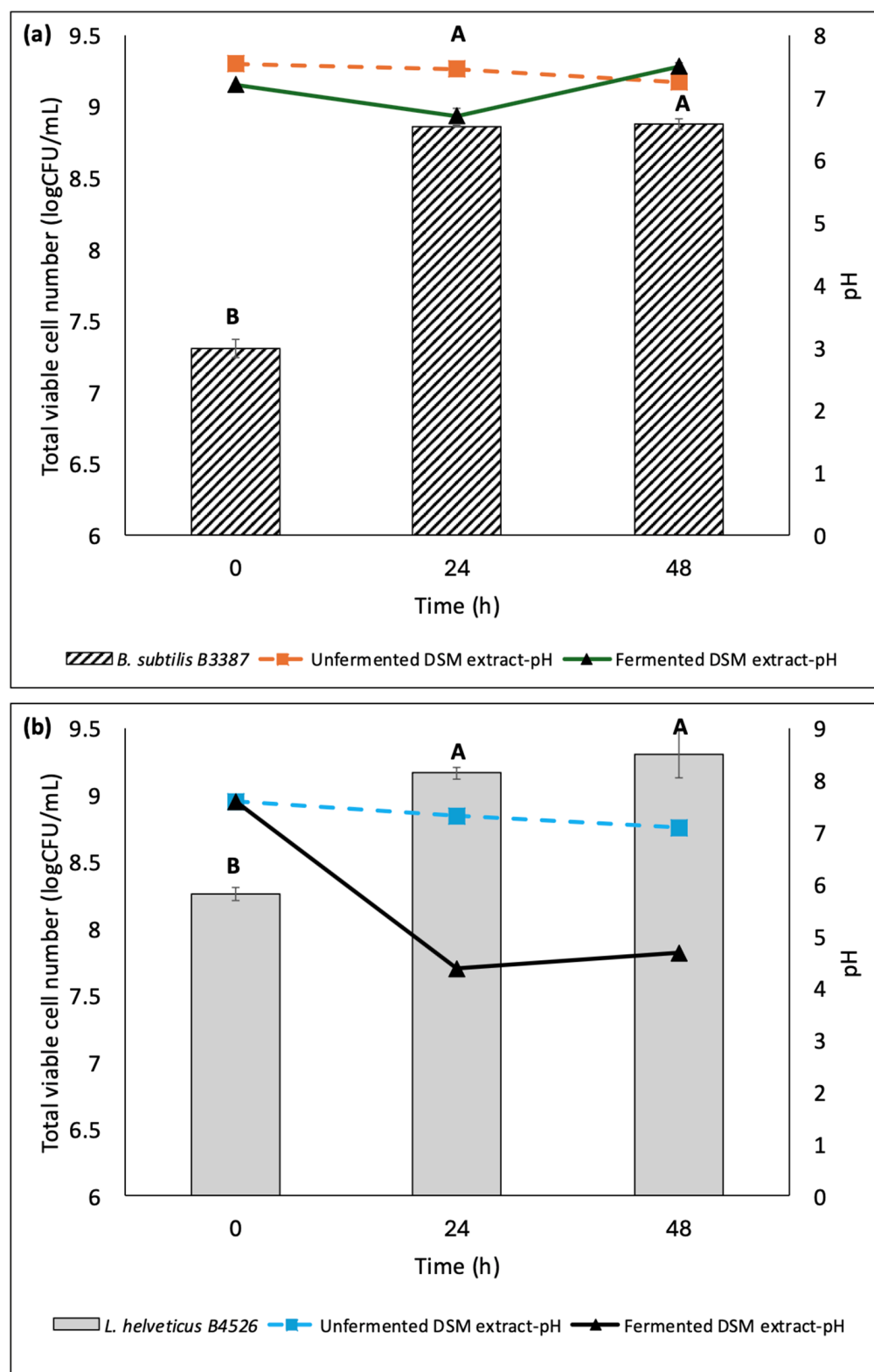


Figure 1. Growth and pH changes of *Bacillus subtilis* B3387 (a) and *Lactobacillus helveticus* B4526 (b) during 48 h fermentation of de-oiled sunflower meal (DSM) protein extracts. ^{A,B} Uppercase letters indicate significant changes in the total viable cell number during fermentation ($p < 0.05$).

3.2. Changes in Total Soluble Protein Concentration

Changes in the total soluble protein concentration (TSP) were monitored during fermentation. At the start of fermentation, the medium contained 12.7 g/L of soluble protein. Following inoculation, TSP decreased sharply to 6.9 g/L in *L. helveticus* fermentations, likely due to the acidic nature of the inoculum, as protein solubility is strongly influenced by pH. After 24 h, TSP further declined to 9.6 g/L in the *B. subtilis* fermentation and 4.6 g/L in the *L. helveticus* fermentation, with little change observed thereafter (9.7 and 4.5 g/L, respectively, at 48 h). These results indicate that the acidic conditions during *L. helveticus* fermentation markedly reduced protein solubility. However, after adjusting the pH to 8.0 post-fermentation, the TSP of the *L. helveticus* medium increased to nearly 8.0 g/L, whereas no statistically significant change was observed for *B. subtilis* (Table S1, Supplementary Materials). This outcome is consistent with the typical U-shaped solubility pattern of sunflower proteins, whereby solubility is minimum at the near-isoelectric point (approximately pH 4) and increases beyond that point (Section 3.8.2) [5,29]. This suggests that, for lactic acid fermentations, washing cells with sterile saline prior to inoculation may be preferable to the direct transfer of culture to minimize the initial loss of soluble protein. Moreover, adjusting pH post-fermentation can enhance the soluble protein content in the final product.

3.3. Protein Profiles by SDS-PAGE

The protein profiles of the unfermented and fermented DSM extracts are shown in Figure 2. Sunflower seeds are primarily composed of 11S globulins (helianthinin) and 2S albumins, with the latter being smaller and water-soluble. Prominent bands between 23 and 46 kDa corresponded to the α and β subunits of dissociated 11S globulins, consistent with previously reported profiles of sunflower protein concentrates [30]. In *B. subtilis* fermentations, inoculation did not alter the protein profile at 0 h, confirming that the inoculation procedure itself did not introduce physical or chemical artifacts. However, at 24 h, the bands between 23 and 46 kDa were completely absent, reflecting rapid and extensive proteolysis driven by the protease activity of *B. subtilis* [26].

Fermentation with *L. helveticus* produced a markedly different pattern, which was complicated by the acidic nature of the inoculum. Following inoculation, bands in the 23–46 kDa region disappeared, suggesting acid-induced protein aggregation as solubility is minimum near the isoelectric point [5,29]. To confirm this, the pH was adjusted to 8 after inoculation, and the bands reappeared, supporting aggregation as the primary cause of band disappearance. During fermentation, the acidic environment generated by lactic acid production further promoted aggregation, with no visible bands at 24 h or 48 h. Post-fermentation pH adjustment restored the 23–46 kDa bands, confirming that *L. helveticus* induced only limited proteolysis compared to *B. subtilis*.

3.4. Total Phenolic Content and Bioactive Properties

The total phenolic content (TPC) increased significantly following fermentation with both *L. helveticus* and *B. subtilis* compared to that in the unfermented control (Table 1). This increase is likely attributable to the release of bound phenolics from phenolic–protein complexes through different microbial enzymatic activities (e.g., carbohydrate-hydrolyzing enzymes, esterases, and tannases) during fermentation [12,31]. Guglielmetti et al. [32] demonstrated that *L. helveticus* possesses cinnamoyl esterase activity capable of hydrolyzing the ester bond in chlorogenic acid (CGA) to release free caffeic acid, thereby supporting the increase in TPC observed in *L. helveticus*-fermented DSM extracts. Pöri et al. [33] further showed that *L. helveticus* fermentation decreases CGA content while increasing caffeic acid. Although the individual phenolic composition was not determined by HPLC in the

present study, these findings provide a mechanistic basis for the observed increase in TPC in *L. helveticus*-fermented extracts. An increase in TPC was observed following *B. subtilis* fermentation, consistent with the results of Karakurt et al. [26], who reported elevated phenolic content in DSM fermented by *B. subtilis*.

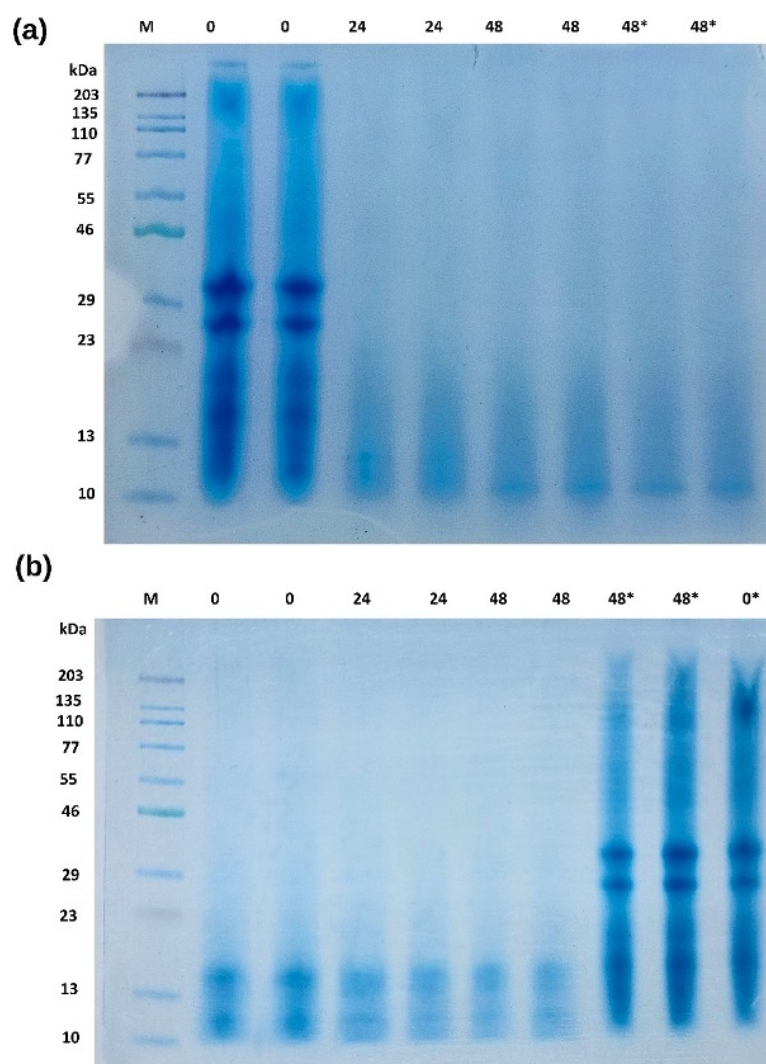


Figure 2. SDS-PAGE profiles of unfermented de-oiled sunflower meal (DSM) protein extracts by *B. subtilis* (a) and *L. helveticus* (b). Lanes correspond to 0 h (after inoculation), 24 h, and 48 h of fermentation. 48* indicates pH adjustment to 8.0 after 48 h of fermentation; 0* represents pH adjustment to 8.0 after inoculation (0 h) in *L. helveticus* fermentation.

All fermentation treatments positively affected the bioactive properties of DSM protein extracts, as assessed by DPPH radical scavenging activity, ABTS radical scavenging activity, CUPRAC, and ACE-inhibitory activity (Table 1). *B. subtilis* fermentation yielded the highest activity in all assays. The enhanced antioxidant capacity observed in the fermented extracts is likely related to the concurrent increase in free phenolic content, as phenolic compounds are well-established hydrogen donors capable of scavenging free radicals [34]. Microbial proteolysis during fermentation can generate bioactive peptides with antioxidant activity that can contribute to enhanced antioxidant activity [35]. The greater bioactivity observed in *B. subtilis* fermentation may be associated with its broader extracellular enzymatic repertoire and higher secretion capacity, which can promote more extensive protein hydrolysis and the release of bound phenolic compounds. In contrast, *L. helveticus* is better characterized as a strong but more specialized proteolytic fermenter [13,14,36].

Table 1. The total phenolic content, antioxidant properties (DPPH inhibition, ABTS Radical Scavenging, and Cupric Ion Reducing Antioxidant Capacity assays) and ACE-inhibitory activity of unfermented and fermented de-oiled sunflower meal (DSM) protein extracts obtained using *B. subtilis* and *L. helveticus*.

DSM Protein Extract	Total Phenolic Content (mg GAE/ g Sample)	DPPH Inhibition Assay (mg Trolox/g Sample)	ABTS Radical Scavenging Assay (mg Trolox/ g Sample)	Cupric Ion Reducing Antioxidant Capacity (mg Trolox/ g Sample)	ACE-Inhibitory Activity (%)
Unfermented	53.7 ± 1.6 ^C	48.5 ± 3.7 ^B	84.7 ± 2.3 ^B	89.1 ± 1.8 ^C	15.2 ± 0.7 ^B
Fermented by <i>B. subtilis</i> B3387	73.9 ± 3.4 ^A	95.5 ± 1.5 ^A	112.5 ± 6.8 ^A	135.4 ± 2.8 ^A	30.2 ± 0.8 ^A
Fermented by <i>L. helveticus</i> B4526	64.5 ± 0.9 ^B	51.0 ± 5.3 ^B	89.4 ± 2.6 ^B	97.9 ± 1.4 ^B	25.9 ± 1.5 ^A

Values are presented as mean ± standard deviation ($n = 3$). ^{A–C} Uppercase superscript letters indicate significant changes in bioactive properties within the same column ($p < 0.05$).

Fermentation of plant protein matrices has been reported to generate peptides with ACE-inhibitory activity through microbial proteolysis, making fermented plant extracts of interest as potential antihypertensive functional ingredients [37]. Consistent with this, ACE-inhibitory activity was significantly enhanced following fermentation with both *B. subtilis* and *L. helveticus* compared to that in the unfermented control (Table 1). This finding suggests that proteolysis may have contributed to the release of compounds with ACE-inhibitory activity from DSM proteins. However, although *B. subtilis* exhibited higher proteolytic activity than *L. helveticus*, both fermentations resulted in similar levels of ACE-inhibitory activity in the final products. This suggests that ACE inhibition is likely influenced not only by the extent of proteolysis, but also the properties of the peptides generated, such as amino acid composition and molecular size. Since peptide profiling was not performed in this study, the specific peptides responsible for the observed activity remain to be identified. Therefore, optimization of ACE-inhibitory activity requires controlling parameters such as degree of hydrolysis, substrate concentration, and other fermentation conditions [38].

3.5. Total Amino Acids

The total amino acid (TA) composition of unfermented and fermented DSM extracts were presented in Figure 3. Glutamic acid was the predominant amino acid in both unfermented and fermented DSM extracts, consistent with the known amino acid composition of sunflower proteins [33]. Fermentation by *B. subtilis* resulted in a marked decrease in total amino acid content relative to the unfermented control, with TA decreasing from 70.8 to 45.2 g/100 g protein across nearly all amino acids (Table S2). In contrast, TA remained largely unchanged after *L. helveticus* fermentation (69.0 g/100 g protein; Table S2). A similar trend was observed for essential amino acids: *B. subtilis* fermentation reduced the total essential amino acid content from 17.4 to 10.13 g/100 g protein, whereas *L. helveticus* fermentation resulted in 18.1 g/100 g protein. Despite the extensive proteolysis indicated by SDS-PAGE, these reductions suggest that a substantial proportion of the liberated amino acids and peptides may have been utilized by the microorganisms as nitrogen and carbon sources for growth and metabolism rather than accumulating in the extract [15]. As lysine is the limiting amino acid in DSM, its reduction from 1.2 to 0.5 g/100 g protein after *B. subtilis* fermentation indicates no improvement in nutritional quality, whereas *L. helveticus* fermentation showed a slight increase to 2.0 g/100 g protein. Overall, *B. subtilis* fermentation appears to enhance bioactivity while reducing amino acid content, whereas *L. helveticus* better preserves the nutritional amino acid profile.

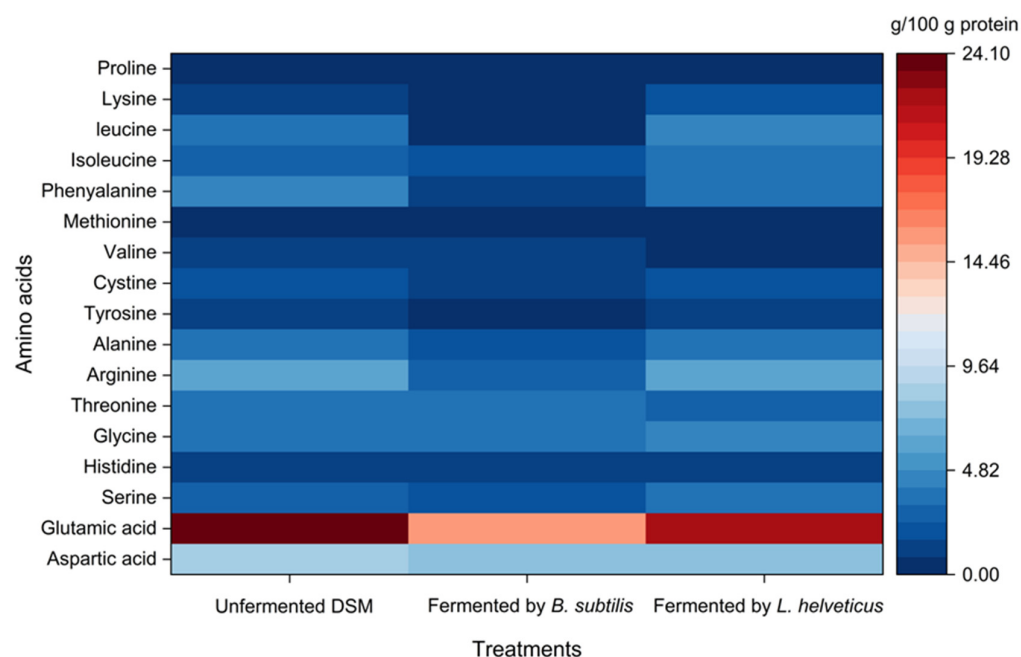


Figure 3. Heatmap reflecting the concentration of amino acids belong to final products derived from unfermented and fermented de-oiled sunflower meal (DSM) protein extracts by *B. subtilis* and *L. helveticus*. Color of the heatmap ranging from red to blue represents higher to lower levels.

3.6. Phytic Acid Content

Phytic acid content was measured for DSM flour, as well as the unfermented and fermented DSM extracts. The content of phytic acid in DSM flour was $3522.8 \pm 49.9 \mu\text{g/g}$ sample. Following the initial alkaline extraction step, as well as incubation and downstream processing, phytic acid levels in the soluble fractions were markedly reduced. In the unfermented DSM extract belonging to *L. helveticus* control, only residual amount ($105.4 \pm 23.2 \mu\text{g/g}$) was detected, whereas phytic acid was found to be below the detection limit in both the fermented extracts and unfermented *B. subtilis* control. Phytate is known to form complexes with proteins and minerals and may reduce its solubility and promote its partitioning into insoluble fractions during extraction and centrifugation processes [39,40]. Consequently, the phytic acid values reported in the present study represent the recovered soluble fractions rather than the total phytate content of the original sunflower meal. These findings suggest that, besides microbial phytase activity, processing steps including extraction, pH adjustment, thermal treatment, centrifugation and recovery of only the soluble fraction likely contributed to the removal of phytate or phytate–protein complexes. Therefore, the observed disappearance of phytic acid cannot be attributed solely to microbial degradation and may also reflect partitioning of phytate into insoluble fractions during downstream processing [39,40]. Nevertheless, the absence of detectable phytic acid in the final fermented samples indicates that the applied extraction–fermentation process did not introduce any phytate-related nutritional drawback and may contribute to improving the nutritional quality of sunflower meal-derived protein ingredients by reducing the content of this antinutritional factor.

3.7. Structural Changes Determined by Fourier-Transform Infrared (FTIR) Spectroscopy

FTIR analysis operates based on the vibrations of functional groups within the macromolecules of materials and reveals structural transformations at the molecular level through changes in wavenumbers [41]. The FTIR spectra of the final products derived from fermented and unfermented DSM protein extracts, as well as the structural changes occurring in the protein structure, are presented in Figure 4. In particular, the peak shifts and intensity

differences observed in the Amide I and II bands between 1700 and 1500 cm^{-1} indicate fermentation-induced degradation or rearrangement of the secondary structures of the proteins. Furthermore, changes in carbohydrate-related signals in the 1200–900 cm^{-1} range may suggest that microbial activity has also affected these structures. Compared to the unfermented extracts used as control groups, the peak intensities decreased. This indicates that the fermentation process can reduce the vibrations of –OH and –CH groups.

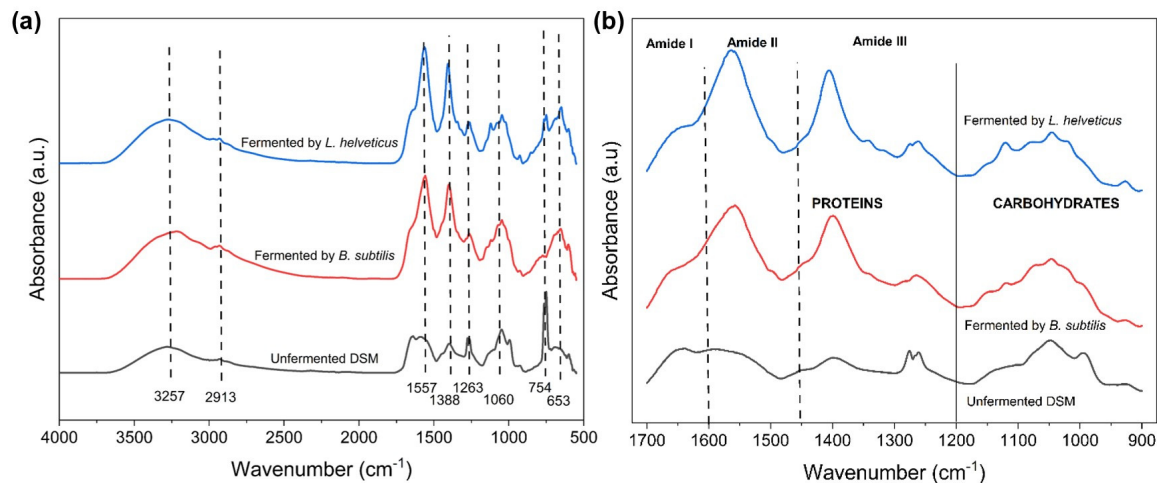


Figure 4. Fourier-transform infrared spectroscopy between 550 and 4000 cm^{-1} (a) and FTIR spectrum signifying changes in the carbohydrate and protein regions (b) for final products derived from unfermented DSM and fermented protein extracts.

To understand the structural order and starch conformation of the unfermented and fermented DSM extracts, the FTIR bands were further analyzed. The peak at 1047 cm^{-1} is generally assigned to the crystalline fraction of starch, while the peaks at 1022 and 995 cm^{-1} represent the amorphous region and hydrated crystals, respectively [42]. The intensity ratio of 1047/1022 cm^{-1} and 995/1022 cm^{-1} is generally used to understand the degree of order (DO) and internal changes in the double-helix structure (DD) within starch molecules, respectively. The DO value of samples fermented by *B. subtilis* and *L. helveticus* (1.29 and 1.27, respectively) was higher than that of the unfermented DSM (1.15). The DD values of the fermented samples (0.93 and 1.20, respectively) decreased significantly compared to that of the unfermented DSM (1.43). Similar fermentation-induced modifications in the starch structure have been reported by Zhao et al. and Xie et al. [43,44]. These changes may be associated with increased molecular ordering of starch structures during fermentation. The shorter starch chains formed during fermentation may have promoted stronger intermolecular interactions and hydrogen bonding [43,45]. *Bacillus* fermentation significantly decreased the ratio of crystalline to amorphous starch. This finding was also supported by the higher intensity observed in the 3000–3600 cm^{-1} region, which indicates the enhanced hydrogen bonding among starch molecules.

3.8. Techno-Functional Properties

3.8.1. Foaming Properties

The effect of pH on the foaming properties of unfermented and fermented extracts is shown in Figure 5a–c. All extracts showed similar foaming capacity (FC) at pH 4.0 (~160%) ($p > 0.05$), but the *B. subtilis*-fermented extract had significantly lower FC at pH 7.0 (105%) and 9.0 (125%) than the unfermented (~210%) and *L. helveticus*-fermented (~200%) extracts. In contrast, the *L. helveticus*-fermented extract maintained relatively stable FC across all pH values. Fermentation significantly affected foaming stability (FS), particularly under alkaline conditions (Figure 5b,c). The superior stability at acidic pH can be attributed to

enhanced protein–protein interactions and the formation of a more cohesive interface film surrounding air bubbles. In contrast, increased electrostatic repulsion at alkaline pH can hinder the formation of a strong protein network resulting in rapid foam collapse [46]. Foams made with *B. subtilis*-fermented extract were less stable and collapsed completely in less than 15 min at pH 7.0 and 9.0. Similarly, fermentation with *L. helveticus* reduced FS at pH 9.0, with stable foam remaining stable for only 12 min.

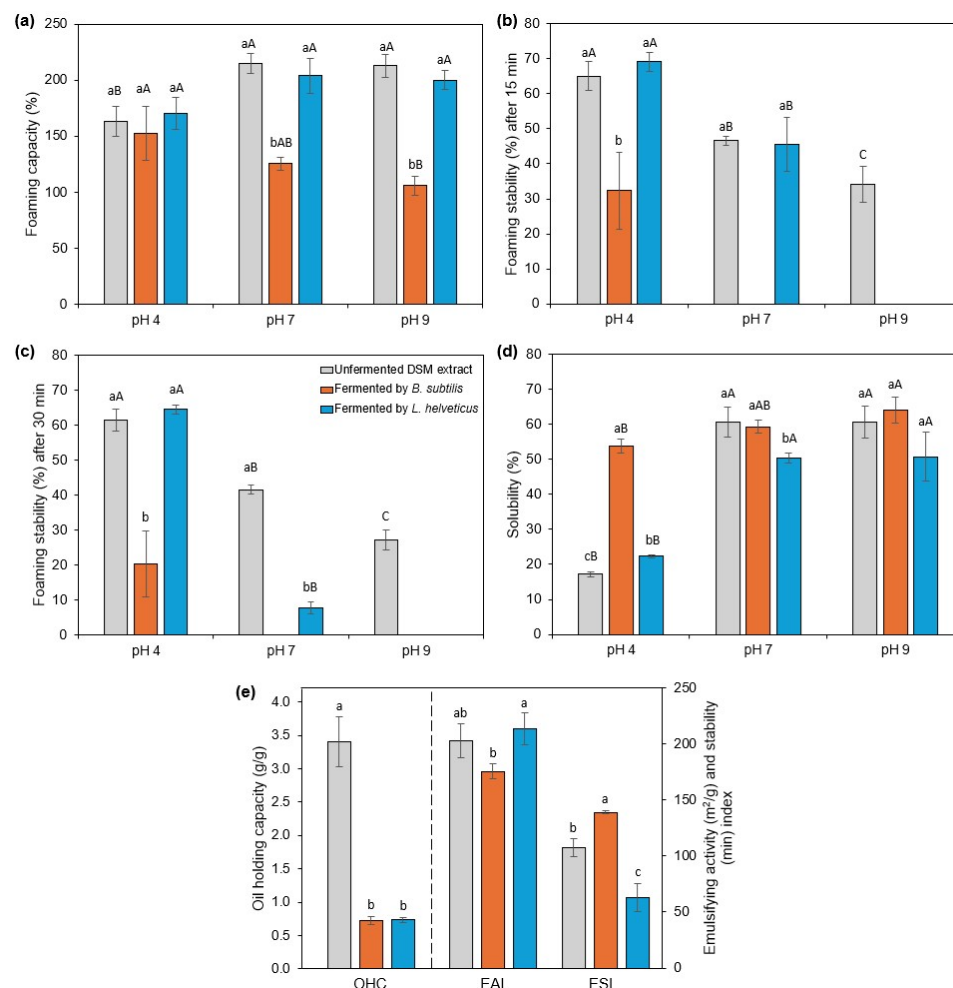


Figure 5. Techno-functional properties of the unfermented DSM extract and fermented samples. Foaming properties (a–c) expressed with respect to different pH values. Solubility (d) at pH 4, 7, and 9 based on 1% (w/v) sample solution. Oil-holding capacity (OHC) expressed as grams of oil per gram of sample, and emulsifying activity index (EAI) and emulsifying stability index (ESI) based on 0.1% (w/v) sample solution at pH 7 (e). ^{a–c} Significant differences at the same pH values ($p < 0.05$) are expressed by different lowercase superscripts. ^{A–C} Significant differences within the same sample ($p < 0.05$) are expressed by different uppercase superscripts.

Osungbade et al. [47] reported a similar decline in FC and FS in sandbox (*Hura crepitans* Linn.) seed flour after 96 h of fermentation. Meinschmidt et al. [48] found that *L. helveticus* fermentation of soy protein isolate increased FC with an increase in fermentation period but did not affect FS. These results indicate that foaming behavior depends on both the extent of protein hydrolysis and the protein source. Extensive degradation can reduce the ability of proteins to maintain stable foam structures despite their improved solubility [49].

From an application perspective, the reduced FC and FS of the fermented extracts may limit their suitability for products that rely on long foam duration, such as whipped toppings, mousses, meringues, and certain aerated desserts [50]. However, this characteristic may not be crucial for bakery products. In these systems, air incorporation is required

only during batter preparation, and the resulting foam structure is stabilized by starch gelatinization and protein denaturation during baking. Therefore, fermented DSM extracts can be used as ingredients in cakes, muffins, pancakes, and different scorched products.

3.8.2. Protein Solubility Profile

The solubility of protein ingredients is a crucial determinant of other functional properties, such as foaming and emulsification, as it directly affects protein dispersion in aqueous systems and the availability of protein molecules to interact at interfacial regions [11,50]. The solubility of the unfermented DSM extract and after fermentation with *B. subtilis* and *L. helveticus* was measured as a function of pH 4.0, 7.0 and 9.0 (Figure 5d). The extracts displayed the characteristic behavior of proteins derived from legumes, oilseeds, and cereals. They typically exhibit a net charge close to zero at pH 4.0–5.0 (isoelectric point), where the reduction in net surface charge favors protein–protein interactions and results in the lowest solubility [51,52]. The unfermented DSM extract exhibited the lowest solubility at pH 4.0 (17%), which markedly increased to ~60% at pH 7.0 and 9.0. An important observation is that fermentation with *B. subtilis* significantly improved protein solubility of DSM at pH 4.0. This represents more than a three-fold increase compared to that of the unfermented DSM extract. At pH 7.0 and 9.0, *B. subtilis*-fermented extracts maintained high solubility values (59–64%) that are comparable to those of the unfermented extract. In contrast, fermentation with *L. helveticus* resulted in substantially lower solubilities at pH 4.0 and 7.0. In other words, solubility values increased from 22% at pH 4.0 to 50% at pH 7.0 but these values remained significantly lower than those observed for *B. subtilis*-fermented extracts at acidic and neutral pH. Furthermore, Meinschmidt et al. [48] reported that fermentation with *L. helveticus* DSM 20075 improved the solubility of soybean proteins under acidic conditions, whereas a reduction was observed at neutral pH.

Fermentation has been widely reported to improve the solubility of legume proteins, including lupin, pea, and lentil proteins. This enhancement could be attributed to microbial proteolytic activity and the disruption of protein aggregates or complexes. The difference in solubility between samples fermented by *B. subtilis* and *L. helveticus* may be attributed to the formation of low-molecular-weight proteins (MW < 15 kDa) that are also supported by the SDS-PAGE results [53].

3.8.3. Oil- and Water-Holding Capacity

Fermentation significantly reduced the oil-holding capacity (OHC) of DSM extracts (Figure 5e). OHC decreased from 3.41 g/g to 0.73 g/g for both fermented products. This was consistent with findings of Toor et al. [54], who reported decreased OHC values in fermented desi chickpea, pigeon pea and soybean flours by *R. oligosporus* while an increase was observed in fermented kabuli chickpea. Given that OHC is strongly dependent on the protein composition, presence of hydrophobic protein regions capable of binding lipids, the decrease in OHC observed in fermented extracts could reflect structural alterations in proteins caused by proteolytic and other enzymatic activities [11,55]. These changes may have reduced the availability of hydrophobic sites involved in oil retention.

Water-holding capacity (WHC) could not be determined for either the fermented or unfermented sunflower protein extracts, as the samples dissolved completely during the analysis. Similar observations have been reported for protein isolates from *Trichosanthes kirilowii* seeds obtained through reverse micelle extraction, where WHC measurements could not be performed due to the nearly complete dissolution of the protein isolate in water [56]. Furthermore, Stone [9] reported that the WHC of commercial egg and whey protein isolates was not detectable because the proteins dissolved entirely under the test conditions. In these studies, the absence of measurable WHC was attributed to the high

solubility of the protein materials and their structural characteristics, particularly the increased exposure of hydrophilic groups on the protein surface. These groups enhance protein–water interactions and promote dissolution rather than water retention, thereby preventing the determination of WHC [9,56].

3.8.4. Emulsifying Properties

Emulsifying properties are important functional characteristics of proteins and are influenced by factors such as protein type and concentration, pH, ionic strength, and medium viscosity. These factors affect the formation and stability of protein stabilized oil–water emulsions [57]. The emulsifying activity index (EAI) ranged from 175 to 213 m²/g (Figure 5e). While the EAI of *L. helveticus*-fermented extract was the highest with the ability of proteins to absorb rapidly at oil–water interface, its stability exhibited the lowest value. A similar trend of decline in both EAI and ESI is reported by Lampart-Szczapa et al. [58], who investigated the effect of lactic acid fermentation of different lupin seed varieties. In their research, a high positive correlation of emulsifying properties with surface hydrophobicity of lupin samples was observed. On the other hand, fermentation with *B. subtilis* significantly enhanced the emulsifying stability index (ESI) but failed to increase the EAI. The low emulsifying performance of *B. subtilis*-fermented extracts could be due to an excessively high hydrolysis degree of extracts [10]. To enhance the emulsifying properties relative to the unfermented extracts, hydrolysis should be carefully controlled. A low hydrolysis is generally preferred as excessive degrees can lead to a substantial decline.

4. Conclusions

In conclusion, microbial fermentation represents a highly effective strategy for the valorization of de-oiled sunflower meal (DSM) protein extracts, with the choice of microorganism dictating the final ingredient functionality. To our knowledge, this is the first study to directly compare *Bacillus subtilis* and *Lactobacillus helveticus* fermentation of a DSM protein extract, revealing how each organism's distinct metabolic strategy shapes its functional and nutritional outcomes. *Bacillus subtilis* fermentation induces extensive protein hydrolysis, substantially boosting antioxidant and ACE-inhibitory activities and improving solubility, despite a reduction in total amino acid content. Conversely, *Lactobacillus helveticus* fermentation preserves amino acid integrity and nutritional quality, though its functional properties are somewhat limited by acid-induced protein aggregation. Consequently, microorganism selection must be tailored to the intended food application: *B. subtilis* is ideal for bioactivity-driven formulations, whereas *L. helveticus* is preferred for applications prioritizing nutritional completeness.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fermentation12080356/s1>, Table S1: Total soluble protein concentration (g/L) changes of de-oiled sunflower meal (DSM) protein extracts during fermentation; Table S2: Amino acid compositions of the unfermented de-oiled sunflower meal (DSM) extract and fermented DSM extracts by *B. subtilis* and *L. helveticus*.

Author Contributions: Conceptualization, M.İ.H.; methodology, C.Y., B.Ö., M.İ.H., Ö.A. and O.G.; validation, C.Y., B.Ö., M.İ.H. and Ö.A.; formal analysis, C.Y. and B.Ö.; investigation, C.Y., B.Ö., Ö.A. and O.G.; resources, M.İ.H., E.A.A.K., Ö.A. and O.G.; data curation, C.Y., B.Ö., Ö.A. and O.G.; writing—original draft preparation, C.Y.; writing—review and editing, C.Y., B.Ö., Ö.A., E.A.A.K., O.G. and M.İ.H.; visualization, C.Y. and B.Ö.; supervision, M.İ.H.; project administration, M.İ.H.; funding acquisition, M.İ.H., E.A.A.K. and Ö.A. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by Turkish Council of Higher Education—Research Universities Support Program (Gebze Technical University, Grant number: 2023-A-113-02) and European Union’s Horizon Europe research and innovation programme (Excel4Pro, Grant number: 101186662).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Materials. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

ABTS	2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
ACE-I	Angiotensin-converting enzyme-I
CGA	Chlorogenic acid
CUPRAC	Cupric ion reducing antioxidant capacity
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSM	De-oiled sunflower meal
EAI	Emulsifying activity index
ESI	Emulsifying stability index
FC	Foaming capacity
FS	Foaming stability
FTIR	Fourier transform infrared
HHL	hippuryl-His-Leu
LAB	Lactic acid bacteria
OHC	Oil-holding capacity
SDS-PAGE	Sodium dodecyl sulfite-polyacrylamide gel electrophoresis
TA	Total amino acid
TPC	Total phenolic content
TSP	Total soluble protein
WHC	Water-holding capacity

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