

Article

Solid-State Fermentation Using *Bacillus licheniformis*-Driven Changes in Composition, Viability and In Vitro Protein Digestibility of Oilseed Cakes

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Abstract: The solid-state fermentation (SSF) efficiency of *Bacillus licheniformis* ATCC 21424 (BL) on various agro-industrial by-products such as oilseed cakes [hemp (HSC), pumpkin (PSC), and flaxseed (FSC)] was evaluated by examining the nutritional composition, reducing sugars, and in vitro protein digestibility (IVPD) for use in animal nutrition. SSF significantly decreased crude protein, along with changes in the total carbohydrates ($p < 0.05$) for all substrates fermented. An increase in crude fat for HSC (1.04%) and FSC (1.73%) was noted, vs. PSC, where the crude fat level was reduced (−3.53%). Crude fiber does not differ significantly between fermented and nonfermented oilseed cakes ($p > 0.05$). After fermentation, neutral detergent fiber (NDF) and acid detergent fiber (ADF) significantly increased for HSC and FSC ($p < 0.05$), as well as for PSC despite the small increase in ADF (4.46%), with a notable decrease in NDF (−10.25%). During fermentation, pH shifted toward alkalinity, and after drying, returned to its initial levels for all oilseed cakes with the exception of PSC, which maintained a slight elevation. Further, SSF with BL under optimized conditions (72 h) increases the reducing sugar content for FSC (to 1.46%) and PSC (to 0.89%), compared with HSC, where a reduction in sugar consumption was noted (from 1.09% to 0.55%). The viable cell number reached maximum in the first 24 h, followed by a slowly declining phase until the end of fermentation (72 h), accompanied by an increase in sporulation and spore production. After 72 h, a significant improvement in water protein solubility for HSC and FSC was observed ($p < 0.05$). The peptide content (mg/g) for oilseed cakes fermented was improved ($p < 0.05$). Through gastro-intestinal simulation, the bacterial survivability rate accounted for 90.2%, 101.5%, and 85.72% for HSC, PSC, and FSC. Additionally, IVPD showed significant improvements compared to untreated samples, reaching levels of up to 65.67%, 58.94%, and 80.16% for HSC, PSC, and FSC, respectively. This research demonstrates the advantages of oilseed cake bioprocessing by SSF as an effective approach in yielding valuable products with probiotic and nutritional properties suitable for incorporation into animal feed.

Keywords: solid-state fermentation; *Bacillus*; proximate composition; digestibility; IVPD; fatty acid profile; soluble proteins; peptides; spores; hemp; pumpkin; linseed; flaxseed



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1. Introduction

Considering the increasing demand of alternative high-protein sources of foods and feeds, oilseed cakes (OSCs), derived from the industry of edible oil extraction as an under-rated by-product, could serve as a drive for sustainability through enhancing the circular economy and food security [1]. Replacing soybean meal, which has long been considered as the golden standard in animal nutrition [2], with locally sourced oilseed cakes could contribute to the amelioration of the negative impact related with its cultivation such as poor socioeconomics, loss of biodiversity, fertilizers, long transport routes, etc. [3]. With a high

protein content, hemp, pumpkin, and flaxseed cakes represent a tempting alternative to traditional feeding systems. However, the presence of anti-nutritional factors such as trypsin inhibitors, phytic acid, and tannins can reduce protein bioavailability and digestibility, impairing OSC efficient utilization [4]. In this context, an integration of the knowledge acquired from traditional fermentation and modern innovation can drive meticulous changes in nutritional and bioactive composition, shaping the path towards precision.

Solid-state fermentation (SSF), a process carried out by one or a diverse array of microorganisms on a solid substrate with a moisture typically between 30 and 80% [5], has gained more attention in recent years due to a variety of advantages that this technology poses in biotransformation. By using solid matrixes, microbial fermentation can create a unique microenvironment that promotes a microorganism's resistance to catabolic repression (i.e., inhibition of enzyme synthesis) [6], the biosynthesis of high yields of enzymes (amylases, proteases, xylanases, phytases, etc.) [7–9], and bioactive compounds' [10] improvement of their nutrient profile and absorption, followed by a reduction in anti-nutritional factors [11]. Despite the numerous advantages of SSF, the process of scaling-up encounters several constraints, including temperature build-up, pH regulation, oxygen transfer, moisture regulation, and an uneven distribution of cell mass and nutrients. These challenges underscore the critical significance of rational design and meticulous process control [12].

Bacillus licheniformis is a Gram-positive, endospore-forming bacterium which is generally regarded as safe (GRAS) and that has a high extracellular protein secretory capacity [13]. It is known for a diverse range of proteases, including alkaline proteases exhibiting optimal activity within the pH range of 8–12, and neutral proteases showing efficacy between pH 5.0 and 8.0 [14]. The enzymatic action of proteases facilitates the hydrolysis of protein substrates in fermentation media into smaller peptides with improved functionality, such as solubility, digestibility, and biologic activity [15].

Furthermore, SSF is considered one of the most efficient techniques used in altering the functional properties and/or bioactive compounds of a target substrate [16]. Moreover, fermentation has the potential to improve the nutritional composition of various substrates or to obtain value-added products. Several studies found that *B. licheniformis* increased peptide content [17] and bioactivity, and significantly improved the nutritional profile of co-substrates of brewer's spent grain and soybean meal (SBM) [18]. In addition, data from the literature present successful results of *B. licheniformis* in SSF, for example, the SSF of SBM and rapeseed meal to increase peptide, soluble protein content, and also to eliminate anti-nutritional factors [19,20]. Through this enzymatic activity, they contribute to the degradation of substrates, the conversion of ingredients, and the synthesis of new compounds [20,21].

Some reports indicate that fermented products derived from *Bacillus licheniformis* exhibit promising effects on gut health. These products have been found to positively influence gut conditions by modulating the microbiota composition. They demonstrate the ability to inhibit the proliferation of harmful enteric pathogens while promoting the growth of beneficial bacteria such as lactobacilli. Additionally, these fermented products have been associated with improvements in nutrient digestibility and have shown potential in alleviating various gut-related diseases [22]. The proposed mechanisms are related to the enhancement of gut barrier functions, enhanced immunity (lymphocyte activation, increased levels of immune globulins, improved cellular or humoral immunity), as well as the production of antibiotic proteins/peptides [23]. Animal studies have shown a positive impact of solid-state fermentation (SSF) using *Bacillus licheniformis* in alleviating necrotic enteritis and improving intestinal morphology in broilers challenged with *Clostridium perfringens* [24,25] as well as in reducing diarrhea incidence in weaning piglets [26].

The next step toward precision agriculture involves targeted probiotic interventions, as described here, owing to the ability of the *Bacillus licheniformis* to form spores under environmental stress; the bacterium demonstrates a unique resilience that enables it to withstand harsh conditions encountered within the gastrointestinal tract of animals [27,28].

Considering these aspects, this study aims to bridge the gap between solid-state fermentation, probiotics, and sustainable protein sources by exploring the dynamics of *Bacillus licheniformis* growth and sporulation patterns throughout the fermentation process. Additionally, we studied the driven changes in proximate composition and fatty acid profiles to gain a deeper understanding of how this process influences these nutritional aspects. By assessing alterations in protein solubility and reducing sugars, we seek to provide an overview of the hydrolysis process. Finally, implementing gastrointestinal simulation allows us to study protein bioavailability and bacterial survival which are crucial factors in developing probiotic-enhanced feed formulations.

2. Materials and Methods

2.1. Chemicals

Enzymes used in this study were α -amylase (10080; 52.2 U/mg), pepsin (P7000, 605 U/mg), and pancreatin (P3292, 4 \times USP) bought from Sigma-Aldrich (Saint-Louis, MO, USA). Culture media and bile salts were bought from OxoidTM (Basingstoke, UK). All other reagents used in the study were of analytical grade, purchased from Sigma-Aldrich (Saint-Louis, MO, USA) and Carl Roth (Karlsruhe, Germany).

2.2. Plant Material and Microorganism

Hempseed cake (*Cannabis sativa* L.) was kindly provided by Canah International S.R.L. (Bihor, Romania), while pumpkin (*Cucurbita* spp.) and flaxseed (*Linum usitatissimum* L.) cakes were kindly supplied by Dachim S.R.L. (Cluj, Romania), both of which resulted as byproducts from oil cold-pressing production processes. The microbial strain used in fermentation, *Bacillus licheniformis* ATCC 21424, was acquired from The American Type Culture Collection (Manassas, VA, USA).

2.3. Solid-State Fermentation (SSF)

SSF was carried out for 3 different timepoints (24, 48, and 72 h) using separate 500 mL Erlenmeyer flasks for each timepoint, where 100 g from each oilseed cake was added individually. Flasks were plugged with cotton wool covered by aluminum foil and autoclaved at 121 °C for 15 min. Frozen bacterial culture was revitalized, and after 2 passages on Nutrient Agar, a working stock culture of *Bacillus licheniformis* was prepared in LB (Luria–Bertani) broth and grown overnight for approx. 20 h at 37 °C, 150 rpm to an OD₆₀₀ of 4.5 (this value was extrapolated from a 10-fold dilution). SSF flasks were inoculated with 18 mL of working stock culture and 82 mL of autoclaved distilled water was added to achieve a final OD₆₀₀ of 0.8/g equivalent to a viable cell number of 7.75×10^8 cfu/g and 50% moisture content. After a thorough mix using a sterile spatula, inoculated flasks were incubated at 37 °C. After the designated incubation period elapsed, samples were collected for viability assessment and spore number quantitation according to a pH of 2.4. Subsequently, the remaining quantity of samples was stabilized for future analysis by subjecting it to freeze-drying; afterwards, the samples were blended for 30 s in order to achieve a powdery consistency and stored at 4 °C.

2.4. Microbial Viability, Spore Number, Colony Morphology, and pH Value

2.4.1. Determination of Microbial Viability

Approximately 1 g of sample was prelevated at 24, 48, and 72 h, mixed with autoclaved distilled water (1:9 *w/v*), and shaken at 150 rpm for 30 min at room temperature. For the determination of viable cell number, 1 mL of supernatant was sampled and serially diluted in 0.85% saline solution, and 0.1 mL was plated on Nutrient Agar and expressed as log cfu/g after 24 h incubation at 37 °C.

2.4.2. Determination of Spore Number

Spore number was assayed at 24, 48, and 72 h, with 1 mL of the supernatant consisting of vegetative cells, and the spores were diluted 10-fold in 0.85% saline solution and

incubated at 80 °C for 15 min [28] in order to eliminate vegetative forms. Afterwards, it was serially diluted and plated on Nutrient Agar, and spore number was determined in the same manner as for microbial viability assay.

2.4.3. Determination of pH Value

The pH of the fermented samples at 0, 24, 48, and 72 h was measured for the supernatant using a calibrated pH meter (pH 7.0 + DHS, XS Instruments, Carpi, Italy).

2.5. Proximate Composition

The proximate composition of oilseed cakes at 0 and 72 h was assayed based on the recommended methods of the AOAC (Association of Official Analytical Chemists) [29]. In brief, dry matter was determined gravimetrically by the drying oven method at 105 °C (Method 925.09); crude protein was determined by the Kjeldahl method (Method 979.09) on a Kjeltac auto 1030 system (Höganäs, Sweden); crude fat was determined gravimetrically by organic solvent extraction (Method 920.39) using a Soxtec 2055 Foss Soxhlet extractor (Höganäs, Sweden); ash content was assayed gravimetrically by calcination in an oven at 600 °C; crude fiber was quantified by successive hydrolysis in alkaline and acidic environments (Method 962.09); neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined by Van Soest extraction using a Raw Fiber Extractor FIWE 6 (Velp Scientifica, Usmate, Italy) [30,31]; and finally, carbohydrate content was determined as a nitrogen-free extract (NFE), as follows: $\text{NFE (\%)} = \text{dry matter\%} - (\text{crude protein\%} + \text{crude fat\%} + \text{crude ash\%} + \text{crude fiber\%})$ [32].

2.6. Fatty Acid Profile

Fatty acids (FAs) from total lipid extract were determined as FA methyl esters (FAMES) at 0 and 72 h based on the ISO/TS 17764-2:2008 [33] standard method [31] by GC-FID using a Perkin Elmer-Clarus 500 (Waltham, MA, USA) gas chromatograph equipped with a TRACE TR-Fame (Thermo Electron, Waltham, MA, USA), 60 m × 0.25 mm × 0.25 µm, capillary column.

2.7. Reducing Sugars

The concentration of reducing sugars at 0, 24, 48, and 72 h was measured by a modified method of Miller's work [34] using DNSA reagent (10 g/L 3,5-dinitrosalicylic acid, 2 g/L phenol, 10 g/L NaOH, and 200 g/L sodium potassium tartrate, 0.5 g/L Na₂SO₃). Samples were prepared by extracting the reducing sugars from 1 g of fermented feeds or controls in 25 mL distilled water in a rotatory shaker at 220 rpm and room temperature for 1 h following a 10 min centrifugation at 10,000 rpm and 4 °C. In a test tube, 1 mL of extract and 2 mL DNSA reagent were vortexed and incubated at 100 °C for exactly 5 min. After incubation, the test tubes were cooled in a water bath, and 9 mL of distilled water was added and recentrifuged. Subsequently, the samples were read on Eppendorf BioSpectrometer (Hamburg, Germany) at 540 nm in comparison to a reagent blank. A calibration curve was constructed across a range of 0.1–2 mg/mL ($R^2 = 0.9982$) using glucose as standard.

2.8. Soluble Proteins and Peptides, Extraction, and Quantification

2.8.1. Determination of Soluble Proteins

Soluble proteins and peptides in neutral solution (pH 7.0), salt solution (0.5 M NaCl), and alkaline environment (pH 10.0) at 0, 24, 48, and 72 h were determined as an indicator of the effectiveness of the fermentation process, providing direct insights on enzymatic activities, extraction efficiency, and protein hydrolysis. The extract was prepared by adding 1 g of fermented feeds or controls into 25 mL of distilled water for neutral extraction, into 25 mL 0.5 M NaCl solution for salt extraction, and for alkaline extraction, the mixture in 25 mL of distilled water was vortexed for 1 min and brought to pH 10.0 using a 3 M NaOH solution. Extraction was performed in a rotatory shaker at 220 rpm and room temperature for 1 h following a 10 min centrifugation at 10,000 rpm and 4 °C, and diluted up to 100-fold.

The soluble protein content of the supernatant of the extract was determined using Pierce BCA Protein Assay kit (Thermo Scientific, Waltham, MA, USA) with bovine serum albumin as standard.

2.8.2. Determination of Peptides

Soluble peptide yield was determined as TCA-soluble peptides; 1 mL of 20% trichloroacetic acid (TCA) was added to an equal volume of extract and incubated for 30 min at 4 °C for protein precipitation, followed by centrifugation [35], and diluted up to 100-fold. Peptide content was quantitated using a bicinchoninic acid (BCA) assay kit.

2.9. *In Vitro* Protein Digestibility (IVPD) and Microorganism Viability under Simulated Conditions

The *in vitro* gastrointestinal (GI) digestion simulation model previously described [36] was employed with some modifications. The GI simulation model consisted of 3 steps that resemble the conditions within a digestive system, namely oral, gastric, and intestinal phases, all of which each fermented (after 72 h) and control (after 0 h) sample underwent. Prior to the experiment, a test was performed to determinate the exact volume of the HCL 3 M/NaOH 3 M needed to adjust the pH of the oral, gastric, and intestinal stages for fermented and control samples as each one has a unique buffering ability. The volume was recorded and the results were adjusted according to it. Simulated solutions containing electrolytes were prepared according to the protocol, pre-warmed to 37 °C, and adjusted to their specific pH in order to mimic physiological conditions. Shortly before the experiment, enzymes and bile salts were added and mixed by employing a magnetic stirrer.

Oral stage: A 2.5 g sample was mixed with 2.5 mL of distilled water and a 5 mL simulated oral solution containing 750 U α -amylase for 2 min at pH 7.0.

Gastric stage: The oral bolus was mixed with 10 mL of simulated gastric solution containing 40,000 U pepsin for 2 h at pH 3.0.

Intestinal phase: The gastric chyme was mixed with 20 mL simulated intestinal solution containing 32 mg/mL pancreatin [37] and 24 mg/mL bile salts for 2 h at pH 7.0 [38].

Samples were taken at different points of the digestion: gastric 1 h (G1), gastric 2 h (G2), intestinal 1 h (I1), and intestinal 2 h (I2). Distinct digestion flasks were prepared per each timepoint. At the end of the prescribed time, samples were taken and immediately diluted and plated (using the method stated at Section 2.4.1) for the viability assay. For IVPD, samples were centrifugated at 4 °C and the soluble peptide content was assayed (Section 2.8.2). IVPD was calculated using the following formula [39]:

$$\text{Protein digestibility (\%)} = B/A \times 100$$

where B is the soluble peptide content after each digestion stage and A is the total protein content. Independently, a reagent blank for each timepoint was prepared and its peptide content was subtracted from the total concentration:

$$\text{Survivability rate (\%)} = B/A \times 100$$

where B is the log cfu/g viability after the I2 stage and A is the initial log cfu/g.

2.10. Statistical Analysis

The results were calculated on dry basis (DW). The data are the means of triplicate experiments \pm standard deviations. Statistical analysis was performed using Minitab v.21.2 (State College, PA, USA) where differences between the means were evaluated by a one-way ANOVA followed by Tukey's test. For analyzing the proximate composition and FA profile, paired *t*-tests were used. Differences were considered significant at $p < 0.05$. Graphics were prepared using Prism-GraphPad v. 9.1.2 (San Diego, CA, USA).

3. Results

3.1. Effect of Fermentation on Proximate Composition and Fatty Acid Profile

Fermentation with *Bacillus licheniformis* has a notable effect on the nutritional composition of the samples as presented in Table 1. Variations in dry weight across samples are attributed to the drying conditions utilized rather than being solely a consequence of the fermentation process itself. Crude protein decreased significantly ($p < 0.05$) for all samples, the most significant being for HSC (−5.40%). The increases in crude fat for HSC and FSC (1.04% respectively 1.73%) and decrease ($p > 0.05$) for PSC (−3.53%) highlights the substrate specificity of this process along with the capacity for de novo lipid biosynthesis from carbohydrates whose lipidic profile will be further explored through FA profile analysis. Nitrogen-free extracts expressed as total carbohydrates significantly decreased ($p < 0.05$) for FSC, suggesting the bacterial capacity to ferment specific carbohydrates presented in flaxseed. Small increases were observed for HSC and were more accentuated for PSC relative to its initial content. Crude fiber does not differ significantly between fermented and unfermented ($p > 0.05$) samples for HSC, and small increases were accounted for PSC and FSC. NDF and ADF significantly increased for HSC and FSC ($p < 0.05$), whereas PSC showed small increases despite ADF (4.46%), and a notable decrease in NDF was observed (−10.25).

Table 1. Changes in proximate composition after fermentation.

Parameter	HSC T 0 h	HSC T 72 h	PSC T 0 h	PSC T 72 h	FSC T 0 h	FSC T 72 h
DM (%)	93.58 ± 0.38	95.82 ± 0.1	90.91 ± 0.1	95.17 ± 0.03	90.54 ± 0.06	96.44 ± 0.05
CP (% DM)	38.00 ± 0.22	35.95 ± 0.08	45.27 ± 0.1	44.65 ± 0.17	33.35 ± 0.13	32.53 ± 0.07
Crude fat (% DM)	8.44 ± 0.11	8.53 ± 0.08	15.60 ± 0.09	15.05 ± 0.1	18.82 ± 0.09	19.15 ± 0.14
Carbohydrates (% DM)	17.10 ± 0.05	18.24 ± 0.27	1.55 ± 0.58	3.45 ± 0.47	34.07 ± 0.149	31.21 ± 0.56
CF (% DM)	30.23 ± 0.09	30.14 ± 0.04	31.18 ± 0.29	32.27 ± 0.15	10.44 ± 0.23	11.78 ± 0.22
NDF (% DM)	43.98 ± 0.13	47.16 ± 0.1	45.28 ± 0.28	40.64 ± 0.33	27.32 ± 0.17	28.22 ± 0.24
ADF (% DM)	29.96 ± 0.25	33.83 ± 0.12	29.60 ± 0.17	30.92 ± 0.19	13.99 ± 0.21	16.82 ± 0.13
Ash (% DM)	6.20 ± 0.06	7.11 ± 0.06	6.37 ± 0.09	4.56 ± 0.05	4.11 ± 0.09	4.48 ± 0.06

DM = dry matter; CP = crude protein; CF = crude fiber; NDF = neutral detergent fiber; ADF = acid detergent fiber. Each value represents the mean for three replications ± standard deviation.

The effect of solid-state fermentation of OSC on the FA metabolism of *Bacillus licheniformis* is displayed in Table 2. The results revealed notable alterations in the levels of saturated, monounsaturated, and polyunsaturated FAs across the substrates. A significant increase in total saturated FAs was observed in FSC with 8.59% ($p > 0.05$), contrasting with a decrease in PSC with 6.61%, while no significant changes were noted in HSC. Palmitic acid, which serves as a readily available energy source, decreased with 12.94% and 7.51% for PSC and FSC, respectively, whereas a notable increase of 7.41% was observed in HSC. Levels of monounsaturated FAs increased significantly with 3.59% and 6.91% for PSC and FSC substrates ($p < 0.05$), but decreased for HSC, with 6.20%. Total polyunsaturated fatty acids (PUFAs) show variation, where small significant increases were observed for HSC and PSC, with 0.69% and 1.21%, respectively, and a notable decrease in FSC fermentation with 3.64% ($p < 0.05$). Fermentation does not induce noticeable changes in the total ratio of omega-6 to omega-3 FAs (n-6/n-3 ratio). However, individual fatty acids such as linoleic acid and omega-6 decreased with 0.33% and 2.04% for HSC and FSC, increasing with 3.61% for FSC after 72 h. Alpha linolenic acid, an omega-3 PUFA, is also affected by fermentation, decreasing with 6.15% for HSC, 22.88% for PSC, and 3.51% for FSC, respectively. Arachidonic acid, which serves as a precursor for pro-inflammatory eicosanoids, exhibited noteworthy changes, increasing approx. 5-fold in HSC and decreasing below undetectable levels for PSC.

Table 2. Changes in fatty acid profile after fermentation.

FA (g/100 g)	HSC T 0 h	HSC F 72 h	PSC T 0 h	PSC F 72 h	FSC T 0 h	FSC F 72 h
Myristic C14:0	0.04 ± 0.01	0.06 ± 0.01	0.12 ± 0.02	0.11 ± 0.01	0.09 ± 0.02	0.05 ± 0.01
Pentadecanoic C15:0	0.04 ± 0.01	0.11 ± 0.02	0.04 ± 0.01	0.19 ± 0.02	ND	0.22 ± 0.02
Palmitic C16:0	8.61 ± 0.05	9.29 ± 0.04	14.53 ± 0.09	12.65 ± 0.15	7.67 ± 0.13	7.51 ± 0.19
Heptadecanoic C17:0	0.72 ± 0.04	ND	0.03 ± 0.0	0.05 ± 0.0	ND	0.08 ± 0.01
Stearic C18:0	2.66 ± 0.04	3.05 ± 0.06	5.03 ± 0.07	5.45 ± 0.06	3.32 ± 0.08	3.99 ± 0.2
Arachic C20:0	1.85 ± 0.03	1.75 ± 0.05	ND	ND	ND	ND
TOTAL SATURATED	13.97 ± 0.08	14.28 ± 0.18	19.76 ± 0.19	18.45 ± 0.22	11.09 ± 0.24	12.04 ± 0.38
Pentadecenoic C15:1	0.12 ± 0.02	ND	0.07 ± 0.02	ND	0.05 ± 0	ND
Palmitoleic C16:1	0.19 ± 0.02	0.12 ± 0.01	0.11 ± 0.03	0.05 ± 0.0	0.08 ± 0.01	0.06 ± 0.01
Heptadecenoic C17:1	0.81 ± 0.07	ND	ND	0.05 ± 0.0	ND	0.73 ± 0.07
Oleic C18:1	11.49 ± 0.05	11.71 ± 0.1	28.4 ± 0.3	29.51 ± 0.13	21.9 ± 0.37	22.77 ± 0.08
TOTAL MONO-UNSATURATED	12.61 ± 0.16	11.84 ± 0.12	28.59 ± 0.31	29.61 ± 0.13	22.04 ± 0.38	23.56 ± 0.13
Linoleic Cis C18:2n6	54.13 ± 0.07	53.96 ± 0.08	48.48 ± 0.41	50.23 ± 0.24	15.05 ± 0.08	14.74 ± 0.06
Linolenic γ C18:3n6	0.09 ± 0.02	ND	ND	ND	0.21 ± 0.03	ND
α Linolenic C18:3n3	17.4 ± 0.04	16.33 ± 0.06	0.39 ± 0.04	0.3 ± 0.04	51.14 ± 0.07	49.34 ± 0.67
Octadecatetraenoic C18:4n3	0.66 ± 0.03	1.7 ± 0.08	0.24 ± 0.02	0.33 ± 0.06	0.16 ± 0.02	0.24 ± 0.02
Eicosadienoic C20:2n6	0.33 ± 0.03	0 ± 0	0.38 ± 0.04	0.14 ± 0.04	0.07 ± 0.0	ND
Eicosatrienoic C20:3n6	ND	ND	ND	ND	ND	ND
Arachidonic C20:4n6	0.07 ± 0.0	0.47 ± 0.04	0.17 ± 0	ND	ND	ND
Eicosapentaenoic C20:5n3	0.02 ± 0.0	ND	0.22 ± 0.04	ND	ND	ND
Docosapentaenoic C22:5n3	ND	0.12 ± 0.03	ND	ND	ND	ND
Docosadienoic C22:2n6	0.22 ± 0.02	0.68 ± 0.04	0.16 ± 0.02	0.14 ± 0	ND	ND
Other Fatty Acids	0.49 ± 0.02	0.68 ± 0.06	0.6 ± 0.08	ND	0.11 ± 0.0	ND
TOTAL POLY-UNSATURATED	73.44 ± 0.22	73.95 ± 0.41	50.81 ± 0.54	51.43 ± 0.42	66.76 ± 0.21	64.33 ± 0.72
n-6/n-3 ratio	0.75	0.75	0.98	0.99	0.23	0.23

ND, not detected; each value represents the mean of three replications ± standard deviation (SD). Results are expressed as g FAME/100 g oil.

3.2. Effect of Fermentation pH and Reducing Sugars

The initial pH levels of the studied substrates were slightly acidic, at 5.9, 6.5, and 6.6 for FSC, HSC, and PSC, respectively, as shown in Figure 1.

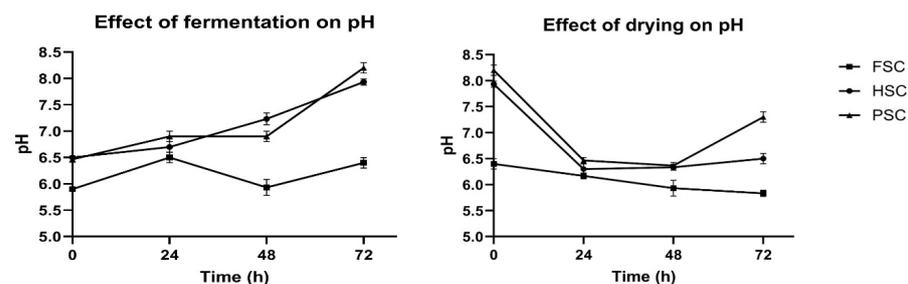


Figure 1. Variation of pH during fermentation and after the drying process.

As fermentation progressed, there was a shift toward alkalinity, with final pH levels reaching 7.9 and 8.2 for HSC and PSC. In contrast, the pH remained slightly acidic for FSC, measuring 6.4 at the end of fermentation. For HSC, the pH increased during fermentation, showing an alkalization trend, while for PSC, alkalization started only after 48 h of fermentation; finally, for FSC, a slower acidification was observed in the first 48 h of fermentation, followed by a slightly rise in pH toward 6.4. After drying, the pH returned to its initial slightly acidic state for HSC and FSC, suggesting the volatilization of alkaline compounds that increased the pH during fermentation. On the other hand, PSC maintained a slight elevation above the baseline pH, measured at 7.3.

As shown in Figure 2, for FSC, reducing sugar content initially decreased from 1.33% in the first 24 h to 1.09%, followed by a subsequent increase to 1.46% at 72 h. For HSC, sugars were predominantly consumed, showing a decline from 1.09% to 0.55%. On the other hand, the PSC exhibited an increase in reducing sugar concentration, rising from 0.36% to 0.86% at 24 h, followed by a further increase to 0.89% at the end of fermentation.

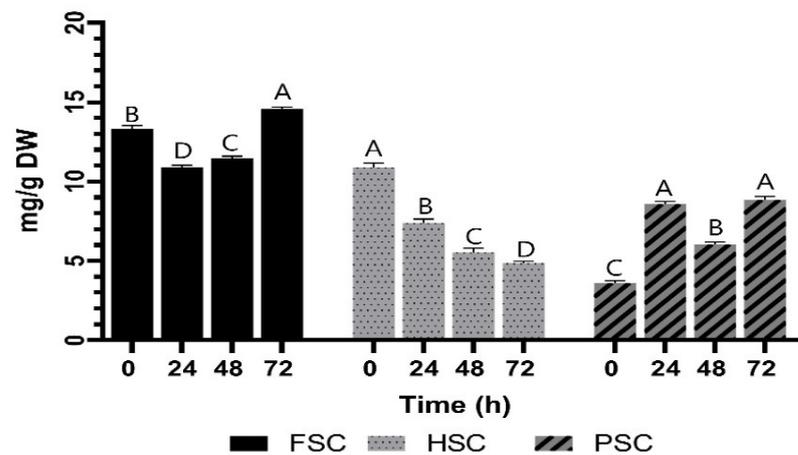


Figure 2. Reducing sugar concentrations at different timepoints. The use of distinct uppercase in the graph corresponds to the results of Tukey's post hoc comparison that highlight differences in reducing sugar concentrations across the four timepoints.

3.3. Effect of Fermentation on Growth Dynamics and Spore Formation

In this case, *Bacillus licheniformis* growth and spore formation exhibited consistency through all examined samples, as demonstrated in Figure 3. Initial count increased in the first 24 h of fermentation from initial 8.57 log cfu/g up to 11.66, 10.97, and 10.17 log cfu/g for HSC, PSC, and FSC, respectively ($p < 0.05$), following a declining phase until the end of fermentation, which was accompanied by an increased spore formation (not determined for T0). Viability reached the lowest point at 72 h of fermentation for all samples, measuring 9.80, 9.47, and 9.32 log cfu/g for HSC, PSC, and FSC ($p < 0.05$). Sporulation rate (%) relative to total viable counts progressed over time (24 h, 48 h, and 72 h), with a rising trend, as follows: from 39.21% and 71.78% to 84.92% for HSC; from 52.99% and 72.19% to 86.36% for PSC; and from 43.60% and 51.61% to 82.26% for FSC ($p < 0.05$). As HSC and PSC displayed an approx. similar pattern, FSC rose from 43.60% to 51.61% in the first 48 h and rapidly surged to 82.26%.

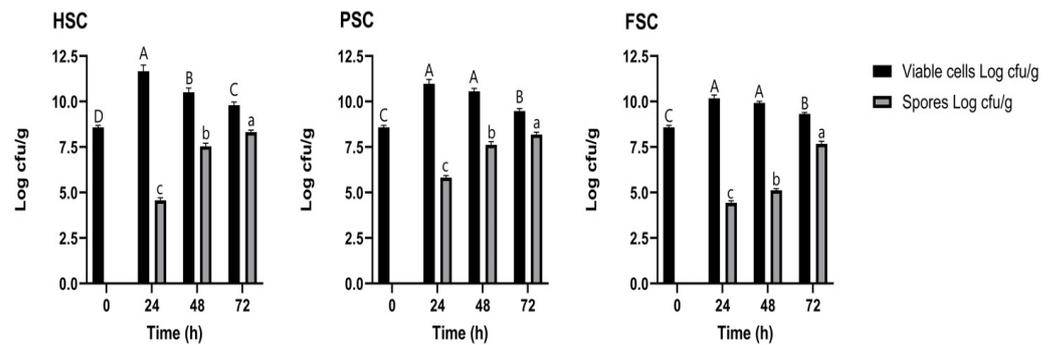


Figure 3. Viability and spore concentrations at different intervals. Bars represent SD of means. The use of distinct uppercase and lowercase letters in the graph corresponds to the results of Tukey's post hoc comparisons. These comparisons highlight differences in viability across the four timepoints and separate differences in spore concentration over the same time intervals.

3.4. Effect of Fermentation on Protein Solubility and Peptide Content

As illustrated in Figure 4, this study indicates a significant improvement in water protein solubility over time due to fermentation ($p < 0.05$) for HSC and FSC, and most notably for PSC, where an approx. 4.8-fold increase was observed after 72 h. Water-soluble protein content (mg/g) increased from 15.87 ± 0.61 to 69.40 ± 1.06 for HSC, from 90.44 ± 0.33 to 127.64 ± 0.67 for FSC, and from 32.46 ± 0.70 to 156.56 ± 0.57 for PSC. Peptide yield, due to *Bacillus licheniformis*' enzymatic activity, increased in the same manner, improving peptide content (mg/g) from 10.62 ± 0.53 to 38.99 ± 0.67 for HSC, 43.73 ± 0.33 to 63.45 ± 0.67 for FSC, and 20.96 ± 0.25 to 45.48 ± 0.88 for PSC.

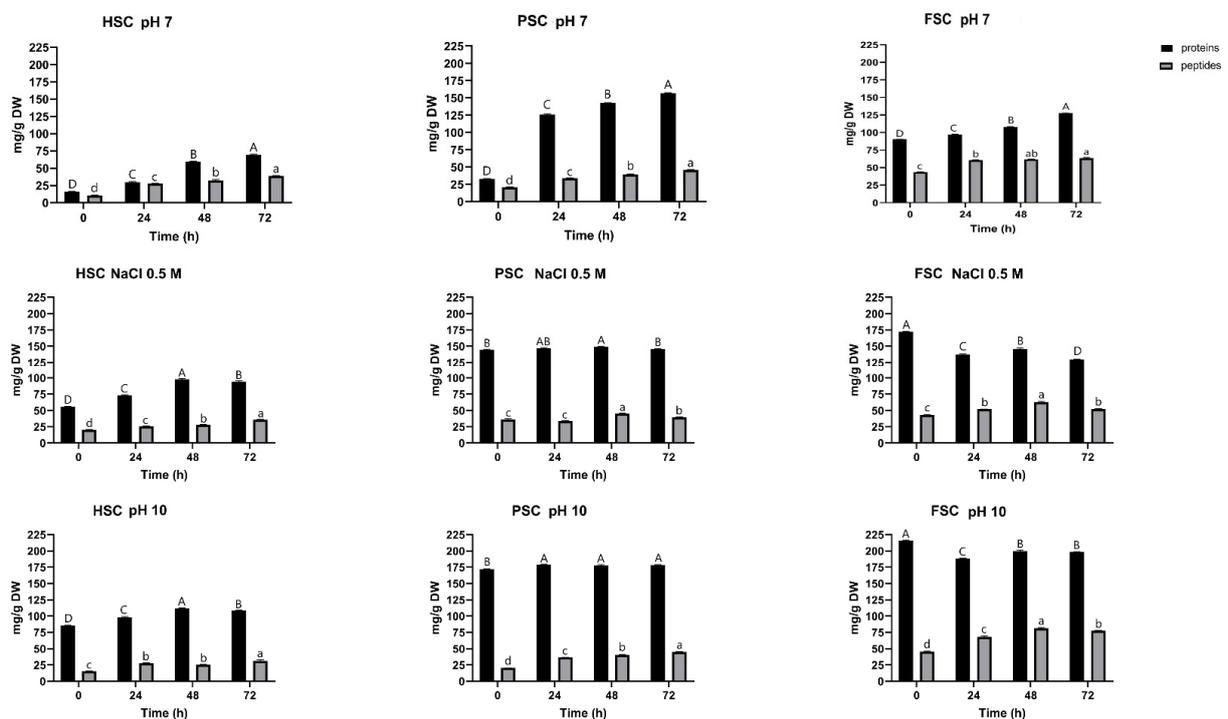


Figure 4. Soluble protein and peptide content during fermentation in water, NaCl, and alkaline solution. Bars represent the mean SD. The use of distinct uppercase and lowercase letters in the graph corresponds to the results of Tukey's post hoc comparisons. These comparisons highlight differences in protein solubility across the four timepoints and separate differences in peptide yield over the same time intervals.

Fermentation positively affected NaCl protein extraction for HSC, increasing the extraction yield (mg/g) from 55.44 ± 0.79 to a maximum of 97.72 ± 1.17 at 48 h. In the case of FSC, a noticeable decrease in protein extracted by salt solution from 172.39 ± 0.72 to 129.53 ± 0.94 was observed. Nevertheless, SSF was not found to be relevant for PSC protein salt extraction. Peptide content (mg/g) increased from 20.36 ± 0.46 to 35.61 ± 0.61 for HSC after 72 h. In contrast, for FSC, an increase was observed from 43.14 ± 0.81 to 62.84 ± 0.95 after 48 h of fermentation, followed by a decrease to 51.78 ± 1.05 at 72 h. Similarly with PSC, peptide yield (mg/g) increased from 36.29 ± 1.13 to 44.96 ± 0.85 mg/g at 48 h, followed by a decrease to 39.39 ± 1.02 mg/g at 72 h, probably due microbial consumption or future hydrolysis to amino acids.

Similar trends were observed in the alkaline extraction as in the salt extraction process. SSF positively influenced HSC, resulting in an increase in extraction yield (mg/g) from 85.53 ± 0.78 to 108.64 ± 0.85 . Conversely, for FSC, there was a decrease from 215.79 ± 0.97 to 198.26 ± 0.44 . In the case of PSC, a marginal increase of approximately 2% was noted within the initial 24 h, rising from 171.63 ± 1.24 to 178.15 ± 0.77 . The lowest content of peptides in comparison with other extractions was obtained through alkaline extraction for HSC, yielding from 15.40 ± 0.71 mg/g to 31.35 ± 1.28 mg/g. PSC's final concentration was similar with water or salt extraction, ranging from 20.31 ± 0.26 to 44.84 ± 0.78 mg/g, and FSC yielded the highest amount at the end of fermentation from 45.38 ± 0.90 mg/g to 77.72 ± 0.56 mg/g, suggesting that FSC exhibited a more pronounced response to enzymatic hydrolysis compared to all other studied samples and conditions.

3.5. *Bacillus licheniformis* Survival under Simulated GI Conditions

In this study, we employed a simulation to consider multiple factors and their interactions simultaneously, rather than isolating them individually, with the viability outcome on different digestion stages, as illustrated in Figure 5. In the first hour of gastric stage, a decline in viability of about 1 log cfu/g was observed for HSC and FSC due to acidic conditions, and 0.12 log cfu/g for PSC. A modest rise in viability from G1 to G2 was observed across all samples, although statistical significance was only noted for FSC ($p < 0.05$). This indicates adaptability to the medium conditions, particularly noticeable in the case of FSC. Transitioning to the intestinal (I1) phase of digestion resulted in a rise in total count by 0.35 and 0.22 log cfu/g for HSC and PSC, respectively. In contrast, there was a small decrease of 0.11 log cfu/g observed for FSC. This decreasing trend persisted in the I2 stage and was particularly significant for HSC and FSC (0.22 log cfu/g, 0.17 log cfu/g, respectively), probably attributable to the presence of bile salts. PSC, in contrast, showed signs of reaching a stationary phase. Despite the modest microbial dynamics observed, *Bacillus* spores demonstrated resilience across the studied conditions. Survivability rate accounted for 90.2%, 101.5%, and 85.72% for HSC, PSC, and FSC when compared to initial counts before simulation.

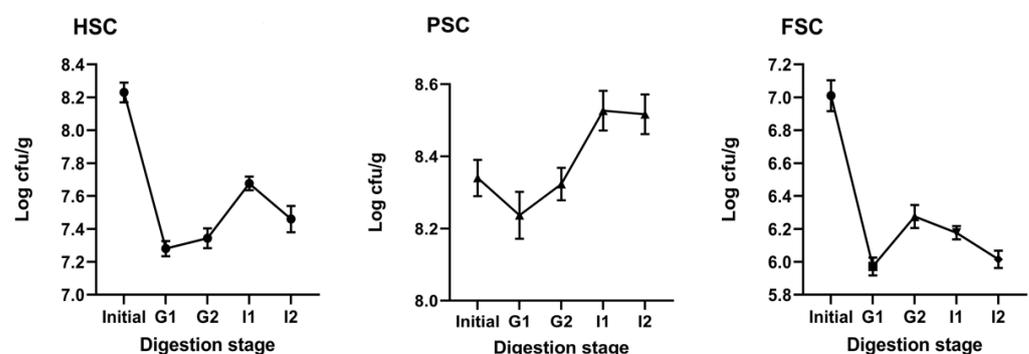


Figure 5. *Bacillus licheniformis* viability during GI simulation on different digestion stages for fermented (T72) samples. Bars represent the mean standard deviation.

3.6. In Vitro Protein Digestibility

Our approach involved measuring the short-chain peptides generated during simulated digestion using TCA-soluble peptides as a marker [39].

Additionally, we employed the BCA assay for the detection and quantification of peptides. The BCA capability to detect peptides at least three amino acids in length and a few dipeptides and amino acids [40] offers a comprehensive assessment of protein breakdown during digestion.

Peptide content of fermented and unfermented OSC, expressed as mg/g DW after each digestion stage, is shown in Figure 6. After 1 h of gastric digestion (G1), the fermented samples (T72) exhibit significantly higher digestibility rates compared to the unfermented ones (T0). Specifically, the digestibility rates for HSC, PSC, and FSC in the fermented samples are 34.73%, 27.48%, and 44.24%, respectively, whereas those in the unfermented samples are 31.28%, 15.20%, and 35.77%. These findings underscore the role of microbial enzymes in facilitating enzymatic hydrolysis under acidic conditions, improving pepsin digestion at low pH.

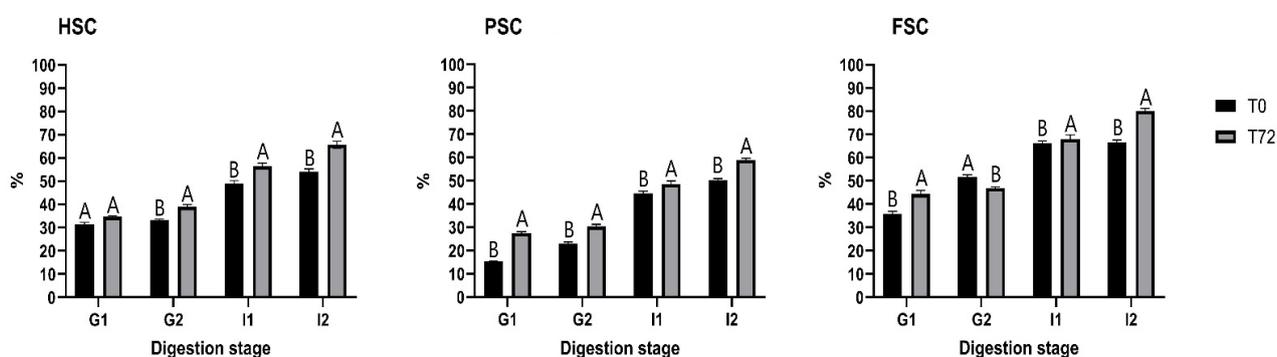


Figure 6. Changes in digestibility (%) during digestion stages. Bars represent the mean SD. The use of distinct uppercase letters in the graph corresponds to the results of Tukey's post hoc comparisons. These comparisons highlight differences in digestibility (%) between unfermented samples (T0) and fermented samples (T72) across the same stages of digestion.

The G2 digestibility results, representing protein breakdown after 2 h of gastric digestion, demonstrate notable improvements across all OCS, where a substantial increase was observed for fermented (T72) HSC and PSC. Following fermentation, the digestibility of HSC increases from 33.30% to 38.98%, while in PSC, it rises from 22.91% to 30.37%. In contrast, FSC demonstrates higher digestibility in the unfermented sample compared to its counterpart, with rates of 51.59% and 46.96%, respectively.

Intestinal digestion markedly increased the amount of liberated peptides, augmented by various proteases, including trypsin from pancreatin composition. SSF improved intestinal (I1 and I2) digestion for all substrates in comparison with unfermented ones, reaching in I1 a digestibility of 56.43%, 48.62%, and 68.03% for fermented (T72) HSC, PSC, and FSC, respectively, in contrast with 48.84%, 44.52%, and 66.41% for unfermented (T0) ones.

At the end of I2, the most pronounced differences were between FSC samples, of about 13.50%, reaching 80.16% in fermented ones, followed by HSC with a difference of approx. 11.60%, reaching 65.67%, and finally PSC, where fermentation improved digestion, with approx. 8.60%, reaching a final protein digestibility of 58.94%.

4. Discussions

In this study, we investigate the dynamic changes of macronutrients and microbial lifecycles through the complete process of the production to consumption of probiotic-enriched feeds by employing chemico-microbiological analysis and in vitro simulated conditions, which allow us to gather comprehensive data within a complex environmental

context. This approach may find relevance in various areas such as animal nutrition, by-product utilization in spore production, or protein and peptide isolation.

Based on the presented results, several notable changes in the nutritional composition of the samples due to fermentation with *Bacillus licheniformis* can be observed. The loss of protein content in all samples can be regarded as a result of the formation of nitrogenous volatile substances, such as ammonia and amines produced during fermentation from protein degradation by microbes [41], that creates an alkaline environment associated with an inhibition in spoilage microorganism and a modification of organoleptic characteristics [42]. As our results show, the drying method could reverse this process, resulting in subsequent loss in volatile compounds associated with a returning of the pH to initial levels.

A potential explanation for crude fiber, NDF, and ADF increases could be related to microbial biomass accumulation through the fermentation process as cells typically contain complex carbohydrates as structural components such as cell walls. A reduction in NDF (composed of cellulose, hemicellulose, and lignin) suggest the presence of carbohydrase activity in PSC. The ability of a bacterial strain to ferment carbohydrates is crucial for various metabolic activities, including growth and reproduction. As the chosen substrates are low in readily available sugars < 1–2% [43–45], the strain is likely to rely on its repertoire of carbohydrase to hydrolyze complex carbohydrates for its carbon metabolism. In this context, different dynamic patterns of production and consumption of reducing sugars can be attributed to substrate composition.

Bacillus sp. possess the ability to alter the composition of FAs within their cells and surrounding environment; this modification of FA patterns is crucial for various cellular functions such as membrane formation and adaptation to different environments. Notably, the process of de novo biosynthesis of FAs has been demonstrated to occur during sporulation [46]. In studies investigating the metabolic adaptation of *Bacillus licheniformis* to stressors such as thermal stress [47] or varying growth stages [48], alterations in cell membrane composition may occur to regulate fluidity. In our study, the dynamic synthesis of specific fatty acids, whether up-regulated or down-regulated, could be attributed to the various stressors encountered during fermentation in the OCS medium. These findings underscore the complex alterations induced by fermentation in the FA profile of OSC, which could have implications for their nutritional value and suitability for various applications in animal feed formulations. However, more studies are required to fully elucidate the influence of different environmental conditions on FA profile modulation by *Bacillus* sp.

When encountering a new life medium, endospore formation is a crucial survival strategy for certain bacteria. It is triggered by various environmental cues (temperature, pH, aeration, presence of certain minerals, carbon or nitrogen concentrations), and one such trigger occurs at the end of the exponential growth phase when nutritional deprivation is recognized, such as during a transition from a nutrient-rich to a nutrient-poor environment. Another factor is population density, as the culture grows, there is an accumulation of a secreted peptide known as the competence and sporulation factor, which acts as an autoinducer for quorum-sensing mechanisms that trigger sporulation [49–51]. Our results suggest that changing culture conditions from inoculum to SSF using HSC, PSC, and FSC create an environmental stress that directly induces the expression of the sporulation mechanism [52,53].

A delay in sporulation in FSC at the 48 h mark suggests that it may harbor substrate-specific nutrients that delay sporulation without increasing in total viable count. Furthermore, a longer fermentation time is required to assess, as Gray et al. [54] suggested, whether the remaining vegetative cells will eventually sporulate or persist in a slow-growing state, rendering them resilient to environmental conditions. Interestingly, based on plated colonies and microscopy observations, new colony morphologies emerged after 3 days of fermentation, typically seen in *Bacillus* spp. biofilms where strains differentiate in different populations [52], suggesting that OSC promotes the apparition of a new subpopulation of genetically identical cells but with different gene expressions [51]. Spore production has been attempted on various subproducts through SSF. Zhao et al. [55] achieved a spore

count of 11 log cfu/g of *Bacillus licheniformis* on wheat bran and straw powder, while Chistyakov et al. [56] reported a notable production of 11.95 log cfu/g *Bacillus amyloliquefaciens* on soybean meal, showing that agricultural by-products are an economical alternative for the production of bacterial spores.

For a functional probiotic-based feed to effectively exert its activity, the probiotic microorganisms must endure harsh or challenging environmental conditions from the GI tract, including low pH, high temperatures, elevated levels of bile salts, and nutrient deprivation [57]. Considering the resilience of *Bacillus* spores in withstanding gastrointestinal-simulated conditions as shown in this study, an intriguing question arises regarding their capacity to transition into vegetative states and sustain their life cycle and potentially colonizing the intestine. The observed increase in viability from G1 to G2 during gastric simulation suggests the strain's capability to proliferate and adapt to initial gastric conditions. However, a subsequent decline in CFU during intestinal simulation indicates a shift into a new adaptation phase for the bacteria, where a lesser reduction viability suggests that PSC has a beneficial food matrix effect over *Bacillus licheniformis* [58]. However, further investigation is needed to clarify if the spore germination versus the remaining vegetative cells is responsible for these dynamics.

In this work, we also conducted a preliminary evaluation on the potential of employing a short fermentation of protein-rich substrates for enhancing protein and peptide recovery. In general, seed storage proteins can be classified based on solubility; they include water-soluble albumins, salt-soluble globulins, hydrophobic prolamins which are commonly soluble in 70% ethanol, and glutenin primarily extracted using acidic solvents or alkali [59,60]; however, some quantity of protein may remain unextracted if not soluble in these solvents [61]. The protein fractions derived from HSC primarily comprise edestin (globulin), which constitutes approximately 60–80%, while albumin around 25% [62]. PSC consists of 87% extractable proteins (glutelin 49%, globulin 20.4%, albumin 13.5%, and prolamins 4.3%) [63] and FSC (albumin 38.1%, glutelin 33.9%, and globulin 27.9%) [64]. An industry-relevant extraction is considered a salt extraction (known as micellization), which is considered a milder extraction associated with less structural and conformational changes [61]. It affects solubility by altering the electrostatic interactions (ionic strength) promoting aggregation [62]. Alkali extraction is widely used at an industrial level; it is suggested that the use of alkali can disrupt disulfide cross-linking in proteins and to ionize neutral and acidic amino acids, therefore increasing solubility [62]. Even though alkali has high efficiency, it favors the racemization of the amino acids L-isomers to D-analogues in a concentration- and exposure time-dependent manner that could lead to toxic and antagonistic actions in animals, thus reducing feeds' nutritional quality [65]. Our results suggest that peptide yield was improved for all OSCs by employing water, alkaline or salt extraction, probably due to enzymes like proteases and cellulases that are widely used in order to improve hydrolysis degree, solubility, and functionality [66]. However, the extraction method and the optimization of strain catalytic activities could further improve the extraction of protein and peptides for OSC. In other studies, hemp seed meal protein solubility increased from 6.07% to 15.15% after *Bacillus subtilis* fermentation [67]. In PSC, a remarkable peptide content of approx. 190 mg/g was achieved using *R. oligosporus*, due to high fungal proteolysis activity [68]. In flaxseed proteins, fermentation with *Bacillus altitudinis* was successfully employed to generate bioactive peptides with antioxidant and antibacterial activity [69].

The evaluation of protein digestibility is crucial for nutritional assessment in terms of the quality and bioavailability of feed products. Therefore, fermentation enhances in vitro protein digestibility possibly by *Bacillus licheniformis* proteolytic activity that can target plant-based proteins, thereby promoting hydrolysis, protein modification [70–72], and remediation of anti-nutritional factors such as trypsin inhibitors [19]. Some studies using lactic bacteria achieved 65–79% IVPD in hemp [73]. Alternatively, indigenous microbial communities' microbiota were used for PSC fermentation where protein digestibility was 68.7% after 7 days of fermentation [74]. Isolation of flaxseed protein can give a digestibility

coefficient of 68% [75], while in other cases, fermentation with *Aspergillus oryzae* was directly correlated with proteolytic activity, thereby enhancing digestibility to values between 70 and 85% after 96 h [76].

5. Conclusions

Fermentation is a dynamic process with substrate specificity orchestrated by the degradation and synthesis of new compounds. While proximate composition provides a broad overview, it may not fully capture the complex chemical transformations occurring during fermentation. We did not find *Bacillus licheniformis* strain ATCC 21424 capable of making extreme changes in proximate composition or fatty acid profile; however, it seems capable of markedly improving water protein solubility and salt solution solubility in the case of HSC, accompanied by the generation of higher peptide yields. The SSF of oilseed cakes induces sporulation, demonstrating that it is suitable for producing probiotic-enriched feeds that can withstand harsh environmental conditions from simulated GI tracts along with a noticeable improvement in protein digestibility. Furthermore, our study underscores the potential of laboratory-domesticated strains like ATCC 21424 in improving feed digestibility and functionality through SSF. Future research focusing on strain adaptation to fermentation media holds promise for enhancing the capabilities of such strains.

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Abbreviations

HSC	hemp seed cake
PSC	pumpkin seed cake
FSC	flaxseed cake

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