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Nutritional and Functional Biovalorization of Potato Pulp through Simultaneous and Two-Stage Fermentations

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Abstract

Background and Objective: Potato pulp, a major agro-industrial by-product, contains valuable nutrients, which are still underused due to their limited bioavailability and functional characteristics. This study aimed to enhance the nutritional and functional qualities of potato pulp through solid-state fermentation (SSF) using *Lactobacillus fermentum* MT-ZH893 (LF) in combination with *Aspergillus oryzae* (AOR) and *Rhizopus oryzae* (ROR).

Material and Methods: Potato pulp was utilized as the substrate for solid-state fermentation. Four strategies combining *Lactobacillus fermentum* with fungi were investigated, including: simultaneous fermentation (LF+AOR and LF+ROR) and two-stage fermentation (fungi inoculated first, followed by the bacteria of; AORLF and RORLF). Key parameters assessed included crude protein content, probiotic viability, total phenolic content, antioxidant capacity (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate and 2,2-diphenyl-1-picrylhydrazyl assays), organic matter digestibility and metabolizable energy.

Results and Conclusion: Simultaneous fermentation with *Aspergillus oryzae* increased crude protein content, whereas treatments involving *Rhizopus oryzae* resulted in a significant decrease in crude protein. Simultaneous fermentation significantly enhanced probiotic viability. In contrast, the two-stage process using *Rhizopus oryzae* produced superior outcomes in total phenolic content (1011 mg Gallic acid equivalents per gram of dry material, antioxidant capacity (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate, 934.9 µmol Trolox; 2,2-diphenyl-1-picrylhydrazyl, 97.01%), organic matter digestibility and metabolizable energy. These results indicated that while simultaneous fermentation optimized probiotic viability and protein content (with *Aspergillus oryzae*), the two-stage approach with *Rhizopus oryzae* significantly enhanced the functional characteristics and digestibility of potato pulp, presenting promising strategies for value-added bioprocessing of agro-industrial residues.

Keywords: Agroindustrial byproducts, *Aspergillus oryzae*, *Lactobacillus fermentum*, Potato pulp, *Rhizopus oryzae*, Solid-state fermentation

What is "already known" on this topic:

- Potato pulp is a lignocellulosic agro-industrial by-product with limited utilization due to high moisture and rapid spoilage.
- Solid-state fermentation (SSF) is a known bioprocess for enhancing the nutritional and functional properties of various food byproducts.
- *Aspergillus oryzae* and *Rhizopus oryzae* produce hydrolytic enzymes that degrade cell wall components and enhance substrate digestibility.

What this article adds:	➢ Simultaneous SSF of potato pulp with *Lactobacillus fermentum* MT-ZH893 *and A. oryzae* maximized crude protein content (15.91% DM) and probiotic viability. ➢ Two-stage SSF (*R. oryzae* followed by *L. fermentum*) achieved the highest TPC (1011 mg GAE/g DM), antioxidant capacity (DPPH: 97.01%; ABTS: 934.9 µmol Trolox), organic matter digestibility, and metabolizable energy. ➢ The sequence of microbial inoculation in SSF of potato pulp critically shapes the final product profile. ➢ Fermented potato pulp, owing to its significantly enhanced nutritional and functional properties, demonstrates considerable potential as a nutraceutical feed supplement in livestock diets.

1. Introduction

Potatoes rank as the world's fourth most significant food crop, contributing profoundly to global food security [1]. With their extensive consumption and industrial processing, vast quantities of wastes, particularly potato peels and pulp, are generated, which are projected to reach over 8 million tons by 2030, emitting nearly 5 million tons of CO_2 equivalents [2]. While such wastes pose serious environmental challenges, these represent a valuable reservoir of bioactive compounds and fermentable substrates with great potential for sustainable bio-valorization [3]. Potato pulp, achieved at roughly 0.75 tons per ton of extracted starch [4], is a lignocellulose-rich byproducts consisting of water, starch, cellulose, hemicellulose, pectin, protein, fibrous carbohydrates, phenolic compounds and minerals. Despite its valuable composition, economic and technical barriers linked to preservation and transport currently limit its industrial use [5].

To overcome these limitations and safely use these agro-industrial residues, various strategies have been suggested. From these suggestions, microbial fermentation has significantly been interested as a highly effective approach for increasing the nutritional values and functional characteristics of such byproducts. By converting complex macromolecules into simpler further digestible components, fermentation not only improves the overall nutritional quality of the residue but also facilitates its stabilization and further use.

Therefore, solid-state fermentation (SSF) has emerged as a compelling solution to increase the added value of potato pulp. Rather than fundamentally solving economic transport barriers, SSF represents an economically viable and environmentally friendly bioprocess characterized by low water and energy consumption. The efficiency of SSF is ruled by several key variables, including moisture content, temperature, fermentation time and inoculation strategy. Use of probiotics such as *Lactobacillus fermentum* with filamentous fungi such as *Aspergillus oryzae* and *Rhizopus oryzae* in SSF systems is particularly advantageous. The *L. fermentum* shows immunomodulatory, anti-inflammatory and microbiota-balancing effects. Complementarily, these fungal species secrete hydrolytic enzymes that facilitate starch and protein degradation, improving substrate digestibility and supporting probiotic viability [6]. This integration promotes circular bioeconomy principles by transforming agri-food wastes into high-value health-promoting products [7].

Furthermore, numerous studies have directly highlighted the efficacy of SSF in valorizing potato byproducts. For example, potato pulp has been used as a substrate for enzyme production using recombinant *A. oryzae* strain [8]. Moreover, SSF of potato peels uses *R. oryzae* enriched fungal biomass with proteins and essential amino acids (EAA) [9]. A two-stage fermentation converted potato starch wastes into high-quality animal feed using mutant *A. niger* followed by *Bacillus licheniformis* [10]. More recently, enzyme-assisted fermentation of potato pulp promoted the growth of *L. plantarum*, increasing organic acid production and altering microstructural characteristics to facilitate rapid drying [11]. Co-fermentation of *Aspergillus* spp. with *B. subtilis* improved physicochemical characteristics and decreased spoilage of potato pulp [12].

Regarding sequential strategies aimed at probiotic enrichment, fungal pretreatment with *A. oryzae* enhanced the viability of *Pediococcus acidilactici* by degrading the rigid lignocellulosic structure of the substrate [13]. Similarly, SSF of industrial potato wastes with *L. plantarum, S. cerevisiae* and *A. oryzae* increased phenolic compounds, antioxidant capacity and simultaneously decreased anti-nutritional factors such as phytic acid [14]. Despite these advancements, studies specifically assessing the SSF of potato pulp that use simultaneous or sequential cultures of *L. fermentum* with *A. oryzae* or *R. oryzae* are still limited.

Accordingly, the overall strategy of this study was to valorize potato pulp through solid-state fermentation by comparing simultaneous and two-stage fermentation systems of *L. fermentum* with either *A. oryzae* or *R. oryzae*.

By focusing on interspecies metabolic interactions, the study aimed to systematically investigate the effects of various inoculation strategies on nutritional quality, antioxidant enrichment, probiotic stability and substrate digestibility. Ultimately, this study was carried out with the goal of developing a value-added bio-based feed additive from this starchy agro-industrial waste; thereby, identifying the optimal approach for its microbial upcycling.

2. Materials and Methods

2.1. Materials

2.1.2. Potato pulp and microorganisms

Potato pulp was sourced from Alvand Starch, Iran, dried at 50 °C for 48 h and stored at -18 °C. The *L. fermentum* MT.ZH893 was isolated from Mazandaran local cheese at the Food Science and Engineering Department, Tarbiat Modares University, Tehran, Iran [15]. The *A. oryzae* PTCC 5163 was provided from the microbial collection of the Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran, and *R. oryzae* PTCC 5176 was provided by Persian Type Culture Collection (PTCC), Iranian Research Organization for Science and Technology (IROST), Tehran, Iran.

2.1.3. Materials and Reagents

The chemicals and culture media used in this study were as follows. Briefly, de Man, Rogosa and Sharpe (MRS) agar media were purchased from Ibresco, Iran; methanol (CH_3 OH) was purchased from Majalli Industrial Chemicals, Iran; 2,2-diphenyl-1-picrylhydrazyl radical [2,2-diphenyl-1-picrylhydrazyl ($DPPH^+$) ≥ 98% purity], 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt [2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate ($ABTS^+$) ≥ 98% purity] and Folin-Ciocalteu phenol reagent were purchased from Sigma-Aldrich, USA. Trichloroacetic acid (TCA) and sulfuric acid were purchased from Merck, Germany. All reagents included analytical grade or higher and were used without further purification unless otherwise stated.

2.2. Methods

2.2. Fermentation process

In the two-stage fermentation, 20 g of dried potato pulp were transferred into a 250-ml Erlenmeyer flask, brought to 60 % humidity and sterilized at 121◦ C for 15 min using autoclave. Then, 1 ml of fungi [*A. oryzae or R. oryzae* (10^8 cfu ml^{-1})] was added to the media and incubated at 30 ◦ C for 24 h. The mixture was autolyzed at 55 ◦C for 24 h. After cooling down, the probiotic *L. fermentum* was added to the potato pulp at a concentration of 1.5×10^8 cfu ml^{-1} and incubated at 37 ◦C for 72 h. In the simultaneous fermentation, fungi and *L. fermentum* were inoculated in a single step. The fermentation condition was microaerophilic [16,17]

2.3. Viability analysis

After the fermentation period, 1 g of the fermented substrate was weighed using conical tube (15 ml) with physiological serum under sterile conditions and diluted serially after complete homogenization. Then, 0.1 ml of each dilution was surface-cultured on MRS agar media, which was incubated at 37 °C for 72 h. With known percentage of moisture in the fermented substrate, the colony count was calculated based on dry matter (g) of the substrate as cfu per gdry weight substrate (cfu $gdws^{-1}$) [18]

2.4. Total protein and total nitrogen

The total nitrogen content of digested samples was assessed using micro-Kjeldahl apparatus. For the analysis, 0.2 g of dried sample and 1 g of Kjeldahl digestion catalyst (sodium sulfate with copper sulfate and/or selenium) were transferred into the designated digestion tube of the system. Then, 7.5 ml of concentrated sulfuric acid (H_2 SO_4 , 98%) were added and digestion was carried out at 360°C for 5–6 h. The total nitrogen value was multiplied by 6.25 to calculate the total protein percentage [19].

2.5. True protein

To assess true protein content, the fresh samples were first dried at 50 °C for 24 h until constant weight using oven. The dried samples were thoroughly mixed and homogenized to ensure uniformity; then, 0.5 g was accurately weighed using laboratory balance. The weighed sample was transferred into a standard laboratory glass beaker and mixed with 50 ml of distilled water (DW). The mixture was set at room temperature (RT) for 30 min. Then, 10 ml of 10% trichloroacetic acid (TCA) were added and the sample was set standing for 30 min at RT. The precipitated proteins were filtered through Whatman filter paper no. 1 and the residue was dried using oven. The nitrogen content of the dried residue was assessed using micro-Kjeldahl method and the non-protein nitrogen (NPN) content was calculated as difference between the total nitrogen and the protein nitrogen from the filtrate [20].

2.6. Assessment of gas production, organic matter digestibility and metabolizable energy

Gas production resulting from fermentation was assessed based on a method of Menke et al. (1979). First, samples were ground using a sieve with a 1 mm pore size. A 200 mg portion of the dried sample (60 °C) was transferred into each calibrated glass syringe. After sample loading, the plunger walls were lubricated with petroleum jelly and the syringes were pre-incubated at 39 °C to equilibrate with the temperature of the rumen fluid–artificial saliva mixture. Blank syringes were included at the beginning, midpoint and end of each incubation series. Additionally, a standard feed substrate with a known gas production value (e.g., standard hay or concentrate) was used for calibration purposes.

Rumen fluid was collected from at least two rumen-fistulated wethers and strained through layers of cheesecloth to remove large particles. The collected fluid, containing solid and liquid fractions, was homogenized thoroughly. Donor animals were maintained on a diet containing 30–50% concentrate to ensure forage quality. Artificial saliva was prepared by mixing macro-mineral, micro-mineral, buffer, resazurin and reducing solutions. The DW, buffer and mineral solutions were mixed together using flask, incubated at 39 °C using water bath and supplemented with resazurin to produce a blue solution. This mixture was stirred continuously while a gentle stream of CO_2 was passed through. A reducing solution was added to the mixture until the color changed from blue (oxidized) to purple and then cleared (reduced). The CO_2 flushing continued for nearly 10 min (occasionally up to 15–20 min) to achieve full reduction. Rumen fluid was added to the artificial saliva at a 2:1 ratio, followed by 10 min of CO_2 flushing. The CO_2 inlet was positioned above the fluid surface and flow was adjusted to maintain an anaerobic atmosphere.

Thirty milliliters of the inoculum mixture were injected into each syringe containing the feed substrate. Excess air was expelled by holding the syringe vertically, opening the clamp and adjusting the plunger. Syringes were incubated at 39 °C and cumulative gas volumes were recorded regularly for up to 120 h, until gas production plateaued (e.g., equal readings at three consecutive time points). Organic matter digestibility (OMD) was calculated from the 24 h gas volume ($GP_{(24)}$) from fermentation of 200 mg DM using Eq. 1:

$$OMD = 14.88 + (0.889 \times GP_{24}) + (0.045 \times CP) + (0.0651 \times XA) \quad (1)$$

Where, $GP_{(24)}$ was gas volume after 24-h incubation (ml/200 mg DM), CP was crude protein (g $kgDM^{-1}$) and XA was ash content (g $kgDM^{-1}$). Metabolizable energy (ME) was calculated based on Eq. 2:

$$ME = 2.20 + (0.136 \times GP_{24}) + (0.0057 \times CP) + (0.00029 \times EE^2) \quad (2)$$

Where, ME was Metabolizable energy, expressed in megajoules per kilogram of dry matter; $GP_{(24)}$ was gas volume produced after 24 h; CP was crude protein concentration, expressed in grams per kilogram of dry matter; and EE was ether extract, expressed in grams per kilogram of dry matter [21].

2.7. Total phenolic content

For the quantification of total phenolic content (TPC) in the samples, Folin-Ciocalteu method was used. In this procedure, the fermented sample extracts were achieved by centrifuging the samples at 26,832 g for 15 min using refrigerated centrifuge (Kubota 6900, Japan). A volume of 200 µl of the prepared extracts was mixed with 500 µl of Folin's reagent (diluted 10-fold with DW) in 2-ml vials, which were set in dark for 5 min. Then, 1 ml of 5.7% sodium carbonate (Na_2CO_3) solution was added to each vial. After vortexing, the samples were set in dark for 1 h. The control sample was prepared similarly with the exception that DW replaced the sample. After the incubation period, absorbance of the samples was measured at 765 nm using spectrophotometer. Gallic acid standard curve was plotted with concentrations ranging 0–250 ppm and the phenolic content was expressed as milligrams of Gallic acid equivalents per gram of dry matter (mg GAE gDM^{-1}) [22].

2.8. The DPPH radical scavenging assay

The antioxidant activity of the samples was investigated using DPPH free radical scavenging method. Briefly, 0.1-mM DPPH solution in methanol was prepared and thoroughly vortexed. The extracts of the fermented samples were achieved by centrifugation at 4000 rpm for 15 min. Then, 500 µl of the sample extract and 500 µl of the DPPH solution were mixed and vortexed using 2-ml vials. Furthermore, a control sample consisting of 500 µl of methanol and 500 µl of DPPH solution was prepared. The vials were set in dark for 30 min; then, their absorbance was measured at 517 nm using spectrophotometer. The radical scavenging activity (RSA) was calculated using Eq. 3 [23].

$$\text{Radical scavenging activity (\%)} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100 \quad (3)$$

Where, $A_{control}$ was absorbance of the control and A_{sample} was absorbance of the sample.

2.9. The ABTS radical scavenging assay

The ABTS cation radical scavenging activity was calculated based on the reduction in the green-blue color of this radical by antioxidant compounds. To prepare the reagent solution, 7 mM ABTS solution and 2.45 mM potassium persulfate were mixed in a 1:1 ratio and set in dark for 12–16 h. Then, 600 µl of the resulting solution were diluted with 40 ml of DW to adjust the absorbance to approximately 0.7 at 734 nm. For the assay, 20 µl of the sample extract were mixed with 980 µl of the ABTS solution and set in dark for 10 min; the control was prepared by replacing the extract with DW. Absorbance of the samples was measured at 734 nm and ABTS radical scavenging ability of the samples was calculated in micromoles based on the Trolox standard curve [24].

2.10. Scanning electron microscopy

The fermented potato pulp samples were frozen at -74 °C for 24 h, followed by freeze-drying using laboratory freeze dryer. The dried samples were ground and sieved through a 40-mesh sieve (0.45-mm aperture). Then, the powdered samples were coated with a thin layer of gold and studied using scanning electron microscope (SEM) operated at an accelerating voltage of 5.0 kV [11].

2.10. Statistical analysis

Data were analyzed using one-way ANOVA. To analyze the significance of the means, JMP Pro 18 software (SAS, USA) was used, providing robust statistical tools to handle the various data sets generated throughout the study. The Tukey test was used to compare the means and to assess the statistical significance of differences in all stages. All graphs were generated using Excel 365 v.2508 (Microsoft, USA). All assessments were carried out in triplicate.

3. Results and Discussion

3.1. Viability of Lactobacillus fermentum

As shown in Figure 1, fungal pretreatment, whether through simultaneous inoculation or two-stage fermentation, generally enhanced the survival of *L. fermentum*. In the simultaneous inoculation method, the fungus and probiotic bacteria were introduced into the culture substrate at the same time, initiating a multi-faceted synergistic interaction. This interaction is environmental and nutritional as *A. oryzae* consumed oxygen during its initial growth phase, creating microaerophilic conditions that stimulated growth of the facultative anaerobic *L. fermentum*. Simultaneously, the fungus secreted a range of hydrolytic enzymes such as cellulase, amylase and protease, which broke down complex substrates into simpler further absorbable monomers for the bacteria [25]. The ability of *A. oryzae* to produce cellulase is particularly effective for the hydrolysis of lignocellulosic wastes [26]. These effects provided conditions that significantly increased the survival of *L. fermentum*.

In the two-stage fermentation method, the fungus was first grown alone, allowing it sufficient time to produce and secrete a maximum level of macromolecule-degrading enzymes; hence, creating favorable conditions for bacterial growth and survival [27]. This fungal pretreatment improved nutrient availability by enzymatically degrading macromolecules while preventing the excessive consumption of fermentable sugars; thereby, minimizing the production of inhibitory compounds such as furfural [28]. Furthermore, as the fungus grew, its mycelia penetrated the substrate, which decreased particle size and created a further suitable physical environment for the bacteria. Then, fungal autolysis could release intracellular nutrients back into the media [17]. These processes collectively improved the growth substrate for the bacteria; however, the accumulation of fungal metabolites and the consequent pH decrease following pretreatment might shift the environment away from the optimal range for *L. fermentum*. This acidic stress could be detrimental, as it might force bacteria into a lag phase or impair growth by decreasing the uptake of essential elements such as iron and phosphorus, even if food sources were available [27]. Overall, the two methods, through the degradation of lignocellulosic compounds and the production of beneficial metabolites, enhanced probiotic survival. Nevertheless, the simultaneous inoculation method in this study demonstrated a statistically significant improvement in the survival of the probiotic bacteria.

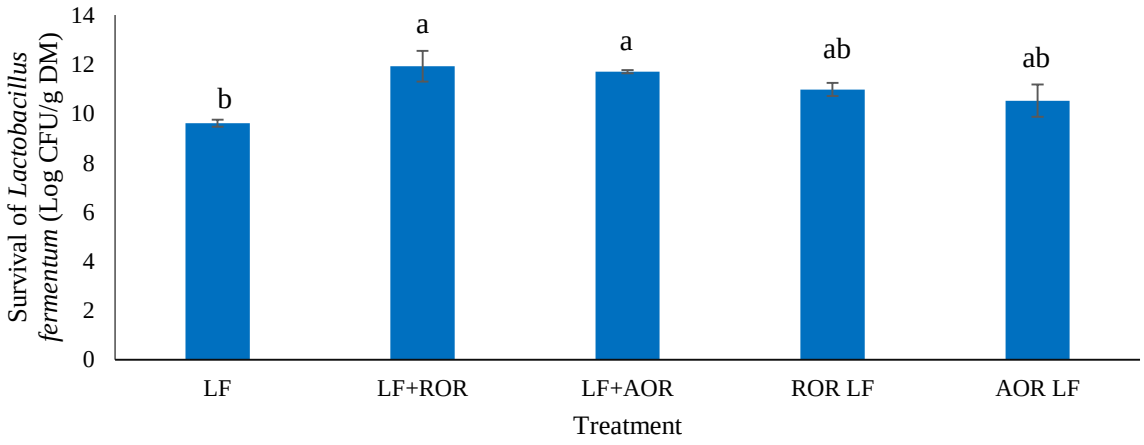

Figure. 1. The viability of *L. fermentum at different treatments on* potato pulp after 48 hours of solid-state fermentation at 37°C. LF (fermented with *L. fermentum*), LF+AOR (simultaneous fermentation with *L. fermentum* and *A. oryzae*), LF+ROR (simultaneous fermentation with *L. fermentum* and *R. oryzae*), ROR LF (two-stage fermentation; initially with *R. oryzae*, followed by *L. fermentum*), and AOR LF (two-stage fermentation; initially with *A. oryzae*, followed by *L. fermentum*). The lowercase letters above the bars indicate statistically significant differences in the means ($P < 0.05$) according to Tukey's test. Data are presented as mean ± SD (n = 3).

3.2. Crude protein and true protein

Based on the results presented in Figure 2, the LF+AOR treatment showed the highest crude protein content (15.91%) in all treatments ($p \leq 0.05$). The LF and AOR LF treatments contained 15.4 and 14.5% of crude protein, respectively. The control treatment (PP) included 13.96% of crude protein, while the lowest value (12.5%) was observed in the LF+ROR treatment ($p \leq 0.05$). The fermentation process could cause significant changes in the protein content of potato pulp. Based on the results, simultaneous fermentation, especially with *A. oryzae*, led to a significant increase in crude protein, compared to the control and other treatments ($p \leq 0.05$). The increase in protein during fermentation could be attributed to various factors such as the accumulation of fungal and bacterial biomass on the fermented potato pulp substrate [29,30] or secretion of extracellular enzymes by microorganisms during cellular metabolism, which were proteinaceous in nature. An increase in protein content played a critical role in improving the nutritional value of animal feed. The results indicated that solid-state and submerged fermentation of fruit and vegetable wastes could produce fermented products with high protein contents. Fermentation by fungi, whether in solid or liquid form, represented a successful strategy for valorizing waste materials to produce valuable and sustainable alternative protein sources for animal feed formulations [31]

The increase in protein content during fermentation could be attributed to several biological and biochemical mechanisms. These included the production of proteolytic enzymes by microorganisms during cellular metabolism, which facilitated breakdown of complex proteins, bioconversion of carbohydrates and lipids into proteinaceous compounds, and growth and proliferation of fungal mycelia that contributed to the overall protein biomass. Additionally, the decrease in pH during fermentation helped prevent nitrogen loss, while the conversion of non-protein nitrogen into microbial protein further enhanced total protein levels. Other contributing factors included the accumulation of fungal and bacterial biomass on the potato pulp substrate after fermentation [29,31,32]. Another study assessed the effect of fermentation on the composition of Irish and sweet potato peels, reporting increases in moisture, ash, fat and proteins with decreases in crude fibers and carbohydrate contents [34].

Based on the results in Figure 2, the highest true protein content (11.87%) was observed in the control treatment (PP), whereas the lowest value (5.97%) was recorded for the LF+ROR treatment ($p \leq 0.05$). The LF and AOR LF treatments contained 8.36 and 8.72% true protein, respectively, the two showing significant differences compared to the control group ($p \leq 0.05$). The LF+AOR treatment demonstrated 9.15% of true protein, while the two-stage ROR LF treatment showed 7.76%. In this experiment, fermentation particularly in combined treatments, resulted in a significant decrease in true protein content, compared to the control ($p \leq 0.05$). The discrepancy between crude protein and true protein suggested that protein structure was modified during fermentation, potentially enhancing amino acid availability, but not all the nitrogen was retained in a form measurable as true protein. Previous studies have reported variable effects of fermentation on true protein content, depending on microbial strains and process conditions. For example, in the solid-state fermentation of sweet potato industry residues, the highest true protein yield was achieved by co-culturing of *A. oryzae* with *B. subtilis* that led to an overall increase in protein components after fermentation [35].

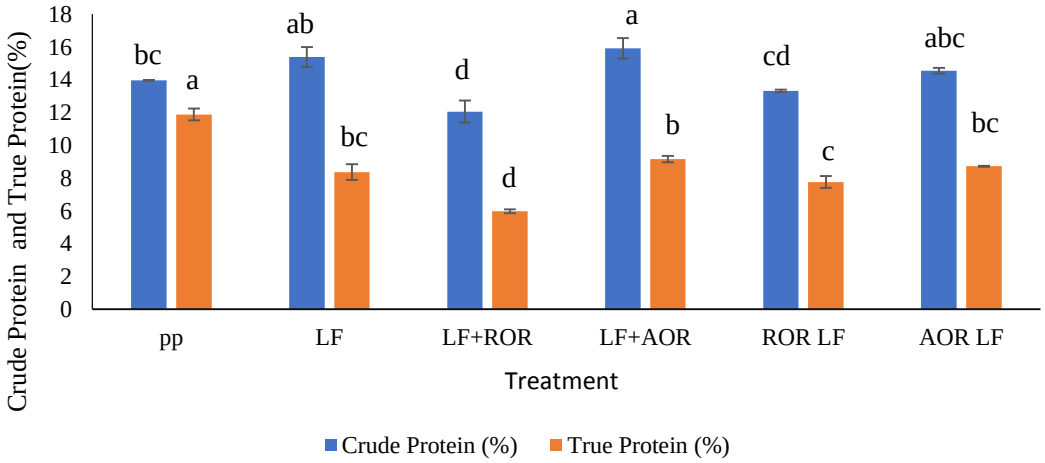

Figure. 2. Total Protein and True Protein (%). PP (unfermented potato pulp), For abbreviations, see Figure. 1. The lowercase letters above the bars indicate statistically significant differences in the means (P < 0.05) according to Tukey's test. Data are presented as mean ± SD (n = 3).

Similarly, Borras et al. (2020) detected that true protein in fermented potato wastes increased by 2.7 U within 24 h at 25 °C [36]. In contrast, Djukic-Vukovic et al. (2015) reported that in lactic acid and animal feed production from distillery stillage using *L. rhamnosus*, protein contents decreased after fermentation while total nitrogen increased, compared to unfermented samples. Similarly, Dadkhodazadeh et al. (2024) observed that solid-state fermentation of fish wastes with probiotic strains led to an increase in non-protein nitrogen accompanied by a decrease in true protein [37,38]. These findings collectively indicated that under certain conditions such as high proteolytic activity or imbalance between protein degradation and microbial protein synthesis, fermentation might decrease measurable true protein despite increasing crude protein, highlighting the need to optimize process parameters to maximize nitrogen retention in its most digestible form.

3.3. Organic matter digestibility and metabolizable energy

Based on the results in Figures 3 and 4, the effects of treatment type on organic matter digestibility (OMD) and metabolizable energy (ME) of potato pulp were clearly evident. Two-stage treatments involving fungal fermentation followed by *L. fermentum* (AOR LF and ROR LF) demonstrated the highest OMD and ME values, which were significantly different from other treatments ($p < 0.05$). The observed increase in two-stage treatments was likely attributed to the effective degradation of cell wall structures by fungal enzymes (e.g., cellulase and hemicellulase) during the first stage, followed by the production of beneficial metabolites (organic acids and proteolytic enzymes) by *L. fermentum* in the second stage, resulting in enhanced nutrient release and digestibility. In contrast, simultaneous co-cultures (LF+ROR and LF+AOR) showed lower performance, possibly due to microbial competition and limited specific enzymatic activities, compared to two-stage treatments. Single-stage fermentation with *L. fermentum* (LF) induced a moderate improvement in OMD and ME, compared to the control (PP). These findings emphasized that the two-stage fungus-bacteria fermentation strategy was more effective in enhancing digestibility and energy value than that simultaneous and single-stage fermentations were, which could be explained by synergistic enzymatic and biometabolic mechanisms.

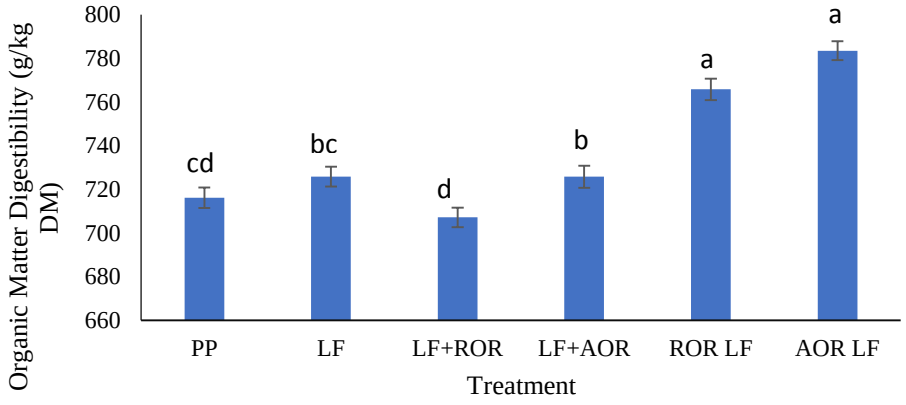

Figure. 3. Organic matter digestibility (g/kg DM) of different treatments.), For abbreviations, see Figure. 1. The lowercase letters above the bars indicate statistically significant differences in the means ($P < 0.05$) according to Tukey's test. Data are presented as mean ± SD (n = 3).

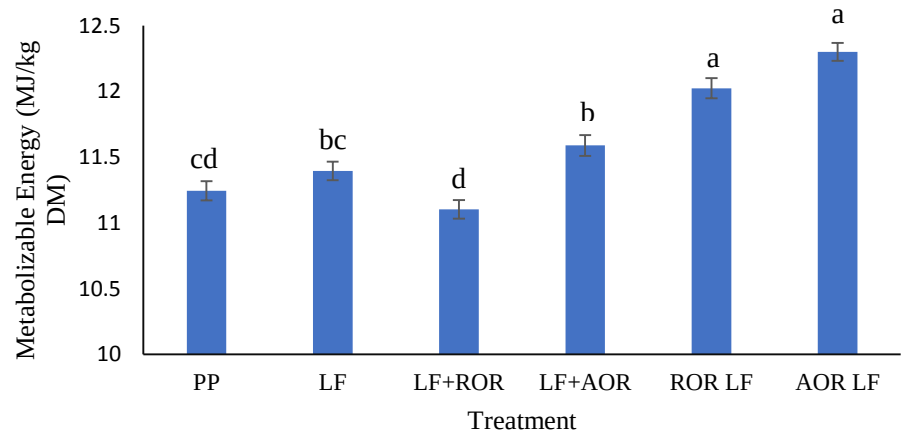

Figure. 4. Metabolizable Energy (MJ/kg DM). For abbreviations, see Figure. 1. The lowercase letters above the bars indicate statistically significant differences in the means ($P < 0.05$) according to Tukey's test. Data are presented as mean ± SD (n = 3).

The results demonstrated that two-stage fermentation, using *R. oryzae* and *A. oryzae* in the initial stage followed *by L. fermentum* in the subsequent stage, facilitated structural cell wall component breakdown and nutrient release through synergistic microbial activities, significantly improving the digestibility and nutritional value of plant-based substrates [38–40]. Fungi secrete enzymes such as cellulose, xylanase [41,42] and proteases for protein hydrolysis [43,44], effectively pretreating the substrate. In the second stage, *L. fermentum* produced lactic acid and organic acids that decreased pH and facilitated complex molecule degradation, improving flavor profile and nutritional indices [45,46]. The findings of this study, which included an increase in OMD and ME after fermenting the samples, were similar to those of previous studies [47–50]. These results indicated that microbial fermentation, improved organic matter digestibility and feed energy use through the degradation of lignocellulosic structures.

3.4. Total phenols and antioxidant capacity

In this study, it was observed that fermentation with *L. fermentum* increased TPC (Figure 5) and antioxidant capacity (Figures 6 and 7). The *R. oryzae* facilitated the enzymatic degradation of cell wall components during solid-state fermentation, releasing bound phenolic compounds. When coupled with *L. fermentum*, the liberated phenolics could be involved in further biotransformation, leading to increased antioxidant activity. This synergistic interaction was especially effective in two-stage fermentations, where initial fungal pre-treatment maximized phenolic release, followed by bacterial fermentation that stabilized and enhanced the antioxidant potential. Such improvements have been verified in various fermented food substrates such as wheat and corn kernels, with significant increases in TPC and antioxidant activity measured through DPPH, ABTS and FRAP assessments [51,52].

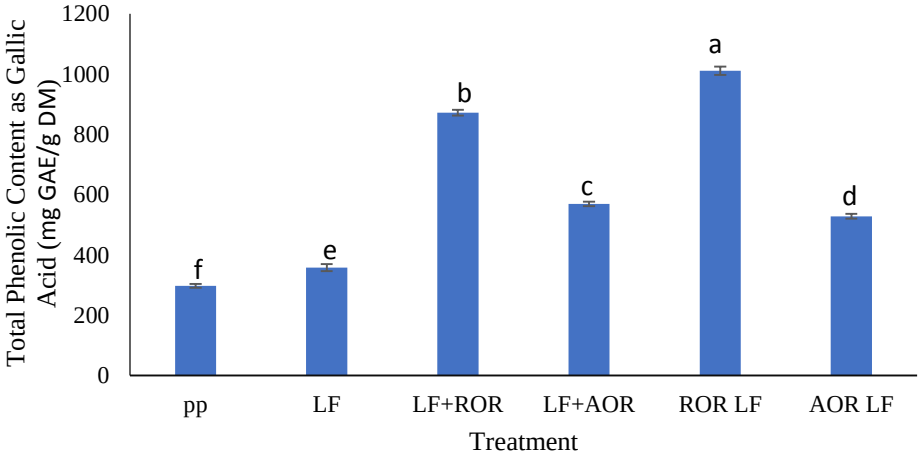

Figure. 5. Total phenolic content as gallic acid (ppm) of different treatments. For abbreviations, see Figure. 1. The lowercase letters above the bars indicate statistically significant differences in the means ($P < 0.05$) according to Tukey's test. Data are presented as mean ± SD (n = 3).

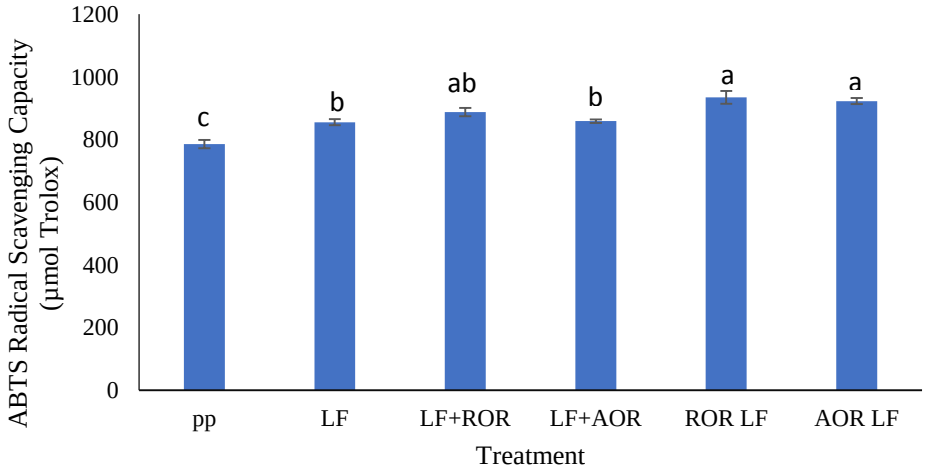

Figure. 6. ABTS radical scavenging capacity (µmol Trolox) of different treatments**.** For abbreviations, see Figure. 1. The lowercase letters above the bars indicate statistically significant differences in the means ($P < 0.05$) according to Tukey's test. Data are presented as mean ± SD (n = 3).

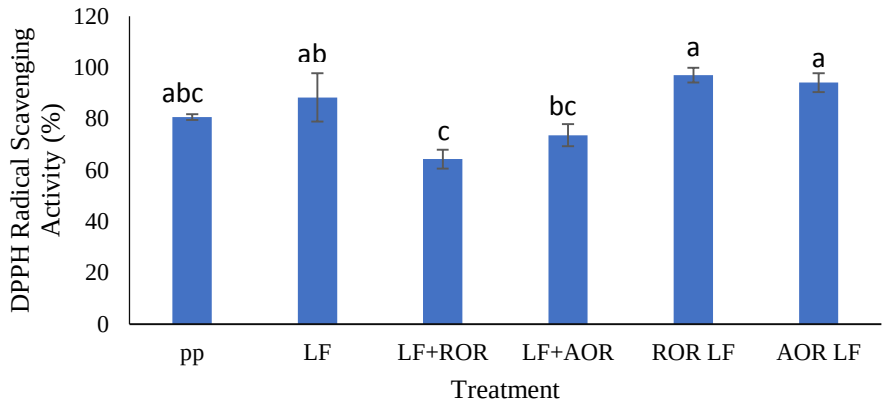

Figure. 7. Inhibition percentage of DPPH free radicals of different treatments. For abbreviations, see Figure. 1. The lowercase letters above the bars indicate statistically significant differences in the means ($P < 0.05$) according to Tukey's test. Data are presented as mean ± SD (n = 3).

The combined fermentation of food matrices with *R. oryzae* or *A. oryzae* with *L. fermentum* significantly enhanced total phenolics, flavonoids and antioxidant indices. These effects were attributed to the enzymatic activity of *R. oryzae* (e.g., β-glucosidase and polysaccharidases) and the strong cell wall-degrading ability of *A. oryzae*, which promoted the release and stabilization of antioxidant compounds [53]. Similar to [54] who reported an increase in TPC and antioxidant activity of fermented malt cake as a result of solid-state fermentation with fungal strains, the present study demonstrated that fermentation of potato pulp samples led to the release of bound phenolic compounds and improvement in free radical scavenging capacity. This effect was likely attributed to the production of hydrolytic enzymes by the microorganisms and the cleavage of bonds between the phenolic compounds and the cell matrix during the fermentation process. Overall, the integration of these microbes in fermentation processes not only boosted antioxidant characteristics but also ensured greater bioactive functionality, as consistently demonstrated through DPPH and ABTS assessments.

3.5. Scanning electron microscopy analysis

The SEM analysis provided mechanistic insights into the structural degradation of potato pulp during fermentation (Figure 8). The unfermented control (Figure 8A; PP) showed intact, smooth starch granules encased within a cohesive matrix, representing a significant physical barrier to nutrient bioavailability, whereas monoculture fermentation with *L. fermentum* (Figure 8B; LF) initiated structural erosion via surface pitting and micro-cracks, indicating early-stage enzymatic and acid-driven disruption. Furthermore, simultaneous co-fermentation strategies (Figure 8C, LF+ROR; and Figure 8D, LF+AOR) revealed synergistic breakdown with profound starch granule collapse, extensive cell wall degradation and network-like microbial clusters, facilitating highly efficient substrate depolymerization. Sequential two-stage fermentations (Figure 8E, ROR-LF; and Figure 8F, AOR-LF) demonstrated the most severe microstructural dismantling, where the original matrix was completely replaced by fragmented debris, proving that fungal pretreatment successfully maximizes subsequent bacterial penetration. These microstructural shifts verified that complex fungal-bacterial fermentation strategies fundamentally dismantled the inherent physical barriers of potato pulp, significantly amplifying substrate accessibility and its ultimate nutritional value.

4. Conclusion

This study highlighted the novelty and efficiency of integrating *L. fermentum* with filamentous fungi (*A. oryzae* and *R. oryzae*) through solid-state fermentation to valorize low-value potato pulp into a functional livestock feed. Comparative analysis of inoculation strategies demonstrated that while simultaneous fermentation maximized probiotic viability, the two-stage process (fungal pretreatment followed by bacterial fermentation) achieved the most comprehensive nutritional enhancement. This approach effectively used fungal enzymatic activity to degrade complex fibers, improving digestibility, energy value and releasing bound phenolics that enhanced antioxidant capacity. Overall, the synergistic and eco-friendly bioprocessing model provided a sustainable framework for converting agro-industrial residues into functionally enriched feed ingredients, supporting waste decrease and the circular bio-economy in animal nutrition.

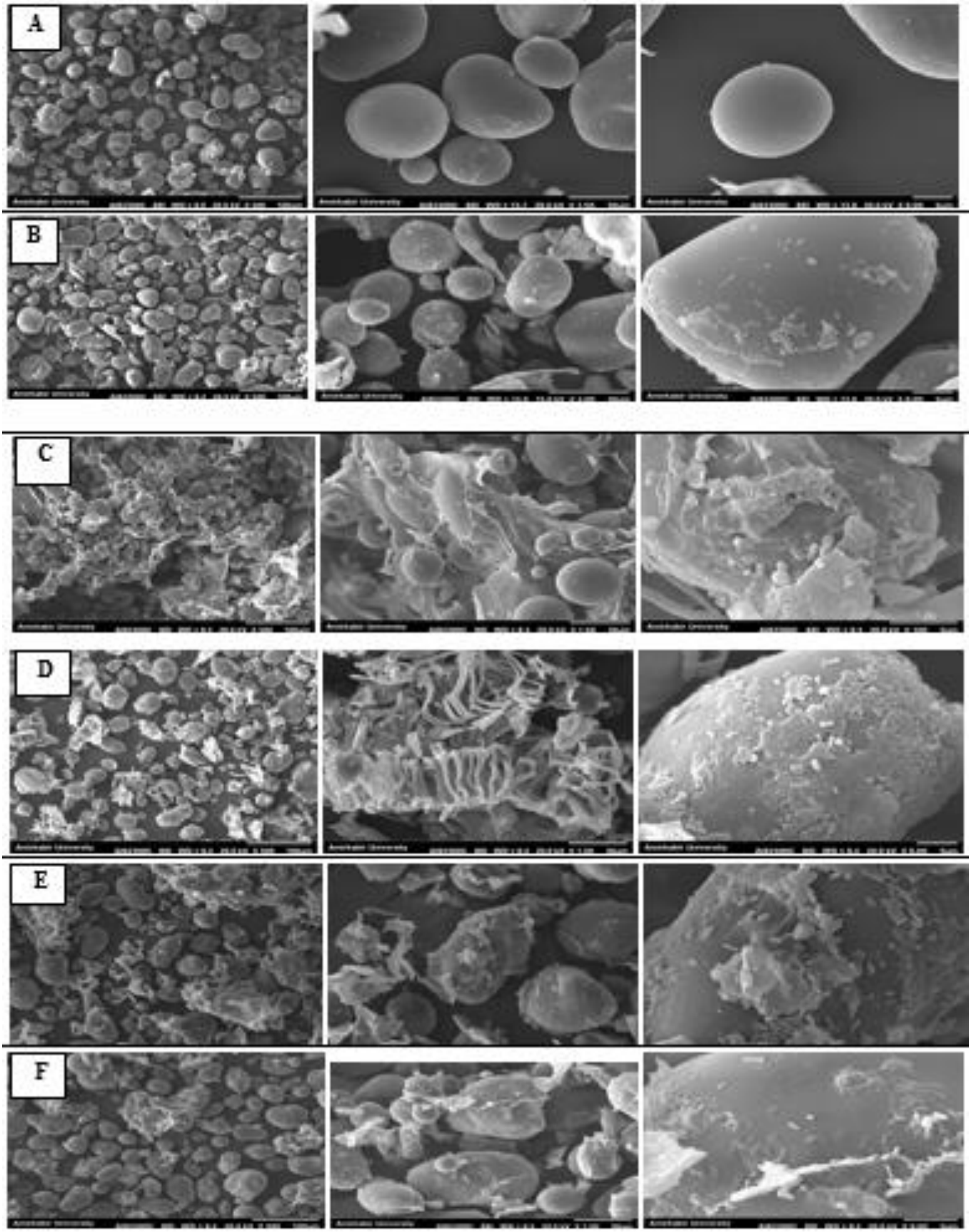

Figure. 8. Scanning electron micrographs (SEM) of different treatments. (A), control sample (non-fermented potato pulp) (B), pulp fermented with *Lactobacillus fermentum* (C), simultaneous fermentation with *Rhizopus oryzae* and *L. fermentum* (D), simultaneous fermentation with *Aspergillus oryzae* and *L. fermentum* (E), two-stage fermentation, initially with *R. oryzae* followed by *L. fermentum* (F), two-stage fermentation, initially with *A. oryzae* followed by *L. fermentum*.

5. Declaration

5.1. Acknowledgements

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5.2. Declaration of competing interest

The authors declare no conflict of interest.

5.3. Authors' Contributions

Hamidi-Esfahani Z. "conceptualization, supervision, methodology, writing - review and editing, validation"; Bagherikia H. "formal analysis, investigation and writing - original draft preparation"; Karimi Torshizi M.A. "visualization, validation, writing - review and editing".

5.4. Using Artificial Intelligent Chatbots

This manuscript was entirely written and developed by the authors based on original experimental data. During its preparation, the authors used ChatGPT (OpenAI) solely to enhance the grammar and language clarity of certain sentences. All AI-assisted outputs were carefully reviewed and edited by the authors, who take full responsibility for the content of this manuscript.

5.5. Ethical Consideration

All authors verify that the study in the manuscript does not involve any human or animal trial experiments.

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ارزش‌افزایی پالپ سیب‌زمینی از طریق تخمیر هم‌زمان و دومرحله‌ای با *لاکتوباسیلوس فرمنتوم* و قارچ‌ها: بهبود ویژگی‌های تغذیه‌ای و عملکردی

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چکیده

سابقه و هدف: پالپ سیب‌زمینی، یک محصول جانبی عمده کشاورزی-صنعتی، حاوی مواد مغذی ارزشمندی است، اما به دلیل فراهمی زیستی محدود و خواص عملکردی آن، کمتر مورد استفاده قرار می‌گیرد. این مطالعه با هدف بهبود کیفیت تغذیه‌ای و عملکردی پالپ سیب‌زمینی از طریق تخمیر حالت جامد (SSF) با استفاده از *لاکتوباسیلوس فرمنتوم*-MT ZH893در ترکیب با *آسپرژیلوس اوریزه* و *ریزوپوس اوریزه* انجام شد.

مواد و روش ها: از پالپ سیب‌زمینی به عنوان سوبسترا برای تخمیر حالت جامد استفاده شد. چهار استراتژی مختلف برای ترکیب *لاکتوباسیلوس فرمنتوم* با قارچ‌ها مورد ارزیابی قرار گرفت: تخمیر همزمان (LF+AOR و LF+ROR) و تخمیر دو مرحله‌ای (ابتدا تلقیح قارچ و سپس باکتری؛ AORLF و RORLF). پارامترهای کلیدی ارزیابی‌شده شامل محتوای پروتئین خام، قابلیت حیات (زنده مانی) پروبیوتیک‌ها، محتوای ترکیبات فنلی کل(TPC) ، ظرفیت آنتی‌اکسیدانی (آزمون‌های ABTS وDPPH) ، قابلیت هضم ماده آلی و انرژی قابل متابولیسم بود.

یافته‌ها و نتیجه‌گیری: تخمیر هم‌زمان با *آسپرژیلوس اوریزه* موجب افزایش محتوای پروتئین خام شد، در حالی که تیمارهای حاوی *ریزوپوس اوریزه* (هم در تخمیر هم‌زمان و هم دومرحله‌ای) کاهش معنی‌داری در پروتئین خام نشان دادند. تخمیر هم‌زمان به‌طور قابل‌توجهی زنده مانی *لاکتوباسیلوس فرمنتوم* را افزایش داد .در مقابل، تخمیر دو مرحله‌ای با *ریزوپوس اوریزه* نتایج برتری در محتوای کل فنولی(۱۰۱۱mg GAE/g DM)، ظرفیت آنتی‌اکسیدانی(ABTS : ۹۳۴/۹ میکرومول ترولاکس؛ DPPH: ۹۷/۰۱ درصد) ، قابلیت هضم مواد آلی و انرژی قابل متابولیزه ایجاد کرد. این نتایج نشان می‌دهد که اگرچه تخمیر همزمان باعث بهینه‌سازی زنده مانی پروبیوتیک‌ها و محتوای پروتئین (در ترکیب با*آسپرژیلوس اوریزه*) می‌شود، اما رویکرد دو مرحله‌ای با *ریزوپوس اوریزه* خواص عملکردی و قابلیت هضم پالپ سیب‌زمینی را به میزان قابل‌توجهی بهبود می‌بخشد؛ که این امر استراتژی‌های امیدوارکننده‌ای را برای فرآوری زیستی و ایجاد ارزش افزوده از پسماندهای کشاورزی-صنعتی ارائه می‌دهد.

واژگان کلیدی: محصولات جانبی صنایع کشاورزی، *آسپرژیلوس اوریزه* ، *لاکتوباسیلوس فرمنتوم* ، پالپ سیب‌زمینی، *ریزوپوس اوریزه* ، تخمیر حالت جامد.