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Improvement in Palm Kernel Meal Quality by Solid-Sate Fermentation with *Bacillus velezensis*, *Saccharomyces cerevisiae* and *Lactobacillus paracasei*

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Abstract: To improve the quality of palm kernel meal (PKM), the effect of solid-state fermentation (SSF) with *Bacillus velezensis*, *Saccharomyces cerevisiae* and *Lactobacillus paracasei* on nutritional components, anti-nutritional factor and antioxidant activity were investigated. The results show that inoculation ratio of three strains 4:2:1, inoculation amount 21%, moisture content 52%, fermentation temperature 34 °C and fermentation time 60 h were the optimal SSF conditions. After 60 h of fermentation, the content of neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose and hemicellulose in PKM were significantly decreased by 22.5%, 18.2%, 20.2%, 17.6% and 32.4%, respectively. Meanwhile, the content of crude protein, soluble protein, peptides, amino acids and reducing sugar were increased significantly by 27.3%, 193%, 134%, 16.3% and 228%, respectively. SSF significantly improved the total phenolic content, DPPH radical scavenging activity, hydroxyl radical scavenging activity and reducing power. In addition, in vitro dry matter digestibility (IVDMD) and in vitro crude protein digestibility (IVCPD) were increased. Scanning electron microscopy (SEM) analysis revealed microstructural alterations in PKM. The results indicate that SSF with *B. velezensis*, *S. cerevisiae* and *L. paracasei* is an effective and promising method to enhance the nutritional value and antioxidant activity of PKM, providing a feasible solution for increasing the utilization of PKM in animal feed.

Keywords: palm kernel meal; solid-sate fermentation; anti-nutritional factor; nutritional value; antioxidant activity

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1. Introduction

In general, agro-industrial processes produce large quantities of by-products annually. If not properly managed, these by-products can negatively impact the environment and result in the inefficient use of valuable resources. Palm kernel meal (PKM) is the by-product of palm oil extraction. The world supply of palm oil and PKM is expected to reach approximately 79.63 million metric tons and 10.72 million metric tons, respectively, by 2024 [1]. To efficiently utilize PKM, its applications have been explored in organic fertilizer, feed for animals, power and steam production and energy production using biogas or combustion [2]. PKM contains approximately 12–21% crude protein as well as some minerals such as calcium, manganese, zinc and sodium, and it is relatively inexpensive, making it a potential protein source for animal feed [3,4]. PKM is commonly used in ruminant diets, but its use is limited in nonruminant diets, such as those for swine and poultry, due to its high fiber content, which is an anti-nutritional factor [3,5]. Dietary fiber retards the digestibility of nutrients in monogastric animals and reduces the surface area, width and height of intestinal villi in poultry [6]. PKM utilization is also limited by other

factors such as low protein, poor amino acid balance and poor nutrient digestibility [3,7]. Therefore, efforts should be focused on exploring techniques for improving PKM quality to enhance its application potential in the feed industry.

The available literature for research on the solid-state fermentation (SSF) with appropriate microbial strains validate that SSF is an efficient way to reduce anti-nutritional factors, improve nutritional components and functional properties of feed resources [8–10]. SSF has generally been carried out using *Bacillus*, *Lactobacillus*, yeast and mold [8,9,11]. For instance, SSF of soybean meal with *Lactobacillus paracasei* subsp. *paracasei* increased the content of organic acids, free amino acids and bioactive isoflavones [4]. SSF with *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Bacillus amyloliquefaciens* enhanced the nutritional content and antioxidant activity, and lowered the content of anti-nutritional factors [12]. *Bacillus velezensis*, a novel species explored in recent years, is a non-toxic probiotic which exhibits significant potential in promoting the growth of aquaculture animals [13,14]. The SSF of soybean meal with *B. velezensis* led to an improvement in nutritional value, revealing the application potential of *B. velezensis* in the fermentation of feed resources [15,16].

The beneficial effects of SSF in enhancing the quality of PKM have been documented, which primarily focused on fiber reduction and crude protein enhancement [17–19]. To the best of our knowledge, SSF of PKM studies related to peptides, amino acids, total phenolics and antioxidant activity have been less investigated. *Trichoderma harzianum*, *Trichoderma longibrachiatum*, *Trichoderma koninggi*, *Aspergillus niger*, *Aspergillus oryzae*, *Aspergillus awamori*, *B. amyloliquefaciens*, *Paenibacillus curdlanolyticus*, *Paenibacillus polymyxa*, *Bacillus megaterium* and *Sclerotium rolfsii* have been employed for SSF of PKM [17–19]. However, the potential of SSF using *B. velezensis*, *L. paracasei* and *S. cerevisiae* to enhance the nutritional value and antioxidant activity of PKM has not yet been explored.

Based on the above, the present study attempted to improve the quality of PKM by SSF with a combination of *B. velezensis*, *S. cerevisiae* and *L. paracasei*. After the essential conditions affecting SSF were optimized, the influence of SSF on nutritional components, anti-nutritional factor and antioxidant activity were investigated. In addition, the changes in microbial count, in vitro digestibility and microstructure of PKM before and after fermentation were evaluated.

2. Materials and Methods

2.1. Materials

Strain selection was primarily based on the ability to hydrolyze cellulose and mannan (using sodium carboxymethyl cellulose and konjac mannan as sole carbon sources) and secondarily on the capacity of producing reducing sugar during SSF. Lastly, *B. velezensis* WZ1, *S. cerevisiae* NJ1 and *L. paracasei* LC86 were selected for the SSF of PKM based on the comprehensive capacity described above. *L. paracasei* LC86 was purchased from Wecare Probiotics Co., Ltd. (Suzhou, China). *B. velezensis* WZ1 and *S. cerevisiae* NJ1 were obtained from Jiangsu Provincial Key Construction Laboratory of Probiotics Preparation, Huaiyin Institute of Technology. PKM was provided by Shandong Changrong Hanyuan Biotechnology Co., Ltd. (Binzhou, China). After drying at 60 °C for 24 h, PKM was milled and passed through a 40-mesh sieve before use. All chemicals used in the experiment were of analytical grade.

2.2. SSF of PKM

For preparation of starters, fresh cultures of *B. velezensis*, *S. cerevisiae* and *L. paracasei* were aseptically inoculated to LB (Luria-Bertani), MRS (de Man, Rogosa and Sharpe) and YPD (Yeast Extract Peptone Dextrose) media, respectively. *B. velezensis* was cultured at 37 °C, 180 rpm for 18 h. *S. cerevisiae* was cultured at 30 °C, 180 rpm for 18 h. *L. paracasei* was cultured at 30 °C, 180 rpm for 18 h. The culture broth of three strains were mixed in a ratio of 1:1:1. Then, the PKM was inoculated with 20% (*v*/*w*) of the mixture of three trains and supplemented with 30% (*v*/*w*) of sterile water. Following thorough mixing, the PKM mixture was incubated at 37 °C for 48 h. After SSF, samples of unfermented PKM and FPKM at 36 h (36 h FPKM), 48 h (48 h FPKM) and 60 h (60 h FPKM) were dried at 60 °C for 18 h for further analysis.

2.3. Optimization of SSF Conditions

The conditions for SSF of PKM with *B. velezensis*, *S. cerevisiae* and *L. paracasei* were optimized using reducing sugar as an indicator. Initially, a single-factor experiment (SFE) was conducted to optimize the inoculation ratio of three strains, inoculation amount, moisture content, fermentation temperature and fermentation time. Subsequently, response surface methodology (RSM) was applied using a Box–Behnken design (BBD) with Design-Expert 13 software (Stat-Ease, Inc., Minneapolis, MN, USA) to further optimize significant factors.

2.4. Chemical Compositions

The crude protein content was analyzed by the Kjeldahl method (990.03) [20]. The lipid content was determined according to the method of Rayaroth et al. [21]. The neutral detergent fiber (NDF), acid detergent fiber (ADF), acid detergent lignin (ADL), cellulose and hemicellulose content were estimated according to the methods described by Iluyemi et al. [17]. The peptide content was detected according to the method described by Wang et al. [22]. The amino acid content was analyzed according to our previous research [12].

To determine the reducing sugar content, sample (1 g) was extracted with 9 mL of boiling water and then incubated at 100 °C for 5 min, followed by shaking at 37 °C, 180 rpm for 15 min. After centrifuging at 5000× *g* for 5 min, the reducing sugar in the supernatant was measured by DNS regent with glucose as standard [23] and expressed as mg reducing sugar/g dry sample.

To analyze the total phenolic content, sample (5 g) was extracted with 40% ethanol (100 mL) by shaking at 70 °C, 150 rpm for 30 min. After centrifuging at 5000× *g* for 5 min, the resulting supernatant was freeze-dried to test the total phenolic content and antioxidant activity. The total phenolic content was analyzed by Folin–Ciocalteu's reagent with gallic acid as standard [24] and expressed as mg total phenolics/g of dry sample.

To measure the soluble protein content, sample (1 g) was extracted with 9 mL of deionized water by shaking at 28 °C, 180 rpm for 60 min. After centrifuging at 5000× *g* for 5 min, the protein in the supernatant was measured by the Bradford assay with bovine serum albumin (BSA) as standard [25] and expressed as mg soluble protein/g of dry sample.

2.5. B. velezensis, S. cerevisiae, L. paracasei Counts

B. velezensis, *S. cerevisiae* and *L. paracasei* counts during SSF of PKM at different time intervals were determined by the plate dilution method. Sample (2 g) was mixed with 20 mL of deionized water and shaken at 180 rpm, 28 °C for 60 min. *B. velezensi* was enumerated on LB agar incubated anaerobically at 37 °C for 36 h. *S. cerevisiae* was enumerated on YPD agar incubated at 30 °C for 48 h. *L. paracasei* was enumerated on MRS (de Man, Rogosa and Sharpe) agar incubated at 30 °C for 60 h. The results are expressed as the logarithm of colony-forming unit per gram of sample (log CFU/g).

2.6. Total Titratable Acidity

The total titratable acidity (TTA) determination was carried out according to the method described by Zhao et al. [26], with little difference. Sample (10 g) was mixed with 90 mL of deionized water and stirred magnetically for 1 h at room temperature. The suspension was titrated with 0.1 M NaOH to an endpoint at pH 8.2. TTA was expressed as the volume (mL) of 0.1 M NaOH per gram of sample utilized.

2.7. In Vitro Digestibility

To evaluate the in vitro dry matter digestibility (IVDMD) and in vitro crude protein digestibility (IVCPD) for nonruminants, a sequential digestion model with pepsin and trypsin was employed, following the method described by Zhu et al. [27]. Essentially, 2 g of sample was blended with 100 mL of phosphate buffer (0.1 M, pH 6.0) and 40 mL of HCl solution (0.2 M). The pH was adjusted to 2.0 with 1 M HCl or 1 M NaOH. Then, 2 mL of chloramphenicol solution (0.5 g/100 mL ethanol) and 4 mL of 2% pepsin solution were added and incubated at 39 °C, 50 rpm for 2 h. Subsequently, 40 mL of phosphate buffer (0.2 M, pH 6.8), 20 mL of 0.6 M NaOH and 4 mL of 10% trypsin solution were added. The

mixture was incubated at 39 °C, 50 rpm for 4 h. At the end of digestion, the undigested residue was collected by filtering through a 50 μm nylon cloth and then washed with ethanol (2 × 25 mL, 95% ethanol) and acetone (2 × 25 mL, 99.5% acetone). Residue was dried at 60 °C for 24 h and used for dry matter and crude protein analysis. IVDMD and IVCPD were computed as follows:

$$\text{IVDMD(IVCPD)} = [\text{dry weight (crude protein) of the sample before hydrolysis} - \text{dry weight (crude protein) of the residue}]/\text{dry weight (crude protein) of the sample before hydrolysis} \quad (1)$$

2.8. Antioxidant Activity Determination

2.8.1. DPPH Radical Scavenging Activity

The 2-2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was carried out according to the method described by Oskoueian et al. [28]. In brief, 1 mL of 0.2 mM DPPH solution was added to 1 mL of sample solution, and the mixture was reacted in the dark at 25 °C for 30 min. The absorbance was measured at 517 nm. The scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (A1 - A2)/A0)] \times 100\% \quad (2)$$

where A0, A1 and A2 represent the absorbance of DPPH in ethanol, the sample mixed with DPPH and the sample in ethanol solution, respectively.

2.8.2. Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging activity was measured by the method reported by Jiang et al. [29] with slight modification. Briefly, 1 mL of sample solution was mixed with 1 mL of 6 mM ferrous sulfate solution and 1 mL of 6 mM salicylic acid ethanol solution (70%). The reaction was initiated by adding 1.0 mL of 6 mM H_2O_2 solution. The mixture was incubated at 37 °C for 30 min and the absorbance was measured at 510 nm. The hydroxyl radical scavenging activity was calculated by the following equation:

$$\text{Hydroxyl radical scavenging activity (\%)} = [1 - (A1 - A2)/A0)] \times 100\% \quad (3)$$

where A0, A1 and A2 represent the absorbance of the H_2O_2 in deionized water, the sample mixed with H_2O_2 and the sample in deionized water, respectively.

2.8.3. Reducing Power

Reducing power was determined following a previously reported method [30]. Briefly, 2.5 mL of sample solution was mixed with 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 1 mL of 1% potassium ferricyanide solution. The mixture was incubated at 50 °C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid solution was added and centrifuged at 4000 rpm for 5 min. Subsequently, 2.5 mL of the supernatant, 2.5 mL of deionized water and 0.5 mL of 1% ferric chloride solution were mixed and reacted for 10 min at room temperature. After centrifugation at 6000 rpm for 10 min, the absorbance of the supernatant at 700 nm was detected.

2.9. Scanning Electron Microscope Analysis

The PKM samples before and after fermentation were dried and crushed through an 80-mesh sieve. Then, the samples were fixed to the specimen holder and sputter-coated with gold. The microstructures of PKM and FPKM were observed using field-emission scanning electron microscopy (SEM; Quanta 250 FEG, FEI, Hillsboro, OR, USA) at ×4000 magnification.

2.10. Statistical Analysis

All experiments were performed in triplicate, and the data were expressed as mean ± standard deviation. Statistical analyses were performed by OriginPro 8 software (OriginLab Corp, Northampton, MA, USA). Data were analyzed for mean differences by one-way analysis of variance (ANOVA) using the Tukey test at a significance level of $p < 0.05$.

3. Results and Discussion

3.1. SFE Optimization

3.1.1. Inoculation Ratio of Three Strains

Appropriate inoculation ratio of microorganisms is essential for effective SSF, as microorganisms work synergistically in multi-microbial fermentation [31]. As shown in Table 1, the optimal inoculation ratio of *B. velezensis*, *S. cerevisiae* and *L. paracasei* was 4:2:1, yielding the highest value of reducing sugar (66.8 mg/g), which was 2.28 times that of the unfermented PKC (29.3 mg/g). Similarly, the inoculation ratio of *B. velezensis*, *Enterococcus faecium* and *Saccharomyces boulardii* was investigated in the SSF of soybean meal with an optimal ratio at 3:2:3 [13].

Table 1. Effect of inoculation ratio of three strains on reducing sugar yield in FPKM.

Group	*B. velezensis*	*S. cerevisiae*	*L. paracasei*	Reducing Sugar (mg/g)
PKM	0	0	0	$29.3^{f} \pm 1.1$
FPKM 1	1	1	1	$57.6^{de} \pm 2.3$
FPKM 2	1	2	2	$58.1^{de} \pm 2.0$
FPKM 3	1	4	4	$56.3^{e} \pm 2.1$
FPKM 4	2	1	2	$59.7^{d} \pm 1.2$
FPKM 5	2	2	4	$60.1^{cd} \pm 2.0$
FPKM 6	2	4	1	$62.4^{bc} \pm 2.1$
FPKM 7	4	1	4	$62.6^{bc} \pm 1.0$
FPKM 8	4	2	1	$66.8^{a} \pm 1.7$
FPKM 9	4	4	2	$64.4^{ab} \pm 1.5$

Note: Different superscript letters above the same row indicated significant difference ($p < 0.05$).

3.1.2. Moisture Content

The reducing sugar content was increased significantly ($p < 0.01$) as moisture content rose from 40% to 50%, reaching a peak value of 68.0 mg/g at 50% moisture (Figure 1a). Then, the reducing sugar content was decreased with further increases in moisture. The result was similar to the finding of Hou et al., who found that the moisture content of 50% was optimal for SSF of rapeseed meal by *B. subtilis* [32]. Lower moisture content reduces nutrient solubility and substrate swelling, thereby inhibiting microbial activity. However, microbial activities were also suppressed due to limited nutrition and oxygen mass transfer caused by upper moisture content [33,34].

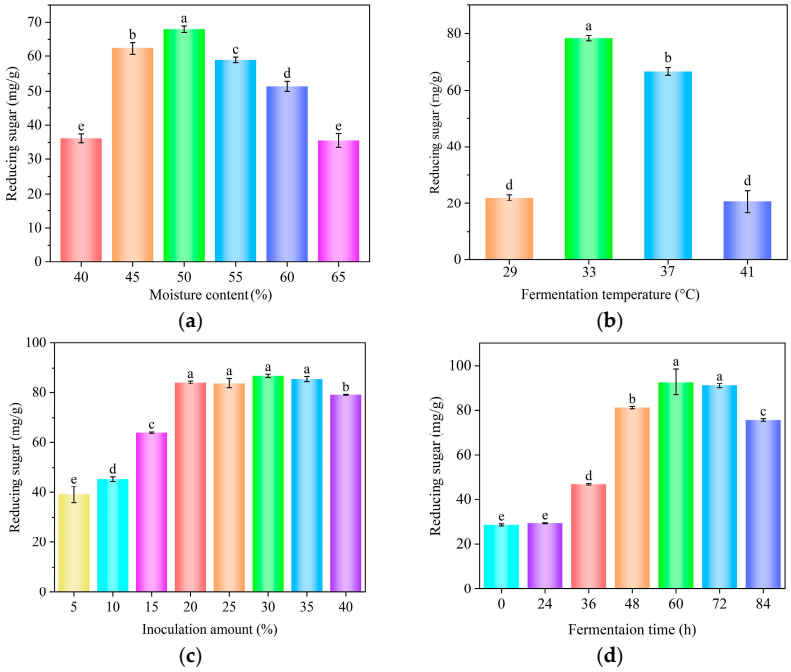

Figure 1. Effect of moisture content (**a**), fermentation temperature (**b**), inoculum amount (**c**) and

fermentation time (**d**) on reducing sugar content. Different superscript letters above the columns indicated significant differences ($p < 0.05$).

3.1.3. Fermentation Temperature

Fermentation temperature significantly influenced the yield of reducing sugar during SSF of PKM (Figure 1b). The reducing sugar content reached its highest value, 78.4 mg/g, at 33 °C. However, the yields of reducing sugar showed a decline at 37 °C and 41 °C. Therefore, it can be concluded that the growth and metabolic activities of *B. velezensis*, *S. cerevisiae* and *L. paracasei* were strongest at 33 °C. The fermentation temperature may vary significantly depending on the substrate and microorganisms. Hou et al. reported that 55 °C was the optimum fermentation temperature for SSF of rapeseed meal using *Geobacillus stearothermophilus* [32].

3.1.4. Inoculum Amount

The reducing sugar content was increased significantly ($p < 0.05$) from 39.1 mg/g to 84.3 mg/g with the increase of inoculum amount from 5% to 20% (Figure 1b). Increasing the inoculum amount beyond 25% did not lead to a significant rise ($p > 0.05$) in reducing sugar content. Then, it decreased to 79.1 mg/g at an inoculum amount of 40%. Low inoculation amount may cause insufficient enzyme secretion by microorganisms, thereby affecting the enzymatic hydrolysis of the substrate. Meanwhile, excessive inoculation amount may accelerate microbial growth and reproduction, depleting nutrients and moisture in the substrate and ultimately affecting product formation [13].

3.1.5. Fermentation Time

Fermentation time is also a crucial factor influencing SSF [9]. As shown in Figure 1c, the reducing sugar content was significantly increased ($p < 0.05$) from 29.6 mg/g to 92.8 mg/g when the fermentation time was increased from 24 h to 60 h, and then it gradually decreased to 75.8 mg/g at 84 h. The decrease in reducing sugar content may be due to the participation of reducing sugars in the Maillard reaction in the later stages of fermentation or the consumption of reducing sugars by microorganisms [35].

3.2. RSM Optimization

Based on the SFE results, four variables, namely inoculum amount (A), moisture content (B), fermentation temperature (C) and fermentation time (D) were chosen for the subsequent RSM. Table 2 shows the design and results of the BBD experiments.

Table 2. BBD design and results.

Run	A	B	C	D	Reducing Sugar (mg/g)
1	20	50	29	48	75.8
2	20	45	33	48	69.6
3	30	50	33	48	71.5
4	10	50	33	48	78.5
5	20	55	33	48	86.1
6	20	50	37	48	79.1
7	20	45	29	60	67.1
8	30	50	29	60	70.3
9	10	50	29	60	75.2
10	20	55	29	60	78.8
11	30	45	33	60	69.3
12	10	45	33	60	69.4
13	10	55	33	60	80.7
14	30	55	33	60	82.7
15	20	50	33	60	92.9
16	20	50	33	60	94.8

Table 2. *Cont.*

Run	A	B	C	D	Reducing Sugar (mg/g)
17	20	50	33	60	94.9
18	10	50	37	60	65.2
19	20	45	37	60	68.7
20	30	50	37	60	78.9
21	20	55	37	60	80.6
22	20	50	29	72	72.7
23	10	50	33	72	71.8
24	30	50	33	72	79.8
25	20	45	33	72	81.3
26	20	55	33	72	86.6
27	20	50	37	72	82.2

The polynomial model for the estimation of reducing sugar (Y, mg/g) in terms of inoculum amount (A), moisture content (B), fermentation temperature (C) and fermentation time (D) was fitted by the following equation:

$$Y = 93.51 + 0.98A + 5.83B + 1.22C + 1.14D + 0.5175AB + 4.64AC + 3.74AD + 0.03BC - 2.80BD + 1.56CD - 11.02A^2 - 7.56B^2 - 10.85C^2 - 5.79D^2 \quad (4)$$

To validate the regression coefficient, the analysis of variance was performed for reducing sugar production (Table 3). The *p*-value of the regression model was less than 0.0001, which indicated that the linear relationship between every variable and reducing sugar yield was extremely significant. Furthermore, the *p*-value of lack of fit for the model exceeded 0.05, indicating that accidental factors might not significantly affect the response values. Overall, this suggested that the model accurately represented the experimental data, accounting for over 95% of the variability in the dependent variables [36]. The coefficient of determination (R^2 = 0.950) indicated that the variations in reducing sugar content could be effectively explained by the selected independent variables, confirming the suitability of the regression model for analyzing response trends. Moisture content was identified as the most critical factor influencing reducing sugar yield ($p < 0.0001$) (Table 3).

Table 3. Analysis of variance in BBD.

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value Prob > F	Significance
Model	1686	14	120	17.8	<0.0001	**
A-Inoculation amount	11.5	1	11.5	1.71	0.216	
B-Moisture content	408	1	408	60.4	<0.0001	**
C-Fermentation temperature	17.9	1	17.9	2.64	0.130	
D-Fermentation time	15.5	1	15.5	2.29	0.156	
AB	1.07	1	1.07	0.159	0.698	
AC	86.2	1	86.2	12.8	0.0038	**
AD	56.0	1	56.0	8.28	0.0139	*
BC	0.0036	1	0.0036	0.0005	0.982	
BD	31.4	1	31.4	4.64	0.0522	
CD	9.77	1	9.77	1.45	0.252	
A^2	687	1	687	102	<0.0001	**
B^2	332	1	332	49.2	<0.0001	**
C^2	667	1	667	98.7	<0.0001	**
D^2	200	1	200	29.6	0.0002	**
Residual	81.1	12	6.75			
Lack of Fit	78.5	10	7.85	6.21	0.147	
Pure Error	2.5	2	1.27			
Cor Total	1766	26				

Note: * $p < 0.05$, ** $p < 0.01$.

Models are plotted as 3D response surface and contour curves within the tested parameters to investigate the interactive effects of the four factors on the yield of reducing

sugar (Figure 2). The steep slope of 3D response surface plot indicated that the interaction between the two factors was significant [37]. The interactions between inoculum amount and fermentation temperature, as well as inoculum amount and fermentation time, were significant ($p < 0.05$). The model predicted that the maximum reducing sugar content (95.3 mg/g) was located at inoculum amount of 20.7%, moisture content of 51.8%, fermentation temperature of 33.3 °C and fermentation time of 60.5 h. Based on the predicted values and considering practical conditions, the optimal conditions were set as follows: inoculum amount, 21%; moisture content, 52%; fermentation temperature, 34 °C; and fermentation time, 60 h. The triplicate test showed an actual reducing sugar content of 96.3 mg/g, which was close to the predicted response, thus affirming the rationality of the model.

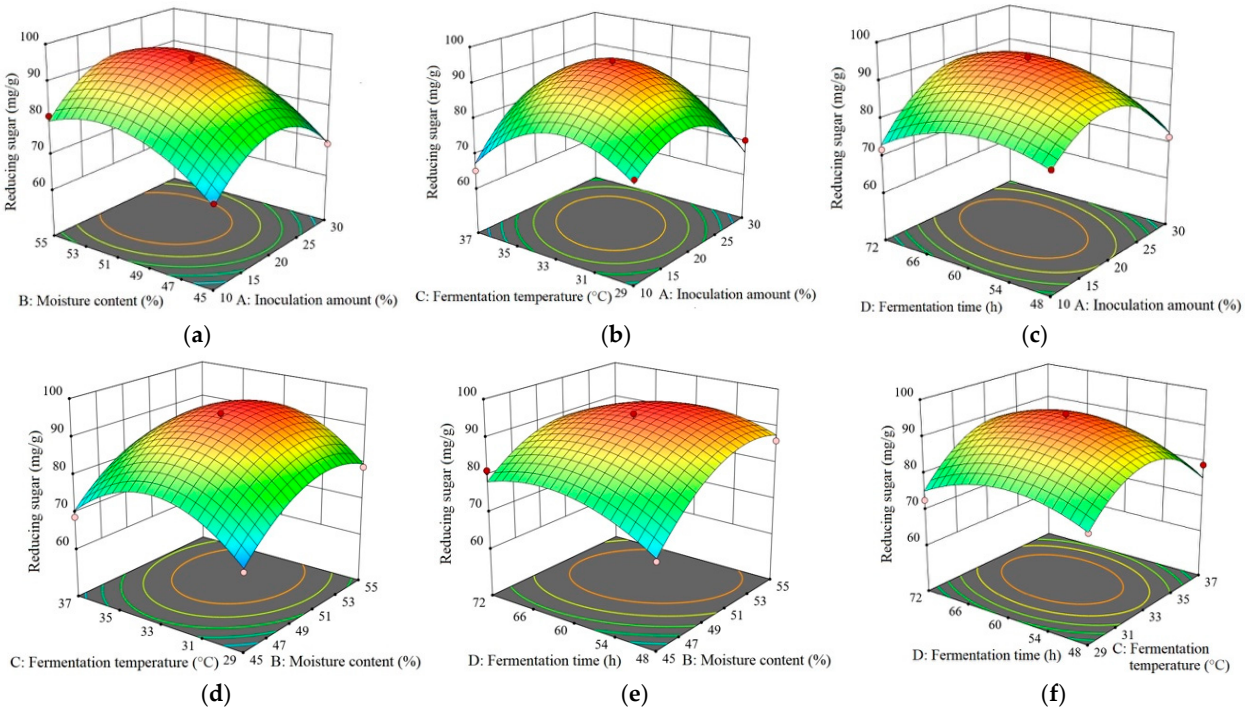

Figure 2. Response surface plots for showing the mutual interactions between (**a**) inoculation amount and moisture content, (**b**) inoculum amount and fermentation temperature, (**c**) inoculum amount and fermentation time, (**d**) moisture content and fermentation temperature, (**e**) moisture content and fermentation time, (**f**) fermentation temperature and fermentation time.

3.3. Anti-Nutritional Factor

While estimating the crude protein, a steady increase in crude protein was observed in FPKM (Table 4). Crude protein content in FPKM of different times showed a variable range rising from 14.4% to 17.4%. Fiber is the anti-nutritional factor of PKM, potentially hindering nutrient digestion and absorption in animals. The fiber fraction primarily originates from plant cell walls and consists of various structural polysaccharides, predominantly cellulose, diverse hemicelluloses and pectic polysaccharides [38]. The main polysaccharide component of PKM is β-mannan (hemicellulose), followed by cellulose [39]. Cellulose and hemicellulose can be hydrolyzed by cellulase and hemicellulase to generate reducing sugars [40]. The NDF, ADF, ADL, cellulose and hemicellulose content of the unfermented PKM and FPKM are presented in Table 4. In the detergent fiber analysis, cellulose is often estimated as the difference between ADF and ADL, while hemicellulose is often estimated as the difference between NDF and ADF [41]. After fermentation of 60 h, the NDF, ADF, ADL, cellulose and hemicellulose content of PKM were decreased significantly ($p < 0.05$) by 22.5%, 18.2%, 20.2%, 17.6% and 32.4%, respectively. Meanwhile, the reducing sugar content was increased remarkably ($p < 0.05$) by 228%. It reflected that *B. velezensis*, *S. cerevisiae* and *L. paracasei* produced cellulase and hemicellulase, biotransforming cellulose and hemicellulose

in PKM into reducing sugars. Similarly, Shi et al. found that SSF of rapeseed meal with *A. niger* reduced the content of NDF and hemicellulose by 6.3% and 25.5%, respectively, due to cellulase and hemicellulase activity [42]. Alshelmani et al. reported that the SSF process, utilizing a combination of four cellulolytic and hemicellulolytic bacteria, including *B. amyloliquefaciens*, *P. curdlanolyticus*, *P. polymyxa* and *B. megaterium*, resulted in a significant reduction ($p < 0.05$) in NDF, ADF, hemicellulose and cellulose compared to the untreated palm kernel cake, whereas there was no significant effect ($p > 0.05$) on the ADL content [18]. In our study, SSF also led to a reduction in the ADL content of PKM, indicating that enzymes capable of degrading ADL were produced during the fermentation process. The substantial reduction in fiber fractions is crucial for improving the quality of PKM, thereby expanding its potential applications in non-ruminant feed.

Table 4. Nutritional components and anti-nutritional factor analysis of PKM and FPKM.

Ingredient	PKM	FPKM (36 h)	FPKM (48 h)	FPKM (60 h)
NDF (%)	$64.7^{a} \pm 1.2$	$61.4^{b} \pm 0.7$	$54.0^{c} \pm 0.5$	$50.1^{d} \pm 0.6$
ADF (%)	$45.1^{a} \pm 1.1$	$43.9^{a} \pm 1.5$	$40.2^{b} \pm 0.6$	$36.9^{c} \pm 0.9$
ADL (%)	$10.2^{a} \pm 0.2$	$10.3^{a} \pm 0.3$	$8.6^{ab} \pm 1.00$	$8.1^{b} \pm 0.8$
Cellulose (%)	$34.9^{a} \pm 1.0$	$33.6^{a} \pm 1.3$	$31.6^{ab} \pm 0.4$	$28.7^{b} \pm 1.7$
Hemicellulose (%)	$19.6^{a} \pm 0.1$	$17.5^{a} \pm 0.0$	$13.8^{b} \pm 1.0$	$13.3^{b} \pm 0.6$
Reducing sugar (mg/g)	$29.3^{d} \pm 0.3$	$47.1^{c} \pm 2.1$	$81.2^{b} \pm 1.3$	$96.3^{a} \pm 2.5$
Crude protein (%)	$13.7^{c} \pm 0.2$	$14.4^{b} \pm 0.2$	$15.3^{b} \pm 0.5$	$17.4^{a} \pm 0.3$
Soluble protein (μg/g)	$542^{b} \pm 17$	$230^{c} \pm 10$	$648^{b} \pm 28$	$1592^{a} \pm 46$
Lipid (%)	6.86 ± 0.20	6.58 ± 0.16	6.82 ± 0.05	6.85 ± 0.06
Peptides (mg/g)	$1.41^{d} \pm 0.04$	$2.26^{c} \pm 0.04$	$2.86^{b} \pm 0.15$	$3.31^{a} \pm 0.22$

Note: Cellulose content was estimated as the difference between ADF and ADL. Hemicellulose content was estimated as the difference between NDF and ADF. Different superscript letters above the same row indicate significant differences ($p < 0.05$).

3.4. Nutritional Components

While estimating the crude protein, a steady increase in crude protein was observed in FPKM (Table 4). Crude protein content in FPKM of different times showed a variable range rising from 14.4% to 17.4%. The highest crude protein content was observed in 60 h FPKM, showing a 27.3% increase compared to unfermented PKM. Similarly, SSF applied to fermented rapeseed meal with *S. cerevisiae* and *S. boulardii* significantly increased the crude protein content by 11.2% and 10.1%, respectively [43]. The increased crude protein was possibly due to the mycoprotein synthesis in the SSF process [44]. The decline in fiber content was also considered as a possible reason for the increase in crude protein, as the decomposed fiber could serve as an energy source for microbial growth and be partially converted into protein [31].

As shown in Table 4, the soluble protein content in unfermented PKM was decreased sharply ($p < 0.05$) from 542 μg/g to 230 μg/g (36 h FPKM). Then, the soluble protein content was increased significantly to 648 μg/g and 1592 μg/g at 48 h and 60 h, respectively, which was 19.4% and 193% higher than that of the unfermented PKM. A similar result was reported by Liu et al., who observed a significant improvement in soluble protein content following SSF of soybean meal with *B. velezensis* [16]. An initial decline in soluble protein during fermentation may be linked to the consumption of soluble protein in PKM by *B. velezensis*, *S. cerevisiae* and *L. paracasei*. As the PKM fermentation progressed, the microorganisms may have synthesized new proteins, which were secreted extracellularly, leading to the rise in soluble protein content. Additionally, the increase in soluble protein could be due to the secretion of proteases by the microorganisms, which degraded insoluble macromolecular proteins into smaller soluble proteins and peptides during fermentation [15,16,45].

The peptide content of PKM was increased significantly ($p < 0.05$) by 135% after fermentation (Table 4). Likewise, Chi et al. reported an increase in the peptide content in soybean meal by SSF with *B. myloliquefaciens*, *Lactobacillus* spp. and *S. cerevisiae* [46]. The rise in peptide content was probably because proteolytic enzymes produced by microor-

ganisms degraded macromolecular proteins into small peptides during fermentation [47]. Low molecular weight peptides hold significant advantages over high molecular weight proteins, as they are more readily digested and absorbed, and display bioactivities such as antioxidant activity, immune activity, antibacterial activity, as well as improvements in the animal intestinal microenvironment [22,48].

3.5. Amino Acids

The amino acid profiles of unfermented PKM and FPKM at different fermentation times are presented in Figure 3. After 60 h of fermentation, the total essential amino acid content in PKC was increased significantly ($p < 0.05$) from 3.74 g/100 g to 4.25 g/100 g. Especially, the content of Lys, which is the major limiting amino acid in PKM [17], was increased significantly ($p < 0.05$) by 53.2%. The content of Thr and Phe in 60 h FPKM were 1.31 and 1.30 times that of unfermented PKM, respectively. The content of total non-essential amino acid (10.1 g/100 g) in 60 h FPKM was observed to be higher than that of unfermented PKM (8.62%). The contents of Asp, Glu, His, Gly, Pro and Cys in 60 h FPKM were 1.37, 1.26, 1.22, 1.27, 1.30 and 1.52 times that of unfermented FPKM, respectively. The total amino acid content was increased ($p < 0.05$) by 16.3% after fermentation for 60 h. The increase in amino acid content after fermentation aligned with previous findings, which reported that SSF of soybean meal with *B. subtilis* 21,927 and 22,983 enhanced the content of total amino acids by 16.1% and 12.9%, and total essential amino acids by 16.0% and 16.5%, respectively [49]. The increase in total amino acid content and changes in the AA profile may be attributed to the rise in protein content and hydrolysis of proteins or peptides [50].

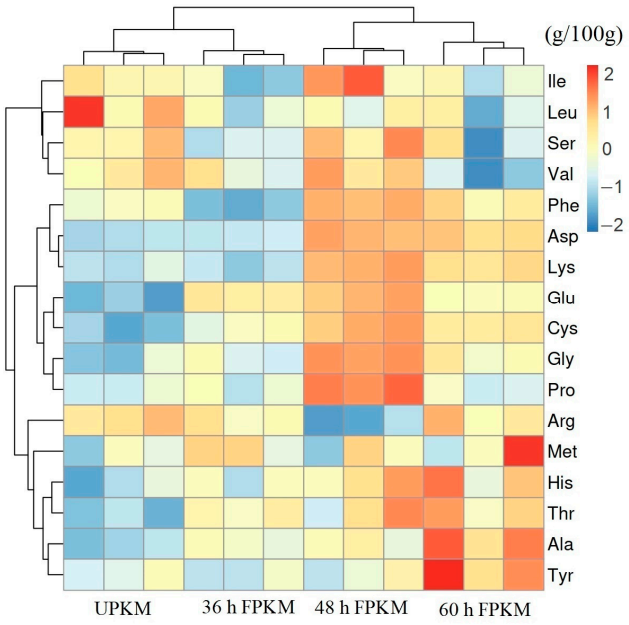

Figure 3. Heatmap representing the relationship effects between fermentation groups and amino acids composition. (UPKM: unfermented palm kernel meal; 36 h FPKM: fermented palm kernel meal at 36 h; 48 h FPKM: fermented palm kernel meal at 48 h; 60 h FPKM: fermented palm kernel meal at 60 h).

3.6. Microbial Counts

In Figure 4a, the initial counts of *B. velezensis*, *S. cerevisiae* and *L. paracasei* inoculated into unfermented PKM and their growth during SSF at different time intervals are shown. *B. velezensis* counts were increased significantly ($p < 0.05$) from 8.65 log CFU/g (unfermented PKM) to 11.1 log CFU/g (60 h FPKM). *S. cerevisiae* started at 7.12 log CFU/g (unfermented PKM), increasing to 9.10 log CFU/g (48 h FPKM), with no significant difference ($p > 0.05$) between 48 h and 60 h. SSF also enriched *L. paracasei* counts, reaching 9.68 log CFU/g at 60 h. The favorable growth of all three strains reflected the suitable environment and appropriate nutritional requirements in PKM for propagation.

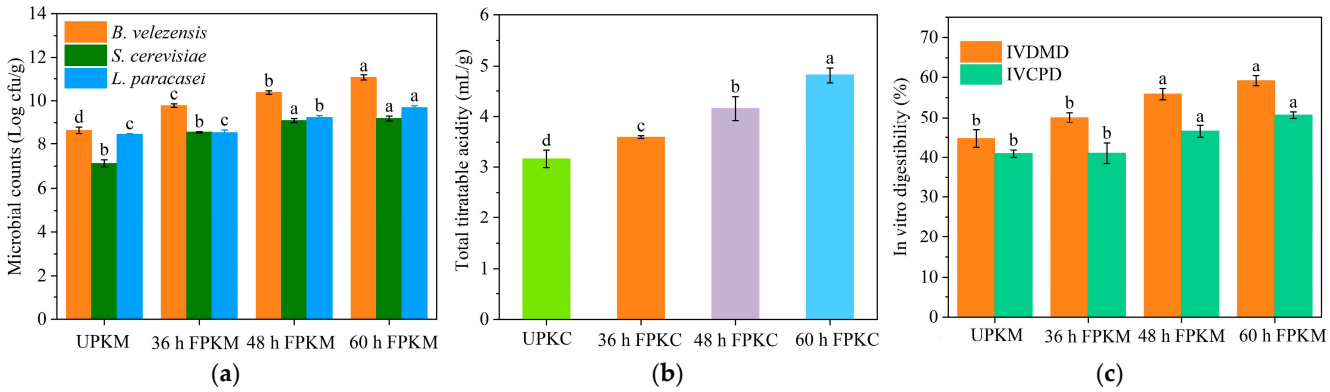

Figure 4. (**a**) *B. velezensis*, *S. cerevisiae*, *L. paracasei* counts; (**b**) Total titratable acidity; (**c**) In vitro digestibility of UPKM and FPKM. (UPKM: unfermented palm kernel meal; 36 h FPKM: fermented palm kernel meal; at 36 h; 48 h FPKM: fermented palm kernel meal at 48 h; 60 h FPKM: fermented palm kernel meal at 60 h). Values not sharing common alphabets in same graph are significantly different ($p < 0.05$).

3.7. TTA

The TTA values of PKM were increased gradually in the process of SSF (Figure 4b). The initial TTA value of PKM was 3.16 mL/g and increased significantly ($p < 0.05$) to the highest value of 4.81 mL/g in 60 h FPKM. The result was in line with a previous study, which showed a rise in the TTA value of defatted adlay after SSF with yeast [51]. The increase in the TTA value was presumably due to the production of organic acids by three strains during the fermentation of PKM. The breakdown of carbohydrates into sugars and organic acids may also contribute to the increase in TTA [52].

3.8. In Vitro Digestibility Analysis

The IVDMD and IVCPD of unfermented PKM and FPKM at different fermentation times are shown in Figure 4c. SSF of PKM for 60 h significantly increased ($p < 0.05$) the IVDMD and IVCPD by 32.4% and 24.1%, respectively. Similarly, SSF demonstrated the ability to improve the IVCPD of lupin flour and IVDMD of sugarcane bagasse [53,54]. The increase in IVDMD and IVCPD may be attributed to fiber-degrading enzymes and proteolytic enzymes produced by microorganisms in SFF. Lee et al. reported that fiber-degrading enzymes improved the IVDMD of canola cake, possibly due to fiber hydrolysis enhancing the availability for pepsin and trypsin digestion [55]. The increase in IVCPD may be linked to the proteolytic enzymes produced during microbial fermentation, hydrolyzing large proteins into smaller molecules [56].

3.9. Total Phenolic Content

The total phenolic contents of unfermented PKM and FPKM at different fermentation times were detected (Figure 5a). The total phenolic content of 36 h FPKM (2.96 mg/g) and 48 h FPKM (4.43 mg/g) was significantly higher than that of unfermented PKM (2.48 mg/g) ($p < 0.05$), with no significant difference ($p > 0.05$) between 48 h and 60 h. The result was in accordance with the finding of Dai et al., who reported that SSF of soybean meal increased the total phenolic content [57]. Phenolic compounds are of great interest due to their antioxidant capacity. Phenolics mainly exist in conjugated forms [58]. Changes in total phenolic content reflected that the free phenolics were liberated from insoluble matrices by the action of enzymes derived from microbial fermentation [35]. In addition, phenolic compounds may be released during lignin degradation in the fermentation process [59]. The increase in phenolic content could also be attributed to the synthesis of more or new phenolics by microorganisms during fermentation [60,61].

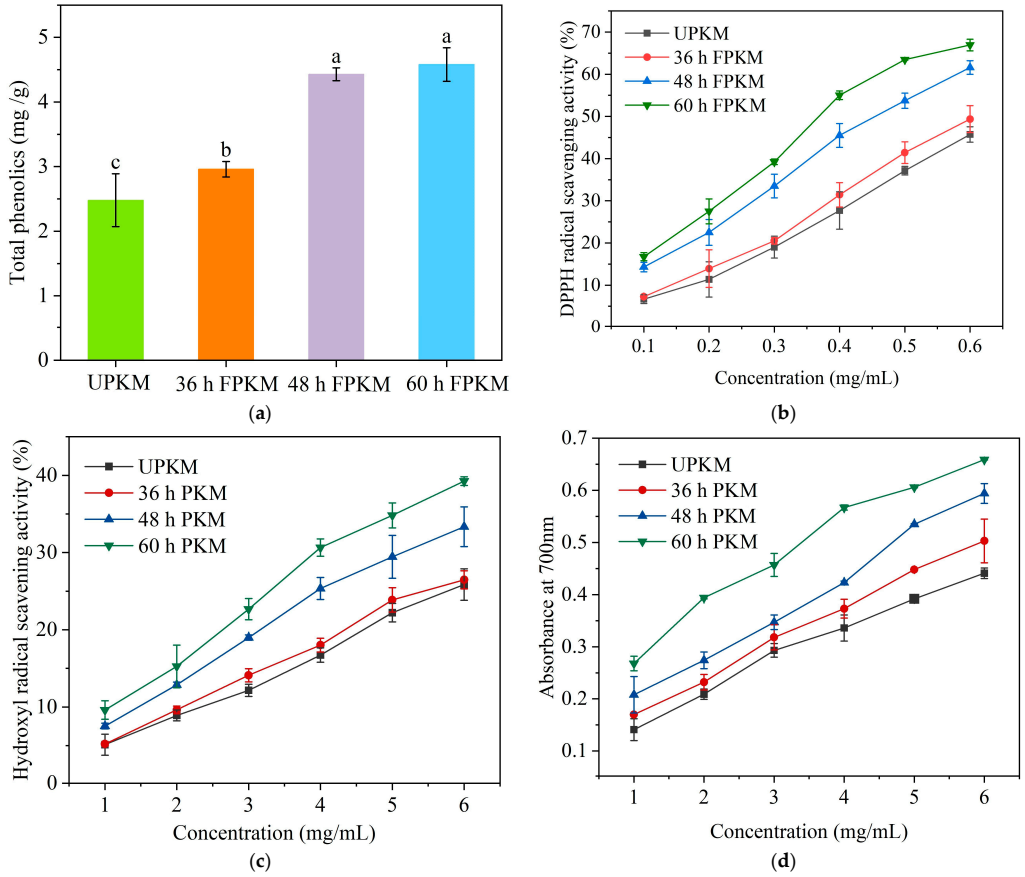

Figure 5. (**a**) Total phenolic content; (**b**) DPPH radical scavenging activity; (**c**) hydroxyl radical scavenging activity; (**d**) Reducing power of UPKM and FPKM. (UPKM: unfermented palm kernel meal; 36 h FPKM: fermented palm kernel meal at 36 h; 48 h FPKM: fermented palm kernel meal at 48 h; 60 h FPKM: fermented palm kernel meal at 60 h). Values not sharing common alphabets in same graph are significantly different ($p < 0.05$).

3.10. Antioxidant Activity

The DPPH radical scavenging activities of unfermented PKM and FPKM at different fermentation times were monitored (Figure 5b). The DPPH radical scavenging activities showed no significant increase in the initial stage of fermentation (0–36 h), but were visibly increased ($p < 0.05$) at 48 h and 60 h. PKM and FPKM showed the highest DPPH radical scavenging activities at the concentration of 0.6 mg/mL, with a rise from 45.7% (unfermented PKM) to 61.6% (48 h FPKM) and 66.9% (60 h FPKM), respectively. The EC_{50} values of unfermented PKM, 36 h FPKM, 48 h FPKM and 60 h FPKM were 0.676 mg/mL, 0.585 mg/mL, 0.439 mg/mL and 0.335 mg/mL, respectively.

Figure 5c shows the hydroxyl radical scavenging activities of unfermented PKM and FPKM at different fermentation times. PKM fermented for 48 h and 60 h was observed to own the superior hydroxyl radical scavenging activities compared to unfermented PKM in the concentration range of 1–6 mg/mL. At the concentration of 0.6 mg/mL, the hydroxyl radical scavenging activity of unfermented PKM was 25.9% and significantly increased to 33.3% (48 h FPKM) and 39.3% (60 h FPKM), respectively. The EC_{50} values of unfermented PKM, 36 h FPKM, 48 h FPKM and 60 h FPKM were 8.96 mg/mL, 6.78 mg/mL, 4.17 mg/mL and 3.7 mg/mL, respectively.

The reducing powers of PKM and FPKM at different fermentation times are shown in Figure 5d. At the concentration of 6 mg/mL, the OD_{700nm} values for unfermented PKM and PKM fermented for 36 h, 48 h and 60 h were 0.441, 0.503, 0.594 and 0.659 respectively. The EC_{50} values of unfermented PKM, 36 h FPKM, 48 h FPKM and 60 h FPKM were 8.73 mg/mL, 8.72 mg/mL, 7.32 mg/mL and 6.74 mg/mL, respectively.

The results for DPPH radical scavenging activity, hydroxyl radical scavenging activity and reducing power confirmed that FPKM had a higher antioxidant activity than unfermented PKM, suggesting that SSF produced more antioxidant compounds. Yang et al. reported that the improvement in antioxidant activity may be attributed to the hydrolysis of proteins into peptides as well as the rise in the total phenolic and flavonoid content after fermentation [62]. Similarly, peptides and total phenolics were observed to be related to the antioxidant capacity of rapeseed meal [12]. In this study, the content of peptides and total phenolics in PKM were increased significantly ($p < 0.05$) from 1.31% to 3.31%, 2.48 mg/g to 4.58 mg/g, respectively, after SSF, which was consistent with the antioxidant activity results.

3.11. Microstructure Analysis

The microstructures of PKM and FPKM at different fermentation times were investigated by SEM (Figure 6). The surface of unfermented PKM was dense and compact. However, the surface of FPKM gradually became rough, loose, porous and irregular with increased fermentation duration. The microstructural alteration and disruption in FPKM may be attributed to extracellular enzymes, particularly fiber-degrading enzymes, secreted during SSF by the microbial strains, which decomposed the fiber and other structural components of PKM [42]. Correspondingly, the decomposition enhanced the contact area between microbial enzymes and PKM, facilitating the release of bioactive substances [52].

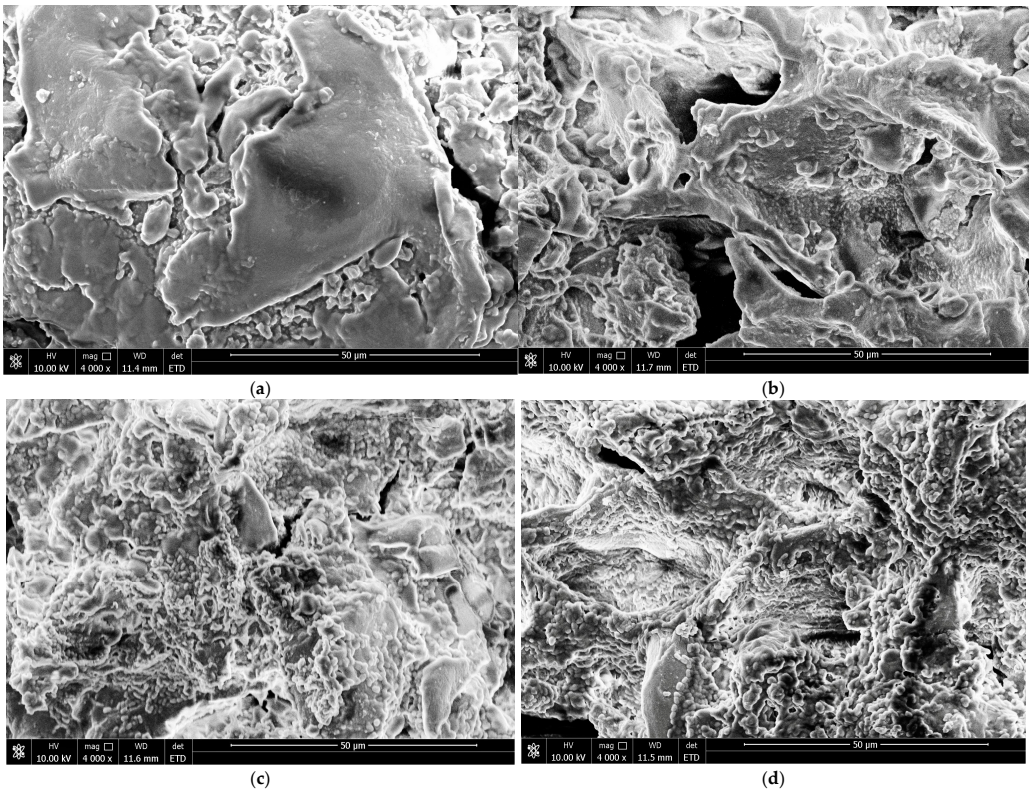

Figure 6. Microstructures of PKM and FPKM at different fermentation times ((**a**–**d**) represent unfermented PKM and PKM fermented for 36 h, 48 h and 60 h, respectively).

4. Conclusions

In the present study, a novel SSF process of PKM with *B. velezensis*, *S. cerevisiae* and *L. paracasei* was developed to transform the agro-industrial by-product PKM into value-added feed resource enriched in nutritional components and bioactive compounds. SSF increased the nutritional components, especially soluble protein content, and reduced the fiber content of PKM. In addition, SSF improved the total phenolic content, antioxidant activity, IVDMD and IVCPD. In summary, SSF with *B. velezensis*, *S. cerevisiae* and *L. paracasei*

is a promising strategy to enhance the nutritional value and antioxidant activity of PKM and FPKM as promising nutritional and functional ingredients for animal feed.

Author Contributions: Conceptualization, X.Z. and X.L.; methodology, X.Z. and S.H. (Shuai He); software, Z.D. and Q.W.; validation, S.H. (Shanxin Hao); formal analysis, X.Z.; investigation, X.Z. and X.L.; resources, P.L. and S.H. (Shuai He); data curation, P.L.; writing—original draft preparation, Z.D. and Q.W.; writing—review and editing, X.Z., P.L., and S.H. (Shanxin Hao); visualization, Z.D. and S.H. (Shanxin Hao); supervision, X.Z. and X.L.; project administration, X.Z. and X.L.; funding acquisition, X.Z. and X.L. All authors have read and agreed to the published version of the manuscript.

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