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Improvement of the Nutritional Quality of Rapeseed Meal through Solid-State Fermentation with *B. subtilis*, *S. cerevisiae*, and *B. amyloliquefaciens*

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Abstract: In this study, the effect of solid-state fermentation with *Bacillus subtilis* GYB6, *Saccharomyces cerevisiae* NJ1, and *Bacillus amyloliquefaciens* Y8 on the anti-nutritional factors, nutritional components, bioactive compounds, antioxidant activity, functional properties, and structure of rapeseed meal (RSM) were investigated. Results showed that the action of three strains in the fermentation of RSM caused a significant decline in glucosinolates, phytic acid, crude fiber, and tannins by 99.18%, 42.41%, 27.21%, and 34.17%, respectively. The amount of crude protein, amino acids, and peptides of RSM increased significantly after fermentation. The SDS-PAGE results showed that 12S globulin and 2S albumin protein were almost entirely degraded. Fermentation considerably increased the concentration of total phenolics and flavonoids, and activated antioxidant activity and functional properties. Furthermore, the structural variation was observed by scanning electron microscopy and FTIR spectroscopy. Thus, these results indicated that the solid-state fermentation process in this study was a promising approach to enhance both the nutritional value and bioactivity of RSM, which could be used as value-added functional animal food ingredients.

Keywords: rapeseed meal; solid state fermentation; anti-nutritional factors; nutritional components; bioactive compounds; antioxidant activity

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1. Introduction

The demand for new protein sources for feed has grown dramatically in recent years. It is very necessary to explore non-food protein sources and develop efficient technologies to enhance the quality [1,2]. The agricultural by-product protein resources are abundant, but underdeveloped. Rapeseed is the third largest source of vegetable oil globally [3]. Rapeseed meal (RSM) is the by-product after the extraction of oil from rapeseed. RSM has attracted significant attention as feedstuff and become the second most widely traded plant protein after soybean meal because of its high potential nutritional value and rich source [4]. RSM has high protein levels (~35%, *w*/*w*) and a well-balanced amino acid profile [5,6]. It is also a good source of calcium, iron, manganese, selenium, and vitamin [4]. Therefore, RSM has been attracted as an essential and potential feedstuff. However, the utilization efficiency of RSM in animal diets is limited due to the presence of anti-nutritional factors such as glucosinolates, phytic acid, tannins, fiber, etc. [7,8]. Glucosinolates, the major anti-nutritional factors, are catalyzed by myrosinase enzyme presented in the rapeseed or animal gastrointestinal tract into isothiocyanate, thiocyanate, and nitrile which disrupt thyroid, liver, and kidney function of animals [7,9]. Phytic acid reduces mineral bioavailability and protein digestibility [10]. Tannins, which are secondary compounds, have been reported to bind proteins and some enzymes, thus affecting the digestion of animals [10,11]. Moreover, RSM contains a relatively high level of fiber which inhibits the absorption of other nutrients,

such as minerals and proteins [12]. Therefore, the nondetoxified RSM not only affects the digestibility and utilization of nutrients, but also causes adverse influence on animal performance, which greatly lowed the application of RSM as feed.

Various approaches, including biological, chemical, and physical detoxification, have been exploited for detoxifying plant protein sources [4,7]. Solid state-fermentation has been recognized as an ideal biological method to reduce anti-nutritional factors because microorganisms can secrete complex enzymes to decompose the toxic components of RSM [4,13]. Solid-state fermentation can also improve the nutrient utilization of RSM, such as the biotransformation of proteins into more readily absorbable and functional peptides, and the production of some bioactive compounds [9,14]. Notably, the studies showed that solid-state fermentation also owned the advantages of improving the palatability of feed, digestibility, and utilization of nutrients, substantially improving animal performance and health [15–17]. Generally, the available literature confirms the feasibility of improving the quality of RSM by solid-state fermentation. Solid-state fermentation with RSM as the substrate using *Rhizopus oligosporus* [18], *Bacillus licheniformis*, *Yeast* and *Lactobacillus* [19], *Bacillus subtilis* YY-1 and *Saccharomyces cerevisiae* YY-2 [20], and *S. cerevisiae or Saccharomyces boulardii* [21] decreased the content of glucosinolates, neutral detergent fiber, and phytic acid, and increased the content of crude protein, lactic acids, and total amino acids. To our knowledge, there are few studies applying *Bacillus amyloliquefaciens* in the solid-state fermentation of RSM. *B. amyloliquefaciens* has been reported to be applied in the improvement in the nutritional quality of soybean meal by solid-state fermentation [22]. *B. amyloliquefaciens* is characterized by producing various enzymes, including α-amylase, protease, cellulase, xylanase, pectinase, and peroxidase, and antimicrobial compounds inhibiting the growth of pathogens, including non-ribosomal peptides and polyketides [23].

Numerous studies have demonstrated that the effect of multi-microbial fermentation is superior to single-strain because multiple microorganisms promisingly produce synergistic effects to improve the quality of the substrates [7]. Multi-microorganism can produce various enzymes facilitating the biotransformation of anti-nutritional factors and macromolecular materials [24,25]. Multiple microorganisms can also produce more abundant metabolites compared to pure culture.

In this study, a novel solid-state fermentation with *B. amyloliquefaciens* Y8, *B. subtilis* GYB6, and *S. cerevisiae* NJ1 was applied to improve the nutritional quality of RSM. The anti-nutritional factors, nutritional components, bioactive compounds, antioxidant activity, functional properties, and structure of RSM and fermented RSM (FRSM) were characterized and compared.

2. Materials and Methods

2.1. Materials

Strain Y8 and GYB6 were isolated from the soil of the rape field. *S. cerevisiae* NJ1 was from our lab. The capacity of degrading glucosinolates was primarily screened and secondarily screened by the power of producing peptides and degrading phytic acid, tannins, and crude fiber in RSM. Lastly, three strains were preferred for the solid-state fermentation of RSM according to the comprehensive capacity described above. Y8 and GYB6 were identified as *B. amyloliquefaciens* and *B. subtilis* by morphological characteristics, physiological and biochemical identification, and 16s rDNA sequence analysis. RSM was provided by Hailong Feed Co. Ltd. (Jiangsu, China). Before the experiment, RSM was grounded and then fractionated with a 40-mesh sieve for further use. All chemicals used in the experiment were of analytical grade.

2.2. Solid-State Fermentation

For inoculation, *B. amyloliquefaciens* Y8, and *B. subtilis* GYB6 were precultured in broth medium at 37 °C for 18 h, while *S. cerevisiae* NJ1 was precultured in yeast extract peptone dextrose medium at 30 °C for 24 h. Then, the biomass was tested and adjusted to 10^8 CFU/mL for the further solid-state fermentation of RSM. Solid-state fermentation

conditions were optimized with the comprehensive index of the decline of glucosinolates and the increase in peptides. The solid-state fermentation experiment was performed under the optimum conditions as follows: inoculation amount of mixed microbial culture 30% (*B. subtilis* GYB6 10%, *S. cerevisiae* NJ1 15%, and *B. amyloliquefaciens* Y8 5%); fermentation temperature 37 °C; fermentation moisture 55%; and fermentation time 96 h. After fermentation, the samples were dried in the oven at 60 °C for related index determination.

2.3. Measurement of Chemical Compositions

The palladium chloride method determined the glucosinolates according to the procedure described previously by [26], which was slightly modified. An aliquot of the ground sample (100 mg) mixed with 80% alcohol (10 mL) was well stirred and shaken at 170 r/min at 40 °C for 4 h. Then, the samples were centrifuged at 4000× *g* for 10 min, and the extract solution was collected. The 1 mL of RSM or FRSM extract solution was added to a tube containing 2 mL of 0.15% sodium carboxymethylcellulose and 1 mL of 4 mM palladium chloride. After incubating at 24 °C for 1.5 h, the absorbance at 540 nm was measured. The mixture with sodium carboxymethylcellulose, palladium chloride, and distilled water was used as a reagent blank. Glucosinolates content was calculated by a sinigrin standard curve. The results were expressed as μmol glucosinolates/g dry sample.

Peptides were analyzed according to the method of Rawdkuen et al. [27], slightly modified. A total of 9 mL of 5% TCA was added to 1 g of samples, and the mixture was shaken at the speed of 150 r/min at 30 °C for 1 h and centrifuged at 8000× *g* for 5 min. TCA-soluble peptides in the supernatant were measured by the method of Lowry.

The phytic acid content in samples was analyzed by the ferric chloride colorimetric method, as described by Sotelo et al. [28]. Tannins content was determined colorimetrically using the Folin–Denis reagent, according to Sugiharto et al. [29]. The crude fiber content was determined according to Van Soest et al. [30]. The crude protein content was analyzed by the Kjeldahl method (984.13, AOAC1990) [31]. RSM or FRSM samples (1 g) were extracted with 40% ethanol (20 mL), and the mixture was centrifuged at 8000 rpm for 10 min. The resulting supernatant was freeze-dried to test total phenolics and flavonoid content as well as antioxidant activity. Total phenolic content was detected using the Folin–Ciocalteu reagent as described previously by Olukomaiya et al. [12]. Total flavonoid content was estimated by a colorimetric method [32]. The amino acids were obtained by hydrolysis of RSM or FRSM at 110 ± 1 °C for 24 h with 6M HCl. The final hydrolysates were analyzed by Sykam automatic amino acid analyzer S 433-D (Sykam GmbH, Eresing, Germany) equipped with cation exchange chromatographic column LCA K06/Na.

2.4. The Molecular Weight Distribution of the Proteins

The molecular weight (MW) distribution of the proteins was determined by SDS-PAGE. The RSM and FRSM were dried at a low temperature of 35 °C and then crushed and screened. The proteins were extracted by NaOH. Briefly, 0.1 g of milled samples were added to 20 mL of 0.1 M NaOH and then shaken at 150 r/min at 37 °C for 3 h. Then, samples were centrifuged at 10,000× *g* for 10 min at 4 °C to collect the soluble fraction for SDS-PAGE analysis.

2.5. Antioxidant Activity

The DPPH radical scavenging activity assay was conducted following the method of Oskoueian et al. [32], with slight modification. Briefly, 1 mL of the DPPH solution (0.1 mM in 95% ethanol) was added to 0.5 mL of sample solutions at different concentrations. The mixtures were incubated at room temperature for 30 min in the dark. The absorbance of the mixtures was detected at a wavelength of 517 nm using a spectrophotometer. The DPPH radical scavenging activity was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1 - (A_2 - A_0)/A_1)] \times 100\%$$

where A_1 is the absorbance of DPPH in ethanol, A_2 is the absorbance of sample mixed DPPH, and A_0 is the absorbance of the sample in ethanol.

Reducing power was conducted following the method of Liu et al. [33] with slight modification. Briefly, 2.5 mL of phosphate buffer (pH 6.6, 0.2 M) and 2.5 mL of 1% potassium ferrocyanide were added to 2.5 mL of samples. The mixture was incubated at 50 °C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged. Subsequently, 2.5 mL of the supernatant, 2.5 mL of distilled water, and 0.5 mL of 1% $FeCl_3$ were mixed and reacted at room temperature for 10 min. The reaction mixture was centrifuged at 6000 rpm for 10 min. The absorbance of the supernatant was measured at a wavelength of 700 nm using a spectrophotometer.

2.6. Functional Properties

The functional properties containing bulk density, water absorption capacity, swelling index, and swelling capacity were evaluated. Bulk density, water absorption capacity, and swelling index were determined according to the method described by Olukomaiya et al. [12]. The bulk density was expressed in g dry sample/mL sample volume. Water absorption capacity was expressed as percentage increase in the sample weight after adding distilled water. Swelling index was calculated as the ratio of the swollen volume to the initial volume after adding distilled water. Swelling capacity was measured according to the method described by Chandra et al. [34] and expressed as the final volume (mL) occupied by the sample after adding distilled water.

2.7. Correlations among Variables Analysis

Principal component (PC) analysis was applied to analyze the correlation between the different variables, including nutritional components, anti-nutritional factors, bioactive compounds, antioxidant activity, and functional properties of RSM and FRSM. Variables with positive correlations cluster together, and variables with negative correlations are distributed at either end of the line passing through the origin. The coordinates of each variable correspond to the correlation and directionality with PC1 and PC2, respectively.

2.8. Structural Analysis

The microstructure of RSM and FRSM was observed using field-emission scanning electron microscopy (SEM; Tescan Mira3, Tescan, Brno, Czech Republic) in different magnifications. FTIR spectra were analyzed with the spectrometer (Nicolet iS5, Thermo Scientific, Waltham, MA, USA). Sample discs were prepared with KBr (1:200, *w*/*w*). The FTIR spectra from 500 to 4000 cm^{-1} were obtained with 32 scans at a resolution of 4 cm^{-1}.

2.9. Statistical Analysis

All experiments were performed in triplicate, and the data were expressed as mean ± standard deviation. The content of phytic acid, tannins, crude fiber, crude protein, peptides, and amino acids was expressed as a percentage of dry sample weight. The content of total phenolics and flavonoids was expressed as mg total phenolics or flavonoids/g dry sample weight. Statistical analyses were performed using 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 $p < 0.05$ and a highly significant level of $p < 0.01$.

3. Results and Discussion

3.1. Effects of Fermentation on Anti-Nutritional Factors of RSM

As the goitrogenic properties of glucosinolates, the detoxification of glucosinolates has constantly been a focus of RSM research [21]. RSM fermented with *B. subtilis* reduced glucosinolates from 38.70 μmol/g to 19.72 μmol/g [35]. *B. subtilis* YY-1 and *S. cerevisiae* YY-2 were applied to fermenting extruded-pretreatment RSM, and the degradation rate of glucosinolates was 88.62% [20]. Predictably, fermentation in the presence of *B. subtilis*,

S. cerevisiae, and *B. amyloliquefaciens* in this study led to the elimination of almost all of the glucosinolate. Compared to unfermented RSM, the glucosinolates content in FRSM was decreased by 99.18% (Table 1). Glucosinolates comprise a glucose molecule and a sulfur-containing group of atoms with various side chains attached [9]. The glucose and sulphur moieties of the glucosinolate compounds presented in the RSM may be consumed by microbial enzymes, which may lead to the decrease in glucosinolates.

Table 1. Anti-nutritional factors content in RSM and FRSM.

Anti-Nutritional Factors	RSM	FRSM	SEM	Degradation Rate (%)	*p*
Glucosiolates (μmol/g)	47.67 ^{a} ± 0.70	0.26 ^{b} ± 0.1	10.60	99.18	<0.01
Phytic acid (%)	3.11 ^{a} ± 0.18	1.79 ^{b} ± 0.12	0.30	42.41	<0.01
Crude fiber (%)	11.25 ^{a} ± 0.26	8.19 ^{b} ± 0.07	0.69	27.21	<0.01
Tannins (%)	2.67 ^{a} ± 0.15	1.76 ^{b} ± 0.02	0.21	34.17	<0.01

RSM = rapeseed meal; FRSM = fermented rapeseed meal; ^{a,b} mean values which do not share a common superscript letter are significantly different ($p < 0.05$); SEM = standard error; and p = value of significance.

Except for glucosinolates, the content of three other anti-nutritional factors also declined significantly ($p < 0.01$) with the fermentation process (Table 1). The phytic acid content decreased significantly from 3.11% to 1.79% after fermentation, consistent with the result reported by Vig et al. [18] that found that the RSM processed with *R. oligosporus* decreased the phytic acid content. The decrease in phytic acid may be attributed to the activity of phytase synthesized by microorganisms [12,26]. The reduction in crude fiber content was also pronounced when the fermentation process was completed. Vig et al. [18] and Hu et al. [36] obtained the same result with fermentation; crude fiber declined by 25.5% and 24.68%, respectively. However, an increase of 9.2% in FRSM was observed by Plaipetch et al. [37]. The difference in the variation of the crude fiber may depend on the fermentation conditions and the capacity of the microorganisms utilized for fermentations to degrade cellulose, hemicellulose, and lignin of RSM [21]. The tannins content of the FRSM decreased to 8.19%, compared to 11.25% in the RSM (Table 1). The reduction of 36.36% in tannins after fermentation of RSM with *B. subtilis*, *Candida utilis*, and *Enterococcus faecalis* was similar to the result observed in this study [36]. Such significant elimination of these anti-nutritional factors is essential from a nutritional point of view and will widen the application of RSM in feed.

3.2. Effects of Fermentation on Nutritional Components of RSM

Crude protein was increased from 33.16% in RSM to 38.56% in FRSM by the fermentation process, with an increased rate of 16.28% (Table 2). Likewise, studies reported an increase in the protein content from 34.84% to 40.72% for 72 h of fermentation with *A. niger* [26] and 34.95% to 38.85% for 72 h of fermentation with *S. cerevisiae* [21]. Crude protein is a crucial index for feed, so increasing crude protein is crucial. The crude protein concentration of RSM was increased, possibly due to the mycoprotein synthesis by microbial fermentation at the expense of fermentable compositions [18,19]. Some authors also considered that gas production during fermentation led to the loss of a portion of the sample, and then the crude protein content of RSM was increased.

Table 2 showed a much higher ($p < 0.01$) peptide content of FRSM, which was 4.00 times RSM. Small peptides have more advantages because peptides are not only easier to absorb but also exhibit bioactive activity such as antioxidant activity, immune activity, and nerve activity [19,36]. The increase in peptides was consistent with the fermentation of RSM with *A. niger* from 2.57% to 8.39% [26] and *B. licheniformis* (1.0813), *Yeast* (ACCC20060), and *Lactobacillus* (ACCC10637) from 11.9 mg/g to 66.0 mg/g [19].

The amino acids profile of RSM and FRSM is presented in Table 2. The total amino acids (TAAs) content in RSM and FRSM was increased from 30.90% to 36.48%. The content of non-essential amino acids (NEAAs) of the FRSM was 1.25 times RSM. Essential amino acids (EAAs) changed slightly with the fermentation ($p > 0.05$). The increase in TAAs was

in harmony with the results of Wang et al. [19] and in partial agreement with the results of Wang et al. [20], in which the content of most of EAAs improved. Among the amino acids, the magnitude of change in the content of Asp, Leu, Thr, Ser, Glu, Gly, His, and Arg was apparent, and the content was increased by 13.57%, 20.08%, 25.00%, 32.32%, 33.86%, 22.66, 44.96%, and 54.20% ($p < 0.01$), respectively. The concentration of Ala and Pro was also upgraded by 12.13% and 7.44% ($p < 0.05$), respectively. Li et al. reported that RSM fermented with *Bacillus subtilis* only increases the Arg, Leu, and Met [35]. Glu is the natural umami amino acid that can enhance the flavor of low-salt foods. Asp, Leu, Thr, Ala, and Pro are hydrophobic amino acids that may relate to antioxidant activity [38]. Leu, Glu, Gly, and Arg are considered to be the functional amino acids to improve growth and health in mammals and fish [39]. The increases in the concentration of certain AAs and the changes in the AA profile of FRSM could be attributed to the rise of protein by fermentation and microbial metabolism during the fermentation [40].

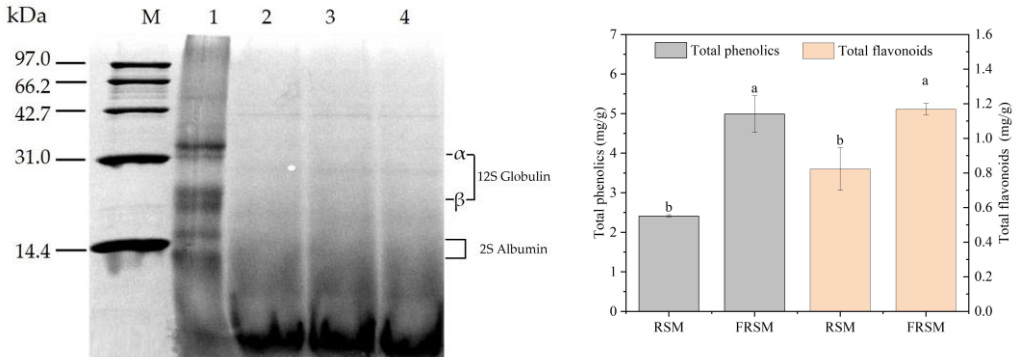
Table 2. Crude protein, peptides, and amino acids content in RSM and FRSM.

Composition (%)	RSM	FRSM	SEM	Increase Rate(%)	*p*
Crude protein	33.16 ^{a} ±1.39	38.56 ^{b} ±1.51	1.32	16.28	<0.05
Peptides	4.13 ^{a} ± 0.17	16.47 ^{b} ± 0.31	2.76	298.79	<0.01
Asp	2.65 ^{b} ± 0.06	3.01 ^{a} ± 0.06	0.08	13.57	<0.01
Ile	1.54 ^{a} ± 0.07	1.51 ^{a} ± 0.05	0.02	−1.74	>0.05
Leu	2.42 ^{b} ± 0.06	2.91 ^{a} ± 0.06	0.10	20.08	<0.01
Lys	1.77 ^{a} ± 0.05	1.84 ^{a} ± 0.05	0.02	4.14	>0.05
Met	0.33 ^{a} ± 0.04	0.37 ^{a} ± 0.05	0.02	10.00	>0.05
Phe	1.84 ^{a} ± 0.06	1.85 ^{a} ± 0.04	0.01	0.54	>0.05
Thr	1.45 ^{b} ± 0.05	1.82 ^{a} ± 0.08	0.08	25.00	<0.01
Val	2.16 ^{a} ± 0.06	2.16 ^{a} ± 0.06	0.02	0.00	>0.05
Ser	1.31 ^{b} ± 0.04	1.73 ^{a} ± 0.02	0.10	32.32	<0.01
Glu	5.69 ^{b} ± 0.15	7.62 ^{a} ± 0.05	0.39	33.86	<0.01
Gly	1.60 ^{b} ± 0.09	1.97 ^{a} ± 0.07	0.09	22.66	<0.01
Ala	1.59 ^{b} ± 0.08	1.79 ^{a} ± 0.06	0.05	12.13	<0.05
Cys	0.70 ^{a} ± 0.06	0.74 ^{a} ± 0.04	0.02	5.74	>0.05
His	0.79 ^{b} ± 0.07	1.15 ^{a} ± 0.03	0.08	44.96	<0.01
Arg	1.47 ^{b} ± 0.07	2.27 ^{a} ± 0.04	0.18	54.20	<0.01
Pro	2.64 ^{b} ± 0.10	2.84 ^{a} ± 0.06	0.05	7.44	<0.05
Tyr	0.93 ^{a} ± 0.06	0.92 ^{a} ± 0.06	0.02	−1.79	>0.05
EAAs	14.16 ^{a} ± 0.35	15.47 ^{a} ± 0.34	0.32	9.18	>0.05
NEAAs	16.74 ^{b} ± 0.56	21.01 ^{a} ± 0.34	0.98	25.58	<0.01
TAAs	30.90 ^{b} ± 0.91	36.48 ^{a} ± 0.69	1.29	18.06	<0.01

RSM = rapeseed meal; FRSM = fermented rapeseed meal; EAAs = essential amino acids; NEAAs = non-essential amino acids; TAAs = total amino acids; ^{a,b} mean values which do not share a common superscript letter are significantly different ($p < 0.05$); SEM = standard error; and p = value of significance.

3.3. Effects of Fermentation on Protein Molecular Distribution of RSM

The SDS-PAGE patterns of the RSM and FRSM are presented in Figure 1a. Studies reported that RSM proteins mainly included cruciferin (12S globulin, α subunit, and β subunit) with six subunit pairs, napin (2S albumin), and some small proteins, such as insulin inhibitors and lipoproteins [12,19,20]. As shown in Figure 1a, 12S globulin and 2S albumin approximately disappeared in FRSM (lanes 2, 3, and 4). It indicated that protease secreted by *B. amyloliquefaciens*, *B. subtilis*, and *S. cerevisiae* successfully hydrolyzed 2S globulin and 2S albumin. Many small-sized proteins or peptides (less than 14.4 kDa) emerged. The result was consistent with the increased content of peptides in this study. Similarity, Fermentation with *B. licheniformis* (1.0813), *Yeast* (ACCC20060) and *Lactobacillus* (ACCC10637) [19], *B. subtilis* YY-1 and *S. cerevisiae* YY-2 [20], *A. sojae* and *A. ficuum* [12], and *A. niger* [26] changed the protein molecular weight distribution of RSM.



(**a**) (**b**)

Figure 1. (**a**) MW distribution presented by SDS-PAGE; M: protein marker; 1: protein sample of unfermented rapeseed meal (RSM); 2, 3, and 4: protein samples of fermented rapeseed meal (FRSM). (**b**) Total phenolic and total flavonoid content. a, b mean values which do not share a common superscript letter are significantly different ($p < 0.05$).

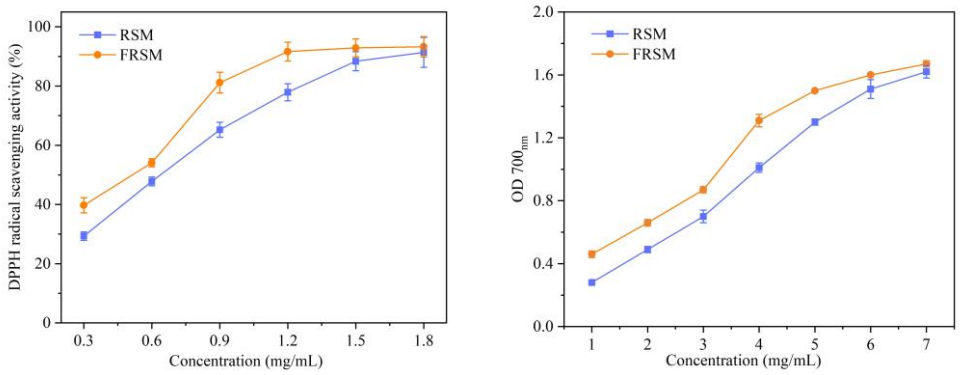
3.4. Effects of Fermentation on Bioactive Compounds of RSM

Phenolics and flavonoids are bioactive compounds, and their content is an important factor in determining the antioxidant capacity of RSM [32]. The effect of fermentation on the content of total phenolics and flavonoids was studied (Figure 1b). The results showed that the fermentation process significantly improved the total phenolic and flavonoid content of RSM ($p < 0.01$). The total phenolic content of FRSM (4.99 ± 0.47 mg/g) was significantly higher than that of RSM (2.41 ± 0.03 mg/g) ($p < 0.01$). The flavonoid content of FRSM (1.17 ± 0.03 mg/g) was also significantly higher than that of RSM (0.82 ± 0.12 mg/g) ($p < 0.01$). During the fermentation, a variety of enzymes, such as cellulase and hemicellulose, were secreted by the microorganism, which could break down the structure of RSM [26] and may liberate phenolic and flavonoid compounds from the RSM. The increase in phenolic and flavonoid content may also be due to more or new phenolics and flavonoids synthesized by the microorganism during fermentation [33,41]. A similar result was reported by Wang et al. [20], that the fermentation enhanced the total phenolic content of extruded-pretreatment RSM. Liu et al. reported that the total flavonoids content of dandelion increased significantly after fermentation [33]. However, reversely, the content of total phenolics declined by 21.58–23.55% using the yeast to ferment RSM [21]. It indicated that the microorganism would influence the metabolism of the substrate.

3.5. Effects of Fermentation on Antioxidant Activity of RSM

The DPPH radical scavenging activities of the RSM and FRSM is presented in Figure 2a. DPPH radical scavenging activities of RSM and FRSM were different in the concentration of 1.5 mg/mL ($p < 0.05$) and significantly different in the concentration of 0.3–1.2 mg/mL ($p < 0.01$). DPPH radical scavenging activity of FRSM was 91.63 ± 3.17%, 81.13 ± 3.52%, 54.08 ± 1.35%, and 39.72 ± 2.56%, respectively, at the concentration of 1.2, 0.9, 0.6, 0.3 mg/mL, while that of the RSM reached 77.90 ± 2.89%, 65.23 ± 4.51%, 47.79 ± 1.47%, and 29.32 ± 1.42%, respectively, at the same concentration. The EC50 of FRSM was 0.72 ± 0.01 mg/mL, while the EC50 of RSM was 0.91 ± 0.07 mg/mL, which indicated that the FRSM displayed a higher DPPH scavenging activity compared to the RSM.

The reducing powers of the different concentrations of RSM and FRSM is presented in Figure 2b. The reducing powers of RSM and FRSM were significantly different in the concentration of 1–5 mg/mL ($p < 0.01$). $OD_{700\,nm}$ of the FRSM was 1.50 ± 0.05, 1.31 ± 0.04, 0.87 ± 0.02, 0.66 ± 0.02, and 0.46 ± 0.01, respectively, at the concentration of 5, 4, 3, 2, and 1 mg/mL, while that of the RSM reached 1.30 ± 0.02, 1.01 ± 0.03, 0.70 ± 0.04, 0.49 ± 0.02, and 0.28 ± 0.01, respectively, at the same concentration. The EC50 of FRSM was 3.53 ± 0.18 mg/mL, while the EC50 of RSM was 4.78 ± 0.65 mg/mL, which indicated that the FRSM owned the better reducing power compared to the RSM.



(**a**) (**b**)

Figure 2. Antioxidant activity of RSM and FRSM. (**a**) DPPH radical scavenging activity; (**b**) Reducing power; RSM = rapeseed meal; and FRSM = fermented rapeseed meal.

According to the reaction mechanisms, methods commonly used to determine the total antioxidant capacity can be divided into two major groups: assays based on a single electron transfer reaction (SET) and assays based on a hydrogen atom transfer reaction (HAT) [42]. SET monitors through a change in color as the oxidant is reduced, while HAT detects the competition between the antioxidant and the substrate for the free radical. The results of two SET reaction-based methods, DPPH radical scavenging activity and reducing power, all confirmed that antioxidant activity of the FRSM was improved compared to that of the RSM, indicating that more bioactive compounds were produced by fermentation. Likewise, RSM inoculated with selected protease-assisting *B. subtilis* and *L. plantarum* showed stronger antioxidant activity than unfermented RSM [25].

3.6. Effects of Fermentation on Functional Properties of RSM

The functional properties of RSM and FRSM are presented in Table 3. The functional properties were all enhanced significantly ($p < 0.01$) by fermentation, except for bulk density. Enhancing the ability of the meal to absorb and retain water is a valuable functional property because it may improve the binding of the structure, strengthen flavor retention, improve mouth feel, and reduce moisture and fat losses of food products [43]. FRSM exhibited a significant increase ($p < 0.01$) in water absorption capacity, the percentage increase ($p < 0.05$) being 24.09% compared to the RSM. This is comparable to that reported canola meal fermented by *A. sojae* and *A. ficuum* [12]. The value of the swelling index and swelling capacity of FRSM was higher than RSM's. The swelling index of FRSM was 1.27 times RSM. The swelling capacity of 14.5 mL of RSM significantly increased ($p < 0.05$) to 24.8 mL of FRSM. The swelling index and swelling capacity values increase consistently with previous reports on fermented cassava flour [44] and fermented canola meal [12].

Table 3. Functional properties of RSM and FRSM.

Parameters	RSM	FRSM	SEM	*p*
Bulk density (g/mL)	$0.54^{a} \pm 0.02$	$0.53^{a} \pm 0.01$	0.00	>0.05
Water absorption capacity (%)	$227.08^{b} \pm 5.07$	$281.78^{a} \pm 10.84$	12.62	<0.01
Swelling index	$1.29^{b} \pm 0.08$	$1.66^{a} \pm 0.05$	0.08	<0.01
Swelling capacity (mL)	$14.00^{b} \pm 0.20$	$24.50^{a} \pm 0.50$	2.35	<0.01

RSM = rapeseed meal; FRSM = fermented rapeseed meal; ^{a,b} mean values which do not share a common superscript letter are significantly different ($p < 0.05$); SEM = standard error; and p = value of significance.

3.7. Correlations among Variables of RSM

The PC analysis of the chemical characteristics, metabolite production, antioxidant activity, and functional properties of the RSM and FRSM samples was shown as a loading plot (Figure 3). Total phenolics, flavonoids, and peptides were closely associated with

a cluster that was positively related to DPPH radical scavenging activity andreducing power, indicating the increment of total phenolics, flavonoids, and peptides might be significant contributors to the upgrade of antioxidant activity of FRSM. Oskoueian et al. reported that phenolics and flavonoids were highly correlated to the field of antioxidant activities [32]. Wang et al. illustrated that the change trends of DPPH radical scavenging activity agreed with those of total phenolic and small peptides [20]. Crude protein and peptides were positively related to water absorption capacity, which agreed with the literature stating that increasing low molecular weight proteins and the number of polar protein groups could increase water absorption capacity [45]. Crude protein, peptides, and total amino acids were closely associated with a cluster. These parameters were negatively associated with glucosinolates, phytic acid, crude fiber, and tannins, highlighting solid-state fermentation's importance for the improvement in nutritional values and degradation of anti-nutritional factors.

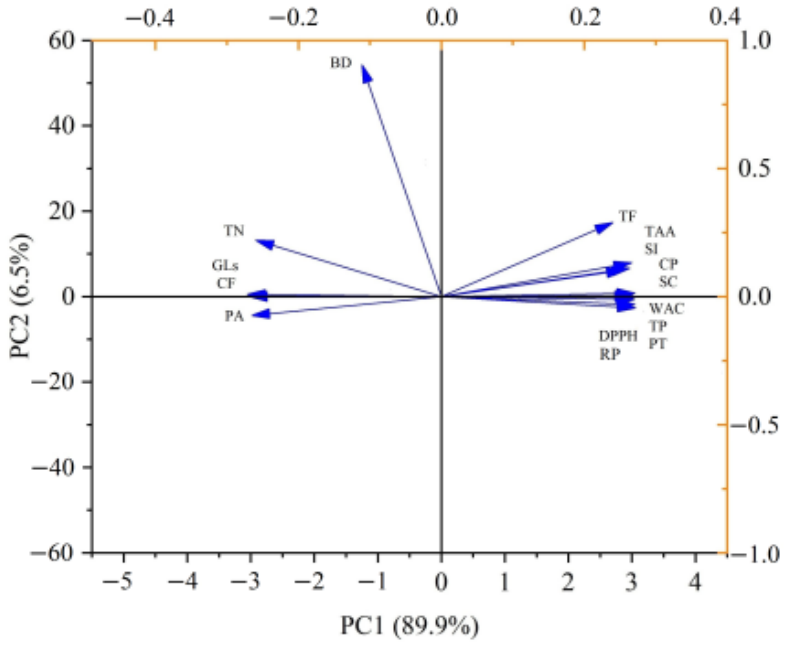


Figure 3. Principal component analysis of the correlation between the different variables during solid-sate fermentation. GLs: glucosinolates; phytic acid: PA; crude fiber: CF; TN: tannins; TF: total flavonoids; TP: total phenolics; CP: crude protein; PT: peptide; TAA: total amino acids; DPPH: DPPH radical scavenging activity; RP: reducing power; BD: bulk density; WAC: water absorption capacity; SI: swelling index; and SC: swelling capacity.

3.8. Effects of Fermentation on Functional Groups and Chemical Bonds of RSM

The alterations in the functional groups and chemical bonds of the RSM and FRSM were observed with FTIR (Figure 4). Broad and robust peaks were evident at 3293 cm^{-1} because of the stretching and bending vibrations of the –OH groups, especially in cellulose and hemicellulose [46]; FRSM showed higher absorbance values as compared to RSM, which may be due to the structural changes in cellulose and hemicellulose in the RSM. Absorption bands at 2800–3000 cm^{-1} were attributable to the C-H stretching of methylene in the polysaccharides [47]. The peaks at 2926 and 2854 cm^{-1} were intensified after fermentation, suggesting the release of shorter-chain polysaccharides. RSM had the characteristic peaks at 1743 cm^{-1} (carbonyl group stretching of ester group) (C–O stretching of COOH in uronic acid). Still, FRSM had no such peak, possibly due to the degradation of uronic acids and pectin [47]. The band at 1448 cm^{-1} to the C–H bending vibration of cellulose and the band at 1068 cm^{-1} to the C–O stretching vibrations of polysaccharides or polysaccharide-like substances [48,49] were shifted to 1447 cm^{-1} and 1071 cm^{-1}, and peak shape was changed, indicating the polysaccharides could be hydrolyzed during the fermentation. The

absorption peak at 1658 cm^{-1}, 1538 $cm^{-1,}$ and 1241 cm^{-1} is the characteristic peak of protein amide I, amide II, and amide III, respectively [50,51]. Compared to RSM, the peak was intensified, and peak positions of amide I, II, and III in FRSM were shifted. These results indicated that the protein structure was changed, and the amount of protein was increased. The present results indicate that the composition of RSM was changed substantially by fermentation. The cellulose, amino acids, proteins, and polyphenolic compounds contain functional groups, including carboxyl, hydroxyl, and methyl groups, which may offer therapeutic effects such as antimicrobial, anti-inflammatory, anticancer, and antioxidant activities [32].

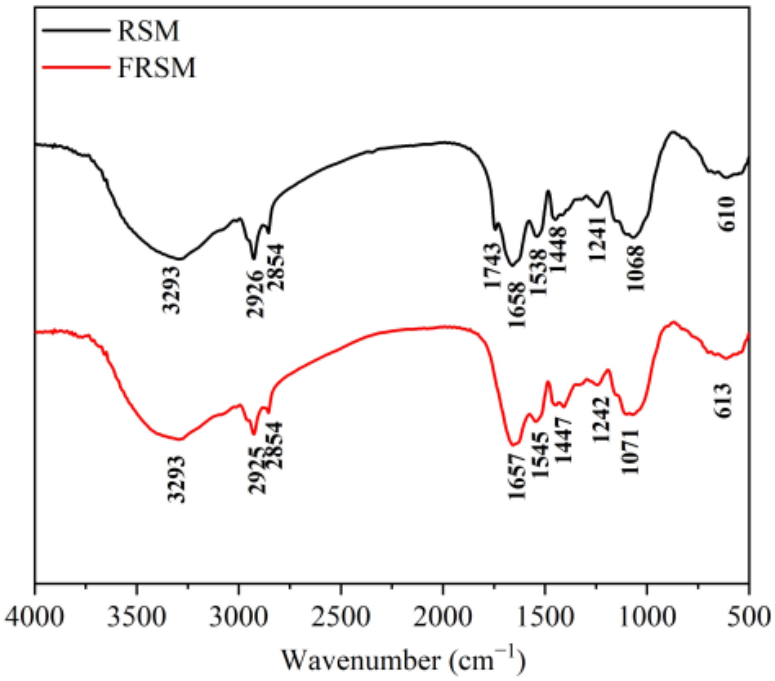


Figure 4. FTIR spectra of RSM and FRSM; RSM = rapeseed meal; and FRSM = fermented rapeseed meal.

3.9. Effects of Fermentation on Microstructures of RSM

The microstructures of RSM and FRSM were observed by SEM (Figure 5). Microstructure was clearly different between RSM and FRSM. The surface structure of the RSM was dense. After solid-sate fermentation, the surface structure was changed substantially to be rough, loose, and porous for FRSM. It demonstrated that the three strains changed and decomposed the structure of RSM during the fermentation, which is consistent with the result of FTIR spectra. The loose and porous nature may contribute to the enhancement of the water absorption of FRSM in this study. Furthermore, RSM had smaller particles than FRSM. Extracellular hydrolases (especially lignocellulolytic enzymes) secreted during solid-state fermentation by microorganisms might have hydrolyzed the fiber and other components of RSM, and the products of hydrolysis were used by the microorganism, which led to the disruption of RSM [26]. Correspondingly, the decomposition may be beneficial in increasing the contact area between microbial enzymes and RSM, and then promoting the effective release of active substances. On the surface of FRSM, a large number of granules existed. This could be due to the production and release of more protein aggregated to become small granules during fermentation [38].

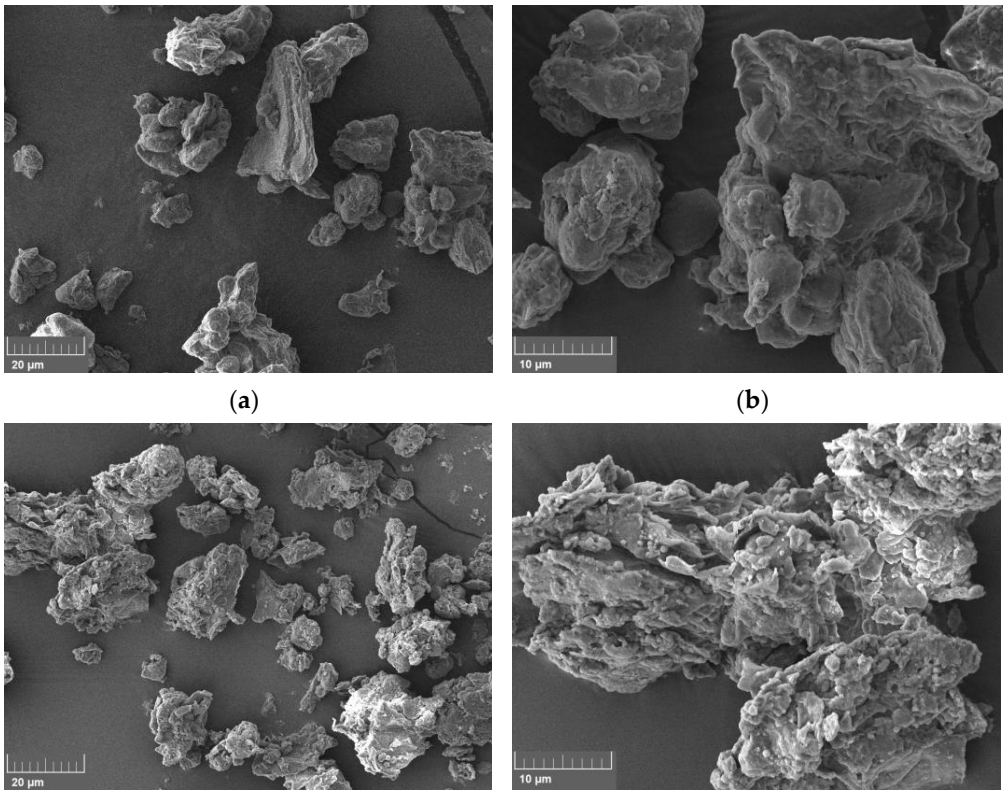


Figure 5. Microstructure of unfermented and fermented rapeseed meal. (**a**,**b**): unfermented. Rapeseed meal (RSM); (**c**,**d**): fermented rapeseed meal (FRSM); Magnifications of SEM photographs (**a**,**c**) and (**b**,**d**) are 2000× and 5000×, respectively.

4. Conclusions

A novel solid-state fermentation conducted by *B. subtilis* GYB6, *S. cerevisiae* NJ1 and *B. amyloliquefaciens* Y8 was developed to transform the agricultural by-product RSM into high-value functional food ingredients rich in nutritional components and bioactive compounds. Concerning the anti-nutritional factors, glucosinolates, phytic acid, crude fiber, and tannins content in FRSM significantly decreased compared to RSM. The nutritional components (crude protein, amino acids, and peptides) and bioactive compounds (phenolics and flavonoids) all increased considerably after fermentation. Fermented RSM revealed better antioxidant activity and functional properties. These results indicated that FRSM could be a promising nutritional food ingredient in animal feed formulations.

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