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Influence of *Lactobacillus* (LAB) Fermentation on the Enhancement of Branched Chain Amino Acids and Antioxidant Properties in Bran among Wheat By-Products

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Abstract: The main objective of this study was to enhance the nutritional properties, including branched chain amino acids (BCAAs), through the solid-state fermentation (SSF) of wheat bran (WB) using lactic acid bacteria (LAB). The physicochemical properties, amino acid profiles, bioactive components, and antioxidant properties of raw and sterilized WB were compared with those of WB fermented with five different LAB strains. The highest level of BCAAs, isoleucine (Ile; 2.557 ± 0.05 mg/100 g), leucine (Leu; 7.703 ± 0.40 mg/100 g), and valine (Val; 7.207 ± 0.37 mg/100 g), was displayed in the WB fermented with *Lactobacillus acidophilus* (L.A WB). In addition, L.A WB showed the highest amount of total phenolic and flavonoid contents (2.80 mg GAE/g and 1.01 mg CE/g, respectively), and the highest Trolox equivalent antioxidant capacity (9.88 mM TE/g). Statistical analysis clearly revealed that L.A WB presented the highest abundance of branched chain amino acids as well as bioactive components. Overall, this study distinctly implemented the possibility of fermented WB with enhanced BCAAs for application in future functional food through experimental and statistical observations.

Keywords: wheat bran; *Lactobacillus*; solid-state fermentation; branched chain amino acids; antioxidant properties

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1. Introduction

Wheat (*Triticum aestivum*) is one of the most commonly consumed grains worldwide as a staple food, and it is commonly utilized as a form of flour without bran. World wheat production increased drastically since the FAO stated that wheat was being harvested at a rate of 219 million ha all over the world, and that the global production of wheat reached 760 million tons by the year 2020 [1]. Of all production, it is estimated that around 25 million tons of wheat are lost as post-harvest waste, and reducing these losses and upgrading the by-products is a major global issue to be prioritized in fulfilling the challenge of food security and to improve the cereal value chain [2]. At the milling stage, wheat bran, germ, and middling are treated as by-products, and among these by-products, wheat bran (WB), which is the outer part of wheat kernel, comprising three layers of testa, aleurone, and pericarp, is considered to be the most important part for its use in human consumption [3,4]. WB accounts for 14.5–25% of wheat, but only 10% of these parts are used in baking industries as kind of dietary fiber supplement, while most of the bran (90%) are discharged or sold to animal husbandry at a cheap value, due to the cost of transportation and environmental issues [5,6]. Despite assuming that WB is an agroindustrial by-product, the consumption of whole wheat has been highly recommended by epidemiological studies due to various health-related nutraceutical characteristics in WB [7]. As WB is rich in phytochemicals such as alkylresorcinol, phytosterols, ferulic acid, bound phenolic compounds and flavonoids, micronutrients, and dietary fiber content, it is

used to enrich dietary fiber and to modify quality in biscuits, bread, pasta, noodles, and doughnuts [4].

However, there has a limitation in the direct use of WB in food factories as it shows rather lower sensory attributes in food products associated with bran, a slower breakdown of exogenous enzymes to phytates due to the presence of an unbroken aleurone layer in bran, etc. [6,8]. To overcome these issues, suitable treatment processes are required to improve the availability and the utility of most bioactive components trapped in complex structures containing lignin, cellulose, and proteins in bran [9]. Various treatment methods such as ultrafine grinding, extrusion, thermal treatment, high-pressure homogenization, enzymatic treatment, and fermentation were suggested for improving the technological accessibility of WB prior to its use as a supplement in food products [6,8,10–12]. Among these treatments, solid-state fermentation (SSF) is a promising way to valorize WB for nutrition [13]. SSF has the ability to improve the bioaccessibility and bioavailability of phenolic compounds in fermented products due to the liberation of bound phenolic compounds and the conversion of bioactive metabolites during fermentation [14]. Lactic acid bacteria (LAB), including *Enterococcus faecalis*, *Lactobacillus rhamnosus*, *Lactobacillus bulgaricus*, *Streptococcus thermophiles*, and *Pediococcus acidilactici,* are promising candidates for SSF to improve the biotransformation of bioactive components into new metabolites in cereal brans [14]. The successful application of LAB in the SSF of WB has also been reported in a previous study that achieved the desired results on the functional and nutritional qualities of WB [9]. However, there are few studies on the increment of BCAAs by SSF of WB.

The BCAAs, namely valine, isoleucine, and leucine, are essential amino acids that are not synthesized or are synthesized insufficiently in animals and humans, and must be taken from the diet to maintain normal growth and development [15]. The production and accumulation of BCAAs have been observed in fermentation with a variety of LAB species [16]. According to clinical evidence, BCAAs-enriched snacks are applied as a supplement for patients with liver disease, to improve hypoalbumineamia and complications such as ascites, jaundice, rupture of the esophageal varix, and liver carcinogenesis [17]. Supplementation with BCAAs prevented age-induced muscle atrophy that mimics sarcopenia in cardiovascular diseases, by enhancing protein synthesis signaling [18]. Supplementing nutrient-enriched food becomes a promising approach to combat nutrient deficiency. In terms of muscle protein synthesis, food intake associated with positive nitrogen balance is considered to be the goal of nutritional support [19]. As the BCAAs serve as substrates for protein synthesis, snacks fortified with BCAAs are an effective nutritional supplement to improve the level of alanine transaminase, aspartate aminotransferase, serum albumin, nitrogen balance, ammonia, and urea, and the general condition of patients for correcting malnutrition, hypercatabolism, and sarcopenia [19]. Thus, supplements enriched with BCAAs might be the better approach for promoting the functionality of wheat by-products.

In order to enhance these BCAAs and bioactive components in WB, the previously mentioned treatment process, SSF, is considered to be a better approach in the valorization of by-products such as WB [13]. In addition, SSF is conceptualized as a cost-effective way of waste upgrading, and the SSF of wheat bran clearly ameliorated the antioxidant and nutritional properties of wheat bran [13,20]. Despite the use of fermented WB with some LAB and yeast for the improvement of bioactivities in bakery products, a better understanding of the effect of LAB types on the characteristics and bioavailability of WB is still required for exploration. In this study, we therefore hypothesized the application of different types of LAB for the fermentation of WB to enrich BCAAs in WB. This research work prioritized the selection of LAB to enhance WB physicochemical characteristics, bioactive components, antioxidant properties, and morphological characteristics. Then, statistical assessment such as principal component analysis (PCA) and cluster analysis were conducted to visualize the clear observations between factors and variables. Ultimately, BCAAs-enriched bran production might be used to produce ingredients with health-promoting properties, and as a way to add value to wheat by-products.

2. Materials and Methods

2.1. Preparation of Samples and Reagents

WB was purchased from a local market in Changwon, Korea. LAB cultures (*Enterococcus faecalis* KCTC 3206, *Lactobacillus plantarum* KCTC 3104, *Streptococcus thermophilus* KCTC 3779, *Lactobacillus acidophilus* KCTC 3164, and *Lactobacillus helveticus* KCTC 3545) were purchased from the Korean Collection for Type Cultures (KCTC), Jeongeup-si, Korea. All chemicals and reagents used in this study were of analytical grade.

2.2. Solid-State Fermentation (SSF)

The growth of the strain and starter inoculum were performed according to the method [9]. Selected LAB strains were grown in MRS broth at 37 °C for 24 h, and LAB cultures were inoculated on MRS agar plates at 37 °C for 24 h in an incubator. The 10^8 CFU/mL calculated with a growth curve was used for further fermentation. Sterilized distilled water was added to sterilize WB to reach a moisture content of 60%. SSF of WB was performed by incubating diluted WB with the inoculated LAB strain at 37 °C for 36 h in an incubator. After completion of the fermentation process, the fermented WB samples were freeze-dried and collected as RWB, raw WB; SWB, sterilized WB; L.A WB, WB fermented with *L. acidophilus*; L.P WB, WB fermented with *L. plantarum*; L.H WB, WB fermented with *L. helveticus*; S.T WB, WB fermented with *S. thermophilus*; E.F WB, and WB fermented with *E. faecalis*.

2.3. Characterization of WB

2.3.1. Physicochemical Characteristics

Moisture, total fat, total ash, and total protein contents were determined according to the Association of Official Analytical Chemists (AOAC) International [21]. The color characteristics of wheat bran samples were determined using a Capsure-RM200 spectrocolorimeter (X-Rite, Inc., Grand Rapids, MI, USA) and the values were denoted with the symbols, L* representing the lightness value, a* representing the redness value, and b* representing the yellowness value.

2.3.2. Bioactive Components and Antioxidant Properties

Phenolic and flavonoid extracts were obtained via extraction with 80% MeOH (1:20, *w*/*v*) and incubation at 25 °C for 1 h in a shaking incubator at 100 rpm. The extracts were centrifuged at 5000 rpm for 10 min at 25 °C. After filtering with 0.45 µm filter paper, the phenolic extracts were stored at −20 °C until use. The total phenolic content (TPC) was analyzed with the Folin–Ciocalteu method [22]. The mixture of 20 µL phenolic extract, 1.5 mL 20% sodium carbonate, and 500 µL 10% Folin–Ciocalteu reagent were placed in the dark at room temperature (25 °C ± 2) for 2 h. The absorbance was determined at 765 nm using an EMC-11D-V spectrophotometer (EMCLAB Instruments, Dusiburg, Germany), and gallic acid was used to construct the standard curve expressing mg GE/g of sample. The total flavonoid content (TFC) was determined based on a published method [20]. The DPPH radical scavenging activity and Trolox equivalent antioxidant capacity (TEAC) of the samples were analyzed through a spectrophotometric method, using Trolox as a standard [23]. The mixture of 190 µL DPPH radical solutions and 10 µL sample extracts were stored at room temperature in the dark for 30 min. The absorbance of the mixture was measured at 515 nm using an EMC-11D-V spectrophotometer (EMCLAB Instruments, Dusiburg, Germany). For the TEAC assay, an equal amount of 7.4 mM ABTS and 2.6 mM potassium persulfate was mixed for 16 h at 25 °C ± 2 under dark conditions. The mixture was diluted with 100% methanol to reach an absorbance of 0.7 at 734 nm. The sample (20 µL) and diluted ABTS solution (2980 µL) were incubated at room temperature (25 °C ± 2) for 7 min, and the absorbance was measured at 734 nm using an EMC-11D-V spectrophotometer (EMCLAB Instruments, Dusiburg, Germany). The results of DPPH radical scavenging activity and TEAC were expressed as µM TE/g and mM TE/g, respectively.

2.3.3. Amino Acid Profiles

Amino acid profiles were determined with a High-Performance Liquid Chromatography (HPLC) Waters instrument (e2695, Waters Corp, Milford, MA, USA) using the Waters HPLC AccQ-Tag method, which is a pre-column derivatization technique. First, 1 g of sample was hydrolyzed with 5 mL of 0.1 N HCl for 15 min, and then the solution was centrifuged and filtered. Chromatographic separation was carried out on a Waters AccQ-Tag column (3.9 × 150 mm) thermostatized at 37 °C. For the mobile phase A, 10% acetate-phosphate buffer (Eluent A, WAT052890, Waters Corp); B, HPLC grade acetonitrile; and C, HPLC grade water were used. The sample temperature was set at 20 °C, and amino acid analysis was performed under the gradient conditions used in the method of Aung et al. [24]. A PDA detector (Waters 2998, Waters Co.) was used to detect the peak elution during HPLC separation. Each amino acid was calculated for the detected peak using the standard amino acids (amino acid standard H, Waters Co., MA, USA).

2.3.4. Morphological Characteristics

The morphological characteristics of the raw and fermented WB were determined using a scanning electron microscope (ZEISS, Merlin Compact, Jena, Germany). Before analysis, samples were lyophilized using a freeze dryer. Each prepared sample was placed on double-sided adhesive tape and then coated with gold. After coating, each sample was subjected to an accelerating voltage of 1 kV. Images were obtained at 3000× magnification.

2.4. Statistical Analysis

The statistical analysis was conducted for each batch of triplicate samples using an analysis of variance (ANOVA) and SPSS software (v. 27.0.0.0; IBM Corp, Armonk, NY, USA), and the results were expressed as the mean ± standard deviation. For the comparison among treatments, Duncan's multiple comparison test was used as a post hoc test expressing significant differences ($p < 0.01$). Multivariate analysis, including principal component analysis (PCA), and cluster analysis, including hierarchical clustering (dendrogram and heatmap), was performed using the online version of MetaboAnalyst (https://www.metaboanalyst.ca, accessed on 3 November 2022).

3. Results

3.1. Physicochemical Characteristics

Physicochemical characteristics, including total fat, protein, and ash content, as well as the color properties of WB fermented with different LAB strains, were investigated by comparing with the raw and sterilized wheat bran (Table 1). The total fat content of unfermented and fermented wheat bran samples showed significant differences ranging from 1.50 ± 0.11 to 3.07 ± 0.08 g/100 g ($p < 0.001$). The samples fermented with *E. faecalis* (E.F WB) and *L. helveticus* (L.H WB) bacteria displayed similar amounts of fat content (3.07 ± 0.08 and 3.06 ± 0.10 g/100 g), followed by the samples fermented with *L. plantarum* (L.P WB), *L. acidophilus* (L.A WB), sterilized wheat bran (SWB), and raw wheat bran (RWB). The lowest content of fat (1.50 ± 0.11 g/100 g) was observed in samples fermented with *S. thermophilus* (S.T WB). E.F WB showed the highest protein content (17.29 ± 0.37 g/100 g) while showing the lowest content (16.13 ± 0.10 g/100 g) in the RWB sample (Table 1). The L.A WB, S.T WB, and SWB samples exhibited similar protein contents amounting to 17.10 ± 0.09, 17.14 ± 0.58, and 17.20 ± 0.18 g/100 g, respectively, followed by L.P WB, L.H WB, and RWB ($p < 0.01$). Higher amounts of total ash content were observed in the fermented samples, showing the highest value (4.93 ± 0.02 g/100 g) in the S.T WB sample and the lowest value (4.74 ± 0.76 g/100 g) in the RWB sample (Table 1) ($p < 0.001$). According to the color characteristics (Table 1 and Figure 1), unfermented samples showed a brighter color representing the highest L* value (64.00 ± 0.76 and 64.33 ± 0.52), a lower redness value (a* value: 7.50 ± 0.34 and 7.07 ± 0.21), and a lower yellowness value (b* value: 21.60 ± 1.07 and 21.00 ± 0.69) in RWB and SWB, respectively. Fermentation resulted in a darker color displaying greater a* values and b* values with respect to the lower L*

values compared to the unfermented samples. E.F WB exhibited the strongest redness level, showing the highest a* value (8.32 ± 0.13). Regarding the yellowness of the samples, the b* values of wheat bran increased significantly, especially for fermented samples compared to unfermented samples.

Table 1. Physicochemical characteristics of raw and fermented wheat bran.

Samples	Fat *** (g/100 g)	Protein ** (g/100 g)	Ash *** (g/100 g)	L* Value ***	a* Value **	b* Value **
RWB	1.99 ± 0.15 ^{b}	16.13 ± 0.10 ^{a}	4.74 ± 0.07 ^{a}	64.00 ± 0.76 ^{d}	7.50 ± 0.34 ^{ab}	21.60 ± 1.07 ^{a}
SWB	2.29 ± 0.27 ^{c}	17.20 ± 0.18 ^{c}	4.85 ± 0.05 ^{ab}	64.33 ± 0.52 ^{d}	7.07 ± 0.21 ^{b}	21.00 ± 0.69 ^{a}
L.A WB	2.89 ± 0.08 ^{d}	17.10 ± 0.09 ^{c}	4.91 ± 0.02 ^{bc}	54.42 ± 1.56 ^{a}	7.19 ± 0.21 ^{b}	22.15 ± 0.44 ^{bc}
L.P WB	2.99 ± 0.08 ^{d}	16.39 ± 0.35 ^{bc}	4.76 ± 0.00 ^{a}	58.93 ± 0.42 ^{c}	7.68 ± 0.22 ^{ab}	22.87 ± 0.94 ^{bc}
L.H WB	3.06 ± 0.10 ^{d}	16.73 ± 0.27 ^{bc}	4.85 ± 0.03 ^{ab}	59.90 ± 2.15 ^{c}	7.93 ± 0.70 ^{bc}	24.13 ± 0.62 ^{d}
S.T WB	1.50 ± 0.11 ^{a}	17.14 ± 0.58 ^{c}	4.93 ± 0.02 ^{d}	59.88 ± 0.98 ^{c}	7.86 ± 0.21 ^{bc}	23.40 ± 0.36 ^{c}
E.F WB	3.07 ± 0.08 ^{d}	17.29 ± 0.37 ^{c}	4.80 ± 0.05 ^{ab}	56.70 ± 0.75 ^{b}	8.32 ± 0.13 ^{c}	22.84 ± 0.22 ^{ab}

RWB: raw wheat bran, SWB: sterilized wheat bran, L.A WB: wheat bran fermented with *L. acidophilus*, L.P WB: wheat bran fermented with *L. plantarum*, L.H WB: wheat bran fermented with *L. helveticus*, S.T WB: wheat bran fermented with *S. thermophilus*, E.F WB: wheat bran fermented with *E. faecalis*, L* value: lightness, a* value: redness, b* value: yellowness. Different letters indicate a significant difference between samples (**: $p < 0.01$, ***: $p < 0.001$).

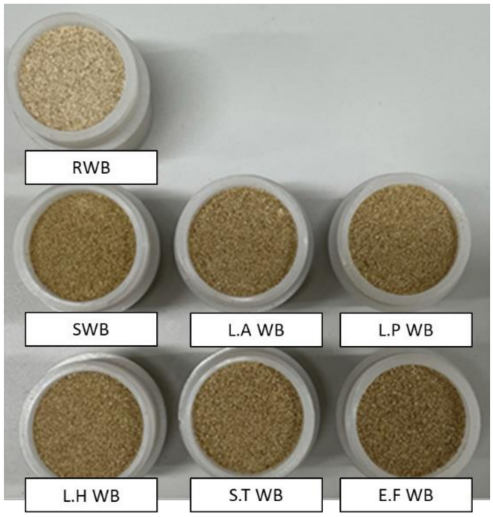


Figure 1. Illustration of raw and fermented wheat bran. RWB, raw wheat bran; SWB, sterilized wheat bran; L.A WB, wheat bran fermented with *L. acidophilus*; L.P WB, wheat bran fermented with *L. plantarum*; L.H WB, wheat bran fermented with *L. helveticus*; S.T WB, wheat bran fermented with *S. thermophilus*; E.F WB, wheat bran fermented with *E. faecalis*.

3.2. Amino Acid Profile

The profiling of amino acids in fermented WB samples was compared to that of raw and sterilized WB samples (Table 2). In this study, 16 amino acids, including eight essential and eight non-essential amino acids, were detected, and samples fermented with different bacterial strains showed different amino acid concentrations ($p < 0.001$). L.A WB exhibited the highest abundance of amino acids, showing the highest amount of histidine (His; 14.59 ± 1.10 mg/100 g), two aromatic amino acids (AAAs), phenylalanine (Phe; 16.46 ± 0.74 mg/100 g) and tyrosine (Tyr; 2.92 ± 0.53 mg/100 g), and three essential branched-chain amino acids (BCAAs), isoleucine (Ile; 2.56 ± 0.05 mg/100 g), leucine (Leu; 7.70 ± 0.40 mg/100 g), and valine (Val; 7.21 ± 0.37 mg/100 g). L.P WB showed the average amino acid concentration, with an above-average glycine (Gly; 6.07 ± 0.19 mg/100 g) and Lys (3.88 ± 0.43 mg/100 g) concentration. Alanine (Ala) and arginine (Arg) presented the highest concentrations in L.H WB (12.16 ± 2.12 and 22.14 ± 1.26 mg/100 g, respectively). Although S.T WB showed a lower amount of most amino acids, the highest glycine

(Gly) and lysine (Lys) concentrations were observed (6.48 ± 0.48 and 4.42 ± 0.25 mg/100 g, respectively). E.F WB displayed the highest glutamic acid (Glu) and threonine (Thr) concentrations (14.24 ± 0.57 and 8.90 ± 0.69 mg/100 g, respectively), while the other amino acids showed average concentrations.

Table 2. Amino acid profiles of raw and fermented wheat bran.

Amino Acid [1]	RWB	SWB	L.A WB	L.P WB	L.H WB	S.T WB	E.F WB
Ala ***	5.74 ± 0.42 ^{b}	5.58 ± 0.20 ^{b}	5.60 ± 0.23 ^{b}	3.40 ± 0.43 ^{a}	12.16 ± 2.12 ^{c}	3.29 ± 0.22 ^{a}	7.12 ± 0.69 ^{b}
Arg ***	15.38 ± 0.45 ^{c}	13.51 ± 0.55 ^{b}	13.52 ± 1.58 ^{b}	19.31 ± 0.33 ^{e}	22.14 ± 1.26 ^{f}	16.90 ± 0.55 ^{c}	3.50 ± 0.12 ^{a}
Asp ***	11.00 ± 0.76 ^{c}	10.94 ± 0.63 ^{c}	3.20 ± 0.17 ^{a}	3.70 ± 0.31 ^{a}	3.37 ± 0.37 ^{a}	3.96 ± 0.25 ^{a}	5.02 ± 0.25 ^{b}
Glu ***	7.31 ± 0.07 ^{b}	6.90 ± 0.13 ^{b}	6.71 ± 0.18 ^{ab}	8.71 ± 0.58 ^{c}	6.06 ± 0.56 ^{c}	8.58 ± 0.50 ^{c}	14.24 ± 0.57 ^{d}
Gly ***	3.33 ± 0.10 ^{a}	3.16 ± 0.07 ^{a}	3.95 ± 0.16 ^{b}	6.07 ± 0.19 ^{d}	6.35 ±0.26 ^{d}	6.48 ± 0.48 ^{d}	5.48± 0.32 ^{c}
His ***	3.12 ± 0.13 ^{a}	2.71 ± 0.09 ^{a}	14.59 ± 1.10 ^{e}	6.81 ± 0.19 ^{c}	9.45 ± 0.11 ^{d}	4.69 ± 0.22 ^{b}	7.13 ± 0.36 ^{c}
Ile ***	1.29 ± 0.03 ^{a}	1.15 ± 0.04 ^{a}	2.56 ± 0.05 ^{d}	1.73 ± 0.25 ^{b}	2.21 ± 0.09 ^{c}	1.31 ± 0.08 ^{a}	1.92 ± 0.11 ^{b}
Leu ***	1.61 ±0.04 ^{a}	1.49 ± 0.07 ^{a}	7.70 ± 0.40 ^{d}	6.25 ± 0.28 ^{c}	5.74 ± 0.32 ^{c}	4.28 ± 0.15 ^{b}	7.48 ± 0.46 ^{d}
Lys ***	3.49 ± 0.12 ^{bc}	3.37 ± 0.12 ^{b}	2.65 ± 0.12 ^{a}	3.88 ± 0.43 ^{c}	3.26 ± 0.39 ^{b}	4.42 ± 0.25 ^{d}	3.39 ± 0.19 ^{b}
Met ***	0.41 ± 0.08 ^{a}	0.29 ± 0.15 ^{a}	3.57 ± 0.08 ^{d}	1.23 ± 0.07 ^{c}	1.26 ± 0.03 ^{c}	0.76 ± 0.02 ^{b}	0.87 ± 0.07 ^{b}
Phe ***	1.36 ± 0.11 ^{a}	1.32 ± 0.21 ^{a}	16.46 ± 0.74 ^{e}	4.30 ± 0.23 ^{c}	3.79 ± 0.22 ^{c}	2.31 ± 0.25 ^{b}	5.16 ± 0.25 ^{d}
Pro ***	19.82 ± 0.91 ^{c}	19.02 ± 0.19 ^{c}	11.67 ± 0.45 ^{b}	10.07 ± 0.66 ^{a}	12.16 ± 0.20 ^{b}	11.45 ± 0.94 ^{bc}	19.19 ± 1.45 ^{c}
Ser ***	28.90 ± 0.15 ^{e}	27.54 ± 0.83 ^{e}	11.48 ± 0.40 ^{a}	17.38 ± 1.32 ^{b}	20.55 ± 0.31 ^{d}	18.47 ± 1.10 ^{bc}	19.44 ± 1.05 ^{cd}
Thr ***	1.86 ± 0.09 ^{a}	1.99 ± 0.42 ^{a}	5.97 ± 0.98 ^{c}	3.97 ± 0.46 ^{b}	6.27 ± 0.60 ^{c}	3.03 ± 0.43 ^{b}	8.90 ± 0.69 ^{e}
Tyr ***	2.06 ± 0.22 ^{c}	1.89 ± 0.10 ^{c}	2.92 ± 0.53 ^{d}	0.49 ± 0.12 ^{ab}	0.74 ± 0.01 ^{ab}	0.15 ± 0.06 ^{a}	0.48 ± 0.11 ^{ab}
Val ***	2.18 ± 0.16 ^{a}	2.12 ± 0.17 ^{a}	7.21 ± 0.37 ^{d}	5.08 ± 0.55 ^{c}	5.02 ± 0.52 ^{c}	3.57 ± 0.35 ^{b}	5.36 ± 0.18 ^{c}

[1] Amino acid (mg/100 g). Different letters indicate a significant difference between samples (***: $p < 0.001$). RWB, raw wheat bran; SWB, sterilized wheat bran; L.A WB, wheat bran fermented with *L. acidophilus*; L.P WB, wheat bran fermented with *L. plantarum*; L.H WB, wheat bran fermented with *L. helveticus*; S.T WB, wheat bran fermented with *S. thermophilus*; E.F WB, wheat bran fermented with *E. faecalis*; Ala, alanine; Arg, arginine; Asp, aspartic acid; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.

3.3. Bioactive Components and Antioxidant Activities

The antioxidant activity of bioactive components, including phenolic and flavonoid compounds, is presented in Table 3. The total phenolic content (TPC) and total flavonoid content (TFC) were the highest in L.A WB (2.80 ± 0.27 mg GAE/g and 1.01 ± 0.08 mg CE/g, respectively), followed by those of L.P WB ($p < 0.001$). L.H WB, S.T WB, and E.F WB showed similar TPCs of approximately 2.3 mg GAE/g, with no significant difference among the samples. The lowest TPC, with respect to the lowest DPPH radical scavenging activity, was observed in the RWB sample. Similarly, E.F WB and L.H WB exhibited the lowest TFC of 0.71 mg CE/g. The antioxidant activities, DPPH radical scavenging activity, and TEAC were analyzed and compared between the unfermented and fermented samples (Table 3). The DPPH radical scavenging activities of L.P WB showed the highest potential with 9.34 ± 0.12 μM TE/g, which is in agreement with the higher TPC and TFC. Unfermented samples showed the lowest DPPH activity compared to that of the fermented samples exhibiting values of 8.08 ± 0.17 and 8.18 ± 0.28 μM TE/g for RWB and SWB, respectively. TEAC ranged from 5.93 to 9.86 mM TE/g, with the highest value in L.A WB and the lowest in L.H WB. Despite the synergistic relationship between TEAC, TPC, and TFC in the L.A WB sample, some fermented samples showed lower TEAC.

Table 3. Bioactive components and antioxidant activities of raw and fermented wheat bran.

Samples	TPC *** (mg GAE/ g)	TFC *** (mg CE/ g)	DPPH *** (μM TE/ g)	TEAC *** (mM TE/ g)
RWB	1.61 ± 0.62^{a}	0.88 ± 0.11^{bc}	8.08 ± 0.17^{a}	8.11 ± 0.33^{bc}
SWB	2.07 ± 0.27^{ab}	0.84 ± 0.06^{ab}	8.18 ± 0.28^{a}	8.47 ± 0.71^{bc}
L.A WB	2.80 ± 0.27^{c}	1.01 ± 0.08^{c}	8.98 ± 0.14^{bc}	9.88 ± 2.07^{d}
L.P WB	2.36 ± 0.16^{bc}	0.91 ± 0.06^{bc}	9.34 ± 0.12^{d}	8.18 ± 0.54^{bc}
L.H WB	1.98 ± 0.21^{ab}	0.71 ± 0.07^{a}	8.86 ± 0.08^{b}	5.93 ± 1.24^{a}
S.T WB	2.23 ± 0.19^{bc}	0.87 ± 0.12^{bc}	9.24 ± 0.10^{cd}	8.06 ± 0.07^{ab}
E.F WB	2.33 ± 0.09^{bc}	0.71 ± 0.08^{a}	8.94 ± 0.02^{b}	9.33 ± 0.42^{c}

Different letters indicate a significant difference between samples (***: $p < 0.001$). RWB, raw wheat bran; SWB, sterilized wheat bran; L.A WB, wheat bran fermented with *L. acidophilus*; L.P WB, wheat bran fermented with *L. plantarum*; L.H WB, wheat bran fermented with *L. helveticus*; S.T WB, wheat bran fermented with *S. thermophilus*; E.F WB, wheat bran fermented with *E. faecalis*; TPC, total phenolic content (mg GAE/g); TFC, total flavonoid content (mg CE/g); DPPH, DPPH radical scavenging activity (mM TE/g); TEAC, Trolox equivalent antioxidant capacity (mM TE/g).

3.4. Morphological Characteristics

Scanning electron microscopy (SEM) was used to identify the morphological characteristics of WB under different LAB fermentation conditions (Figure 2). The SEM image of RWB showed typical integrity without visible fractures and almost no pores on the surface (Figure 2a). SWB contained many granules, with an amorphous structure resulting from the degradation of the cell wall and hydrolysis of protein in WB during sterilization (Figure 2b). After fermentation by LAB, the microstructure of fermented WB displayed embossed patterns with a large, loose network (Figure 2c–g). L.A WB and L.P WB showed similar microstructures, depicting a crimped structure with a few granules on the smooth surface (Figure 2c,d). Honeycomb structures with a higher number of holes and cracks were observed in the L.H WB, S.T WB, and E.F WB (Figure 2e–g).

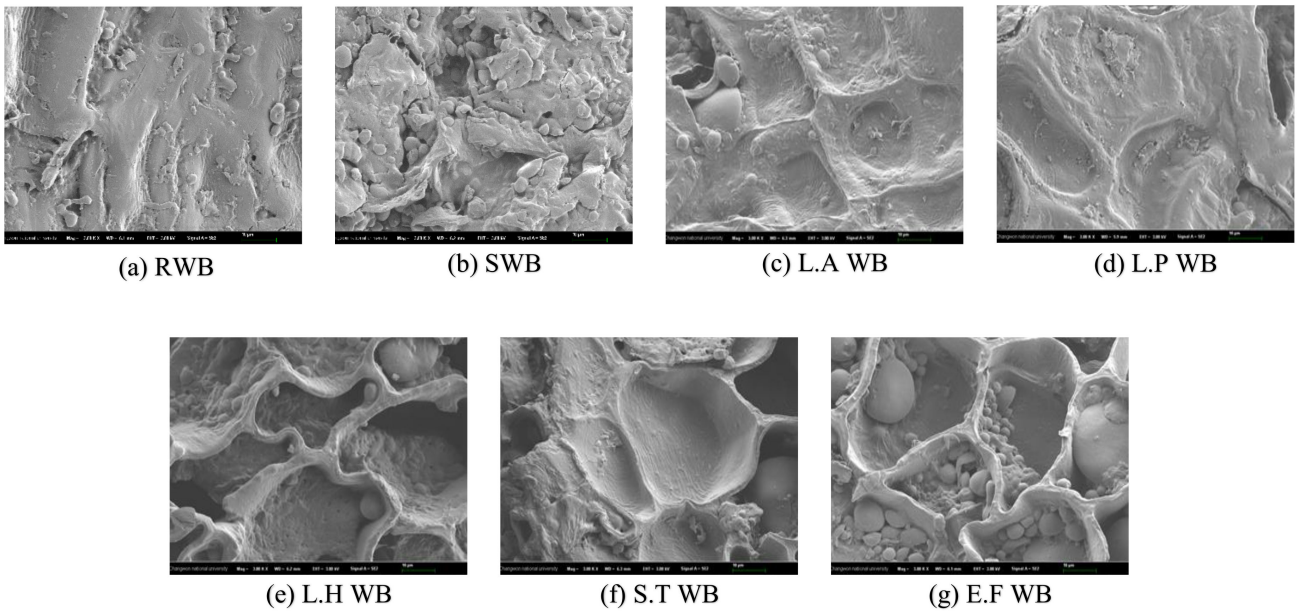
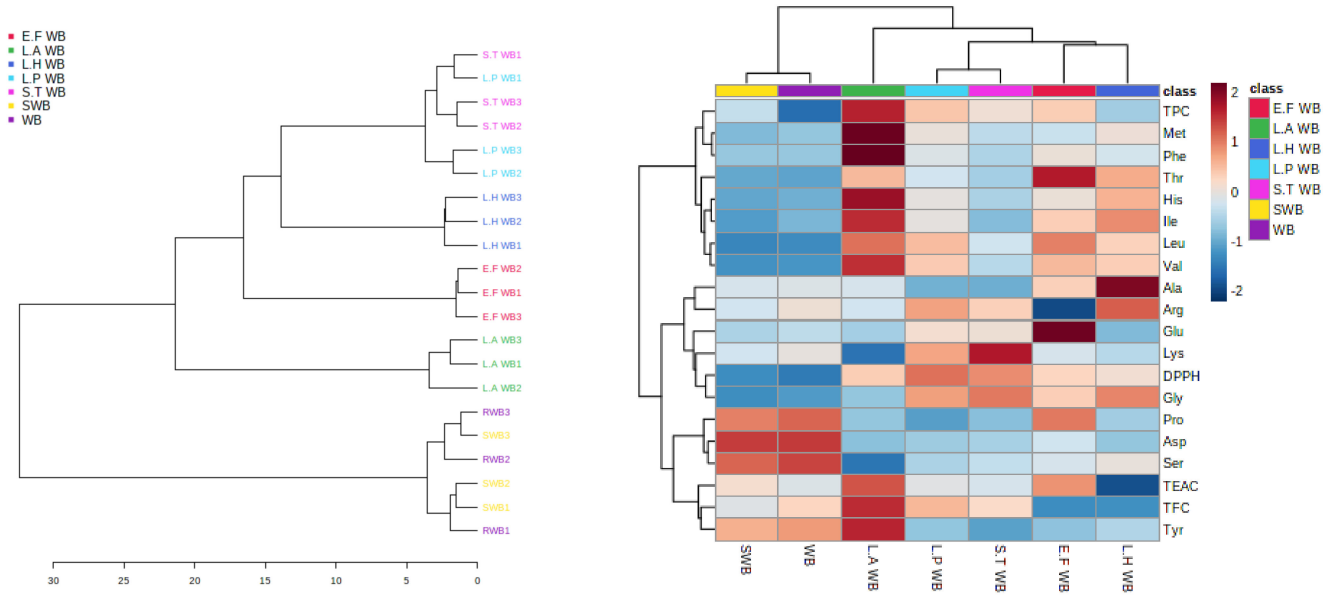


Figure 2. SEM images of raw and fermented wheat bran. RWB, raw wheat bran (**a**); SWB, sterilized wheat bran (**b**); L.A WB, wheat bran fermented with *L. acidophilus* (**c**); L.P WB, wheat bran fermented with *L. plantarum* (**d**); L.H WB, wheat bran fermented with *L. helveticus* (**e**); S.T WB, wheat bran fermented with *S. thermophilus* (**f**); E.F WB, wheat bran fermented with *E. faecalis* (**g**) (3000× magnification).

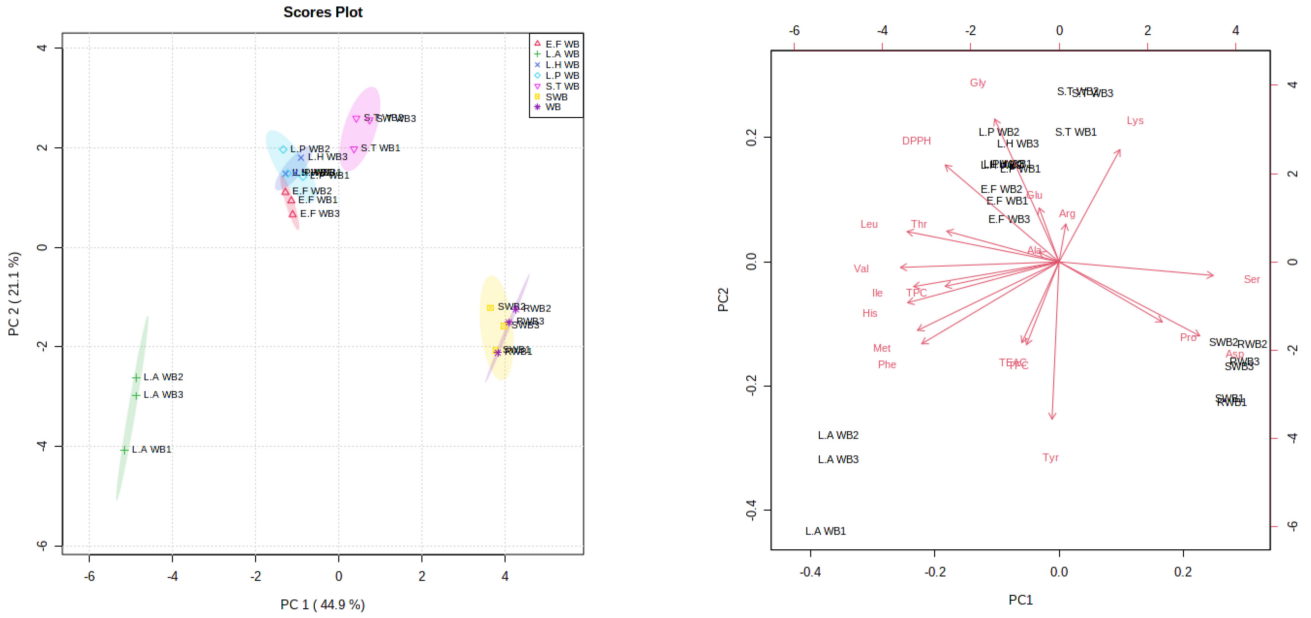
3.5. Statistical Assessment

To obtain perceptible statistical data with easily readable illustrations, clustering and classification assessments were conducted using MetaboAnalyst 5.0 [25] (Figure 3). In Figure 3a, the hierarchical clustering dendrogram shows two primary clusters clearly separated between the fermented and unfermented samples. L.A WB is present as a separate cluster, while the other fermented samples displayed one cluster at the secondary cluster level. According to the heatmap, the abundance of functional and physicochemical parameters differed substantially between the unfermented and fermented samples (Figure 3b). The color gradient displayed from dark blue to dark red represents the low to high abundance of the parameters found in the samples. Generally, fermented samples exhibited a higher abundance of most amino acids, bioactive components, and antioxidant potential than unfermented samples. To identify the difference between the fermentation treatments, a classification assessment was conducted through PCA analysis illustrating the score plot and biplot in Figure 3c,d. The PCA score plot explained the significant separation between the fermented and unfermented samples, showing three clear groups. Although WB fermented with different LAB (*E. faecalis*, *L. plantarum*, *S. thermophilus*, and *L. helveticus*) was not fully distinguishable, those fermented with *L. acidophilus* showed clear separation (Figure 3c). As shown in Figure 3d, the PCA biplot was constructed with an abundance of metabolites and parameters with respect to the WB treatment.



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

Figure 3. *Cont.*



(**c**) (**d**)

Figure 3. Hierarchical clustering dendrogram (**a**), heatmap (**b**), principal component analysis (PCA) score plot (**c**), and biplot (**d**) of raw and fermented wheat bran clustered with Ward's algorithm with Euclidean distance measure. RWB, raw wheat bran; SWB, sterilized wheat bran; L.A WB, wheat bran fermented with *L. acidophilus*; L.P WB, wheat bran fermented with *L. plantarum*; L.H WB, wheat bran fermented with *L. helveticus*; S.T WB, wheat bran fermented with *S. thermophilus*; E.F WB, wheat bran fermented with *E. faecalis*.

4. Discussion

Regarding the physicochemical characteristics, the levels of fat, protein, and ash in the fermented samples increased after fermentation. A significant increase in fat content was observed, except for S.T WB, and it has been reported that there are higher amounts of fat in the fermented samples than sterilized non-fermented samples in the study of wheat bran fermentation for bread preparation [12]. They explained the reason for fat enhancement, this being the synthesis of short-chain fatty acids from free sugars during fermentation. S.T WB showed the lowest content of fat due to the limited fermentation of sugars, including lactose, fructose, sucrose, and glucose, without fermenting galactose by *S.thermophilus*. The release of protein from the cells due to the degradation of cell wall structures of bran during fermentation can be explained as the reason for the increased amount of protein in the fermented samples, while raw wheat bran shows the lowest protein content [11]. The similar result of the highest amount of ash in the fermented samples has also been observed in the study of fermentation of WB, and these results may be due to the accelerated release of organic compounds into the soluble phase after fermentation [12]. The darker color was visualized and determined in the fermented WB samples than the unfermented ones. This is probably due to the presence of carotenoids in wheat bran and the level of these pigments, especially leutin, which increased significantly during LAB fermentation [26].

The production of amino acids is dependent on the types of LAB strain, as the proteinase activity was influenced by bacterial strains. Although RWB and SWB presented similar amino acid contents, a slightly lower content was observed in SWB. This result indicated the consumption of free amino acids for the Maillard reaction of WB during high temperature sterilization [9]. A similar result was observed in the study of SSF WB by *Enterococcus faecalis* M2 showing the lower amounts of amino acid in sterilized WB [9]. Both unfermented samples showed higher asparagine (Asp), proline (Pro), and serine (Ser) concentrations than those of the fermented samples. After fermentation, these amino acids are converted into secondary metabolites and are catabolized into other amino acids. Gen-

erally, the fermentation process includes the production of proteases by microorganisms that hydrolyze WB proteins via the activation of endogenous protease activity, which in turn, increases amino acid content [9]. The increase in amino acids is related to the steps of proteolysis in LAB, including protein degradation, peptide transport, peptide degradation, and amino acid catabolism [27]. Fermentation with LAB involves the degradation of high molecular weight proteins into low molecular weight peptides, thus providing a sufficient nitrogen source for LAB growth and the promotion of LAB proliferation [28]. AAAs include three aromatic amino acids, tyrosine (Tyr), phenylalanine (Phe), and tryptophan (Trp). In this study, two AAAs were detected; Trp was not detected, probably due to its rare abundance and occurrence in plants and microbes [29]. BCAAs play crucial roles in human health because of their therapeutic effectiveness, especially in the improvement of skeletal muscle, the production of human growth hormone, wound healing, and the regulation of blood sugar levels [30]. Moreover, the supplementation of BCAAs in the diet has been conceptually accepted because of their proven protein anabolic effects [31].

The effect of LAB fermentation resulted in the increase in TPC and TFC, due to the release of bioactive compounds through the hydrolysis of raw WB during acidic fermentation, and these results were in line with those reports [28]. The reason for the increased antioxidant activity could be the release of bioactive components, including phenolic compounds, peptides, and amino acids by LAB during fermentation [32]. The aleurone layer of WB is composed of the complex form of lignans, proteins, and amino acids and phytochemicals, and the fermentation of WB could reduce the formation of these complexes and release these components [4]. Another explanation for the increase in antioxidant activity might be related to the release of peptides and free amino acids during fermentation via electron-transfer chain reaction [28]. As the TEAC test is dependent on the reactions between antioxidants and oxidants, a reaction between radicals and any compound that has a redox potential that is lower than that of ABTS may occur [33]. Collectively, the TPC, DPPH, and TEAC results were in line with other reports on the functionality of fermented WB [9,32,34]. Therefore, these results exhibited that SSF with *L. acidophilus* clearly provided the enhanced bioavailability of WB for the potential application of L.A WB as a functional food ingredient.

In regard to SEM investigation, the morphology characteristics of WB depended on the use of LAB strains for fermentation. WB comprises three distinct layers: testa, aleurone, and pericarp. It is mainly composed of dietary fiber, including xylans, lignin, cellulose, galactan, and fructans; bioactive compounds such as alkylresorcinols, ferulic acid, flavonoids, lignans, carotenoids, and sterols; and other components, including vitamins and minerals [4]. The compact structure of unfermented WB samples is mainly represented by the lignified cell walls of the aleurone layer, which are attached tightly to the starchy endosperm [35]. These network deformations with collapsed cell walls indicated the degradation of aleurone through the hydrolysis of the gluten protein into peptides, accompanied with the generation of amino acids through microbial enzymes during LAB fermentation [36]. The cavities with deep holes and ligatures may be responsible for the rupture of dietary fiber upon digestion by microbes exposing the inner gel-like network, which is related to the leaching of amylose upon gelatinization and interaction with proteins, followed by structural deformation [37]. This observation indicates that fermentation had a significant impact on the morphological properties of WB.

Upon statistical approach, the fermented samples were clearly separated from the unfermented samples, and the major abundance of metabolites and antioxidant activities were determined in the fermented samples, especially in L.A WB. In line with these statistical assessments, it can be predominately assumed that WB fermented with *L. acidophilus* can produce bioactive components and important amino acids, and it might serve as a functional ingredient for the enrichment of BCAAs and the bioactive component.

5. Conclusions

In summary, the SSF of WB using different LAB cultures exhibited different nutritional profiles, including amino acids, bioactive components, and antioxidant capacities. The fermentation of WB with *L. acidophilus* resulted in a significant abundance of BCAAs, TPC, and TFC, as well as higher antioxidant capacities compared to those with other LAB strains. Therefore, the use of WB prepared by SSF with *L. acidophilus* might be a promising approach towards value-added products valorizing wheat by-products. However, further analysis is required to determine the factors influencing the fermentation of WB on the production of bioactive components. Nevertheless, this study clearly demonstrated that WB should be efficiently utilized by fermenting with LAB for the purpose of BCAAs enrichment, and fermented WB might be a possible ingredient in functional food preparation.

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