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Multi-Omics-Based Functional Characterization of Hybrid Fermented *Broussonetia papyrifera*: A Preliminary Study on Gut Health of Laying Hens

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Abstract: More attention has been paid in recent times to the application of *Broussonetia papyrifera* (BP) silage in ruminants, owing to its nutritional value. This study aimed to characterize the functionality of fermented BP and preliminarily explore its dietary effects on the gut health of laying hens. In this study, we characterized the antioxidant and antibacterial activities, bioactive compound profile, and bacterial community in *Lactobacillus plantarum*-fermented BP (FBP), as well as its dietary effects on intestinal morphology, microbiota and gene expression of laying hens. Improved contents of protein, total polyphenol and flavonoids as well as antioxidant and antibacterial activities were found after fermentation of BP. Untargeted metabolomics displayed more abundant apigenin, luteolin, diosmetin, and quercetin within the FBP, which may contribute to its functionality. Microbiome demonstrated increased abundance of Firmicutes at the expense of Cyanobacteria phylum, accompanied with raised levels of *Lactobacillus* genus. The results of a feeding trial showed dietary FBP supplementation increased the serum superoxide dismutase, but down-regulated gene expression of aryl hydrocarbon receptor (AhR), mucin2, and ZO-2, without obviously affecting the intestinal morphology and colonic microbiota. These findings suggest that FBP warrants further investigation as it may serve as a functional dietary supplement in laying hen feed.

Keywords: *Broussonetia papyrifera*; functionality; metabolomic; microbiota; laying hen; intestinal health

Citation: Niu, K.; Khosravic, S.; Wang, Y.; Zhai, Z.; Wang, R.; Liu, J.; Cai, L.; Li, J.; Deng, L.; Wu, X. Multi-Omics-Based Functional Characterization of Hybrid Fermented *Broussonetia papyrifera*: A Preliminary Study on Gut Health of Laying Hens. *Fermentation* **2022**, *8*, 547. https://doi.org/10.3390/fermentation8100547

Academic Editor: Hiroshi Kitagaki

Received: 27 September 2022
Accepted: 12 October 2022
Published: 16 October 2022

1. Introduction

Broussonetia papyrifera (BP) L. is a perennial, shallow-rooted, broad-leaf tree with rapid growth, strong adaptability, and high production capacity, and it can be continuously harvested for several years [1,2]. BP is widely distributed in Asia-Pacific countries [3] and has been historically used in paper making, traditional Chinese medicine, and livestock feed [1]. BP has high crude protein content ranging from 18% dry matter (DM) to 22% DM and high yield about 120 tonnes per hectare, thus showing potential to be a new feedstuff to alleviate the protein shortage in China [1]. Nevertheless, the multi-cutting nature and fast growth ability of BP result in huge biomass accumulation bringing large pressure on stable preservation and feed conversion [4].

Ensiling, one of the best methods to preserve fresh BP mass, shows advances in minimal dry matter loss, bioactive compounds enrichment, palatability improvement, and undesirable substance reduction [5,6]. During the natural ensiling process, water-soluble carbohydrates (WSC) are converted to organic acids by the epiphytic lactic acid bacteria (LAB) lowering the pH, which in turn inhibit the growth of detrimental microbes [7]. However, insufficient LAB cells and WSC contents may accelerate the clostridial activity and increase the concentration of butyric acid and ammonia-N, which could negatively affect the fermentation quality [8]. In order to solve this, a wide variety of chemical and biological additives, including molasses, sucrose, and probiotics, are commonly used during ensiling [9–11]. There are several probiotic inoculants used in silage such as *Lactobacillus*, *Saccharomyces*, *Bacillus*, and *Enterococcus* [7]. Among these, *Lactobacillus plantarum* is more commonly used in BP silage fermentation [12,13]. Novel additives are also continuously explored to meet the requirement for making high-quality silage. Yeast culture by-product powder (YB) comprises residues of post-yeast cell harvest with 20% CP content, and it is rich in amino acids, minerals and water-soluble humic acid. YB is produced in large amount annually and cost effectively, with potential to be a good additive in fermented BP product.

Since 2015, BP has been widely planted as a part of the development-oriented poverty program [6]. Single ensiling is not sufficient to consume the huge mass of fresh BP that could cause wasting of resource if not well preserved. BP is rich in active phytochemicals including polyphenols, flavonoids, alkaloids, terpenes, and polysaccharides which render its antioxidant, anti-inflammatory, antimicrobial, and immune modulatory properties [14–16]. Naturally drying fresh BP to make functional feed supplements is a possible way to solve the above-mentioned fact. Over the last decade, nutrient digestibility, proximate composition, viable cell count, and pH were commonly determined in BP silage rather than to evaluate its biological activity, microbial profile, and active metabolite content. The development of the high throughput technology in recent years offers features that make the evaluation process more efficient and thus more widely acceptable by the industry [5,11,17,18].

BP silage is mainly applied in farmed ruminants for improving their performance, yield, and welfare [2,6,19]. To date, the suitability of the developed fermented BP dry power on monogastric farm animals such as laying hens is still unknown. Gut, as the most important organ on nutritional digestion and absorption and immune functions, should be firstly investigated when introduce newly prepared feed stuff. Therefore, the present study mainly aimed to investigate the functionality of fermented BP dry powder with addition of *L. plantarum* and YB by integrative microbiome and metabolomic analyses and the dietary effects of the developed fermented BP on the intestinal health of laying hens.

2. Materials and Methods

2.1. Microbes and Reagents

The BP (Zong ke No. 101) powder, mainly prepared from stem and leaves, was purchased from the local market (Pingxiang, China). The yeast culture by-product powder (YB) was obtained from Angel Yeast Co., Ltd. (Yichang, China). The three probiotic inoculates, including *Lactobacillus plantarum* (Lp), *Bacillus velensis* (Bv), and *Saccharomyces cerevisiae* (Sc), assessed in this study are believed to be the most popular ones in the feed industry. The Lp was originally isolated from *Artemisia princeps* and cultured in MRS (deMan, Rogosa and Sharpe, Difco, NJ, USA) medium at 37 °C. The Sc was obtained from Angel Yeast Co., Ltd. and cultured in YM (Yeast Malt, Difco, NJ, USA) medium at 30 °C. The Bv was isolated from *Artemisia argyi* and cultured in Luria Bertani medium (LB; Difco, NJ, USA) at 37 °C. Four pathogenic strains including *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* PAO1, *Staphylococcus aureus*, and *Salmonella* Typhimurium were cultured in the LB medium, at 37 °C, and used to evaluate the antibacterial activity of the final fermented BP (FBP).

2.2. Selection of the Suitable Probiotic Strain for B. papyrifera Fermentation

Viable cell count, pH, reducing sugar, and free protein content were used as the criteria to evaluate the fermentation potential of the three selected probiotic strains. For this, each strain was cultured (initial cell number, 10^7 CFU/mL) in a sterile BP (10%, wt/vol) liquid medium, at 30 °C, and subsequently sampled at 0 h, 6 h, 12 h, and 24 h. The viable cell count, reducing sugar, and free protein concentration were determined by drop plate method, DNS assay, and bicinchoninic acid (BCA) (Pierce BCA protein assay kit; Thermo Scientific™, Waltham, Massachusetts, USA) assay, respectively [20]. The pH was measured by a pH meter (PHS-3E, INESA Scientific Instrument Co. Ltd., Shanghai, China).

2.3. Effects of Yeast By-Product (YB) Supplementation on B. papyrifera Fermentation

The impact of incremental levels, 5%, 10%, 20%, 30%, 40%, and 50% (wt/wt), of YB supplementation on fermentation performance of the two probiotic strains (Lp and BV), used in the present study, was evaluated based on the viable cell count and pH values (Figure S1). Two model solid-state fermentation culture media: 50% (wt/wt) BP (0YB) and 45% (wt/wt) BP + 5% (wt/wt) YB (5YB), with 50–60% moisture content, were prepared and sterilized. The sterilized media were then inoculated with 10^7 CFU/g (initial cell number) of Lp and incubated at 30 °C for 7 d. Samples were collected at 0, 1, 2, 4 and 7 d to evaluate the general fermentation parameters, following the methods in Section 2.2.

2.3.1. Proximate Composition

The proximate composition including the contents of dry matter (DM), ash, neutral detergent fiber (NDF), crude protein (CP), crude fat (CF), and gross energy (GE) was determined according to the previously described methods [21]. Briefly, DM was determined by oven drying, at 105 °C, for 24 h, and the ash was measured by combustion, at 600 °C, for 4 h. CP was determined using an automatic Kjeldahl System (K9860, Hanon, Shandong, China). CF was estimated gravimetrically by extraction with petroleum ether, using a Soxhlet extractor (VELP Scientifica, Milano, Italy). The GE was measured using an automatic calorimeter ZDHW-5c (Xianfeng instrument and instrument Co., Ltd., Zhengzhou, Henan, China).

2.3.2. Functional Properties

The collected samples were lyophilized and then (1 g) dissolved in 9 mL of MeOH (95%) with strong shaking for 30 min. The resultant supernatants were separated by centrifugation (9000 rpm, 10 min, 4 °C) and collected to analyze the antioxidant activities. DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS (2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid)), and FRAP (ferric-reducing antioxidant power) assays were used to evaluate the antioxidant activity, and total polyphenol content (TPC) and total flavonoid content (TFC) were also measured. DPPH assay was conducted according to a previously reported method [22]. Briefly, 100 µL of the prepared supernatant was mixed with 400 µL of methanol, following the addition of 2 mL DPPH solution (1.5 mmol/L), and incubated in a dark place for 15 min. Methanol was used as the blank and ascorbic acid (1 to 100 µg/mL) was used as the standard. The absorbance was read at 517 nm using a UV–Vis spectrophotometer. The ABTS and FRAP assays were performed using A015-2-1 and A015-3-1 (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) kits, respectively, following the manufacturer's instructions. The TPC was determined by the Folin and Ciocâlteu assay, as described previously [23], with some modifications. Briefly, 100 µL amounts of the prepared supernatant, blank (methanol) and standard (Gallic acid) samples were thoroughly mixed with 200 µL of F-C phenol reagent (1 mol/L) in a macro-tube, following the addition of 800 µL of sodium carbonate solution (700 mmol/L), and incubated for 30 min, at room temperature. Subsequently, 200 µL of the samples was transferred to a 96-well microplate and read with a microplate reader (Spark 10M, Tecan, Switzerland) at 765 nm. The TPC was calculated as µg equivalent gallic acid/mL and expressed as GAE µg/mL. The TFC was determined by a microplate-dependent assay with slight

modifications [24]. Briefly, 25 µL of the test samples, 100 µL of distilled water (DW), and 10 µL of sodium nitrite solution (5%, w/v) were added to a 96-well plate, and kept at room temperature for 5 min. After 6 min incubation with 15 µL of an aluminum chloride solution (10%, w/v), 50 µL of NaOH (1 mol/L) and 50 µL of DW were added to the reaction mixture and kept for 10 min, at room temperature, with shaking at 6 rpm. Absorbance of the samples were measured against the methanol at 510 nm, using quercetin as the standard. The TFC was calculated as µg equivalent quercetin/mL and expressed as QE µg/mL. The supernatants were 5 times concentrated by a rotary evaporator (RE 2008, Shengye, Shanghai, China) equipped with a vacuum and cooling system (DLSB-10L-10, Yuhua, Hunan, China), and used in the antibacterial assay. The antibacterial activity was determined by the agar-well diffusion method [22] against *E. coli* O157:H7, *P. aeruginosa* PAO1, *S. aureus*, and *S. typhimurium*. Briefly, the overnight grown pathogens were swabbed on the LB agar plates containing concentrated supernatant (100 µL), and the antibacterial activity was determined by measuring the clear zone (mm) formed after 24 h of incubation, at 37 °C, using 95% methanol as a blank.

2.4. Characterization of the Practical Fermented B. papyrifera

As part of efforts to practice what we have learned in the previous section and in order to facilitate cost-effective production of the fermented *B. papyrifera*, the fermentation process was carried out under unsterilized condition (at room temperature for 4 d) using *L. plantarum* (10^6 CFU/g) as the fermentation agent and 5% (wt/vol) YB as the fermentation booster. We then proceeded to evaluate the quality and functional properties of the resultant practical fermented *B. papyrifera* (FBP) as well as its bacterial community and active metabolites, as compared to the raw un-fermented *B. papyrifera* (CBP).

2.4.1. Bacterial Community Analysis

The genomic DNA of the lyophilized samples (0.2 g) was extracted and measured for concentration and purity using NanoDrop1000 (Thermo Fisher Scientific, Wilmington, DE, USA) and checked by gel electrophoresis. The primers 515F and 806R were used to amplify the V4 hypervariable region of the bacterial 16S rRNA gene. The NovaSeq6000 platform (Illumina Inc., San Diego, CA, USA) was used to conduct the sequence by a commercial service in Servicebio Technology Co., Ltd. (Wuhan, China). The raw sequence data were changed, processed, and qualified to analyze the operational taxonomic unit (OTU) data using QIIME (v1.9.1) following the methods described previously [25].

2.4.2. Metabolomics Analysis

The lyophilized samples were dissolved in methanol: water solution (8:2, vol/vol), vibrated, centrifuged (20,000× *g*, 4 °C, 10 min), and filtered (0.22 µm filter) to collect the supernatants. Then, the supernatants were subjected to the UPLC-ESI-Q-Orbitrap MS/MS (ultra-performance liquid chromatography-electrospray ionization-Q–Orbitrap-tandem mass spectrometry) platform using a commercial service in Servicebio Technology Co., Ltd. (Wuhan, China). The supernatant (10 µL) was injected into a UPLC system (Vanquish binary pump H system, auto-sampler, RP-C18 column: 150 × 2.1 mm, 1.8 µm particle size) with column temperature at 35 °C and flow rate of 0.3 mL/min. The mobile phase consisted of solvent A (0.1% formic acid in water, vol/vol) and solvent B (0.1% formic acid in acetonitrile, vol/vol) was run as follows: 0 to 1 min, 2% B; 1 to 5 min, 2% to 20% B; 5 to 10 min, 20% to 50% B; 10 to15 min, 50% to 80% B; 15 to 20 min, 80% to 95% B; 20 to 25 min, 80% to 95% B; 25 to 26 min, 95% to 2% B; 26 to 30 min, 2% B. The MS data were collected using an Orbitrap system, which was equipped with a triple-quadrupole mass spectrometer and HESI-II probe. Mass spectra were acquired over the range of 100 to 1500 m/z. MS parameters were: sheath gas temperature at 350 °C; capillary temperatures at 300 °C; and spray voltage of 3.8 kV in positive mode. The collected raw data were pre-processed by the CD2.1 (Thermo Fisher), and then checked and compared in the mzCloud, mzVault, and ChemSpider database. The Venn plot and volcano plot were made using R software.

2.5. Dietary Effects of Fermented B. papyrifera on Intestinal Health of Laying Hens

The animal experiment was conducted according to the regulations of Animal Care and Use Committee of Institute of Subtropical Agriculture at the Chinese Academy of Science (No.ISA-2020-18). A total of 288 healthy 23-day-old Hy-Line brown laying hens were used in the feeding trial. The birds were randomly allocated to 3 dietary treatments feeding with a basal diet (Con), basal diet supplemented with 1% (wt/wt) FBP (FBP1), and basal diet supplemented with 5% (w/w) (FBP5). Each dietary treatment had 8 replicates, and each replicate included 12 birds placed in a 3-layer complete wire cage (384 m^3) with free access to water and feed. The basal diet was mainly composed of corn and soybean meals and formulated to meet the nutritional requirements of the laying hens (National Research Council, 1994). The feeding trial lasted 64 d, including a 7 d pre-adaptation period and 54 d of formal test. The feeding trial was conducted at 28 ± 2 °C, under a relative humidity (40% to 60%), and a 16 h:8 h light–dark cycle. At the end of the trial, 1 bird per cage (8 birds per dietary treatment) was randomly selected and sacrificed for further study.

2.5.1. Serum Antioxidant Activity

Blood samples (5 mL) were taken from the wing vein of the hens and centrifuged (4 °C, 3000× *g*, 10 min) to obtain serum samples for subsequent analysis of total antioxidant capacity (T-AOC), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activities, using commercial kits (Suzhou Keming Biotech Co., Ltd., Suzhou, China) according to the manufacturer's instructions.

2.5.2. Intestinal Morphology

The entire small intestine of the birds was dissected out, and samples (2 cm in length) were taken from the middle portion of the duodenum, jejunum, and ileum, washed gently with sterile saline solution, and fixed in 4% neutral-buffered formalin (Wuhan Seville Biotech Co., Ltd.). A standard H&E (hematoxylin and eosin) staining was conducted to observe the morphological changes in the small intestine. A light microscope with an image analyzer (VistarImage) was used to measure the villus length (VL), and crypt depth (CD), and calculate the VL-to-CD ratio. VL was measured from the tip of the villus to the villus–crypt junction, and the crypt depth was defined as the depth of the invagination between adjacent villi [26].

2.5.3. Colonic Microbiota

The collected mucus from the colonic section was used for intestinal bacterial community analysis. The genomic DNA was extracted using CTAB (cetyl trimethyl ammonium bromide) extraction buffer. The sequencing and data analysis was conducted following the procedures described in the Section 2.4.2.

2.5.4. Ileum Antioxidant-, Immune-, and Intestinal Barrier-Related Gene Expression

Total RNA was extracted from intestinal mucosal tissue by using a Trizol Up Reagent (Beijing Trans Gen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instruction. The purity and concentration of total RNA were determined by spectrophotometer detection (Nanodrop 2000, Thermo Scientific, Hercules, CA, USA). The concentration and integrity of the RNA were analyzed using 1% agarose gel electrophoresis. The total RNA (1 μg) was reverse transcribed into cDNA using Prime Script RT Reagent Kits (TaKaRa, Dalian, China). The gene expression was conducted by real-time quantitative PCR using 2×Q3 SYBR qPCR Master Mix (Universal) reagents (Shanghai Tolo Biotech Co., Ltd., Shanghai, China) and Bio-Rad CFX-96TM Real-Time PCR Detection System (Bio-Rad, Laboratories, Inc., USA). The PCR conditions as follows: 95 °C for 30 s, followed by 40 cycles of 5 s at 95 °C and 20 s at 60 °C. The information of the primers used in the study is shown in Table S4. Housekeeping gene β-actin was selected as the internal reference gene. Additionally, the relative expression levels of the target gene were calculated using the

$2^{-\Delta\Delta ct}$ method. The calculation formula of ΔΔCt was as follows: ΔΔCt = ($Ct_{target} - Ct_{\beta\text{-Actin}}$) treatment − ($Ct_{target} - Ct_{\beta\text{-Actin}}$) control.

2.6. Statistical Analysis

The data from the in vitro experiments were analyzed by independent Student's *t*-test at 5% significant level. Of these, the antibacterial and antioxidant results under the sterile condition were compared between 0YB and 5YB, in terms of different items among the samples taken from 0, 4 and 7 d. The results from practical conditions were compared between the CBP and FBP. The data recorded from in vivo evaluation of the dietary supplementation of the FBP on laying hens were subjected to one-way analysis of variance (ANOVA) followed by Duncan's multiple range test at 5% significant level. All the data were analyzed using SPSS (version 24.0, USA) and all the figures were made by GraphPad Prism 7.

3. Results

3.1. Suitable Fermentative Microbe for B. papyrifera Fermentation

Although all the selected strains were able to grow well in the liquid BP culture, Lp exhibited the fastest growth and highest viable cell count (9.6 ± 0.6 log10 CFU/mL) after 6 h of incubation (Figure 1A). Meanwhile, the pH of the Lp-inoculated BP medium dropped quicker than the pH in the Bv- or Sc-inoculated BP media (pH 4.3 ± 0.1) (Figure 1B). Reducing sugar content in the Lp-inoculated BP medium decreased after 6 h of incubation and reached a plateau thereafter. In the Bv-inoculated BP medium, however, reducing sugar level sharply increased up to 6 h of incubation, and thereafter, it gradually decreased (Figure 1C). After an initial sharp decline, protein concentration gradually recovered after 6 h and 12 h in the Bv- or Lp-inoculated BP medium, respectively (Figure 1D). Based on these results, Lp was selected as the suitable strain for BP fermentation.

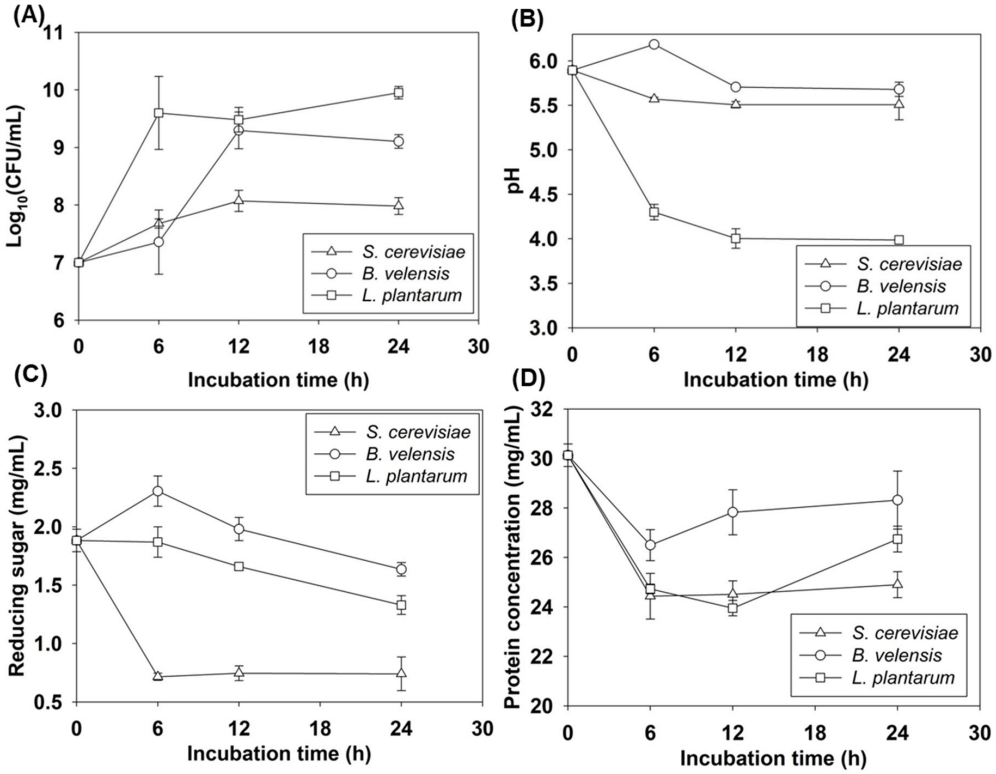

Figure 1. Fermentation performance of *S. cerevisiae*, *L. plantarum*, and *B. velensis* in the sterile liquid *B. papyrifera* (10%, wt/vol) culture. (**A**) Growth ability, (**B**) pH change, (**C**) reducing sugar content, (**D**) protein concentration.

3.2. Effects of Yeast By-Product (YB) Supplementation on B. papyrifera Fermentation

Supplementation of the solid-state Lp-fermented *B. papyrifera* medium with 5% (w/w) YB improved Lp cell growth after 4 d, and it enhanced the protein concentration in the culture medium (Figure 2). After 4 d of incubation, the NDF content of the YB supplemented BP culture medium was significantly lower than those values recorded in the un-supplemented medium up to 4 d of incubation (Table S1). After 7 d of incubation, YB-supplemented medium exhibited significantly higher inhibitory effect against *S. Typhimurium*. However, the presence of YB in the solid-state Lp-fermented *B. papyrifera* medium significantly decreased its antibacterial effect against *S. aureus* and *P. aeruginosa* PAO1 after 7 d of incubation (Figure 3A). While YB addition did not cause a significant change in DPPH and ABTS radical scavenging and in FRAP activities at both 4 d and 7 d, respectively, it increased and decreased the TFC at 4 d and 7 d of incubation, respectively (Figure 3B).

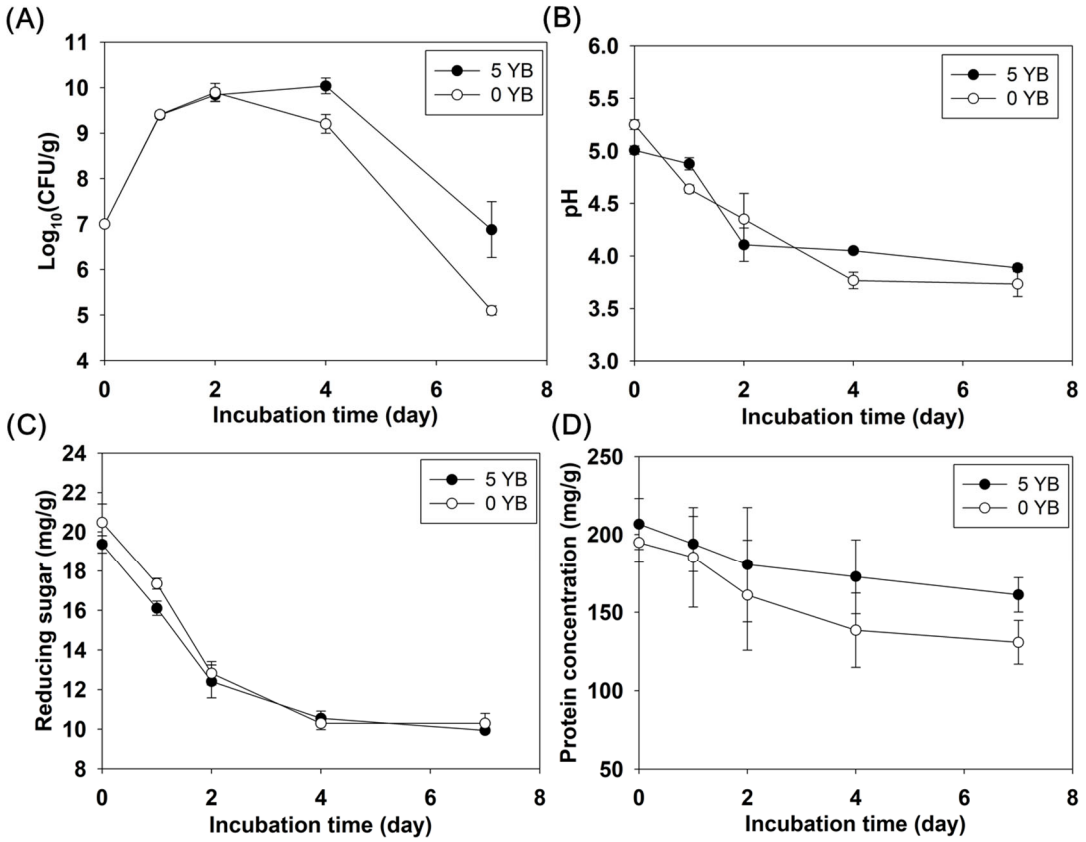

Figure 2. General characteristics of the sterile solid-state Lp-fermented *B. papyrifera* with (5YB) or without (0YB) 5% (wt/vol) yeast by-product supplementation. (**A**) Growth ability, (**B**) pH change, (**C**) reducing sugar content, and (**D**) protein concentration.

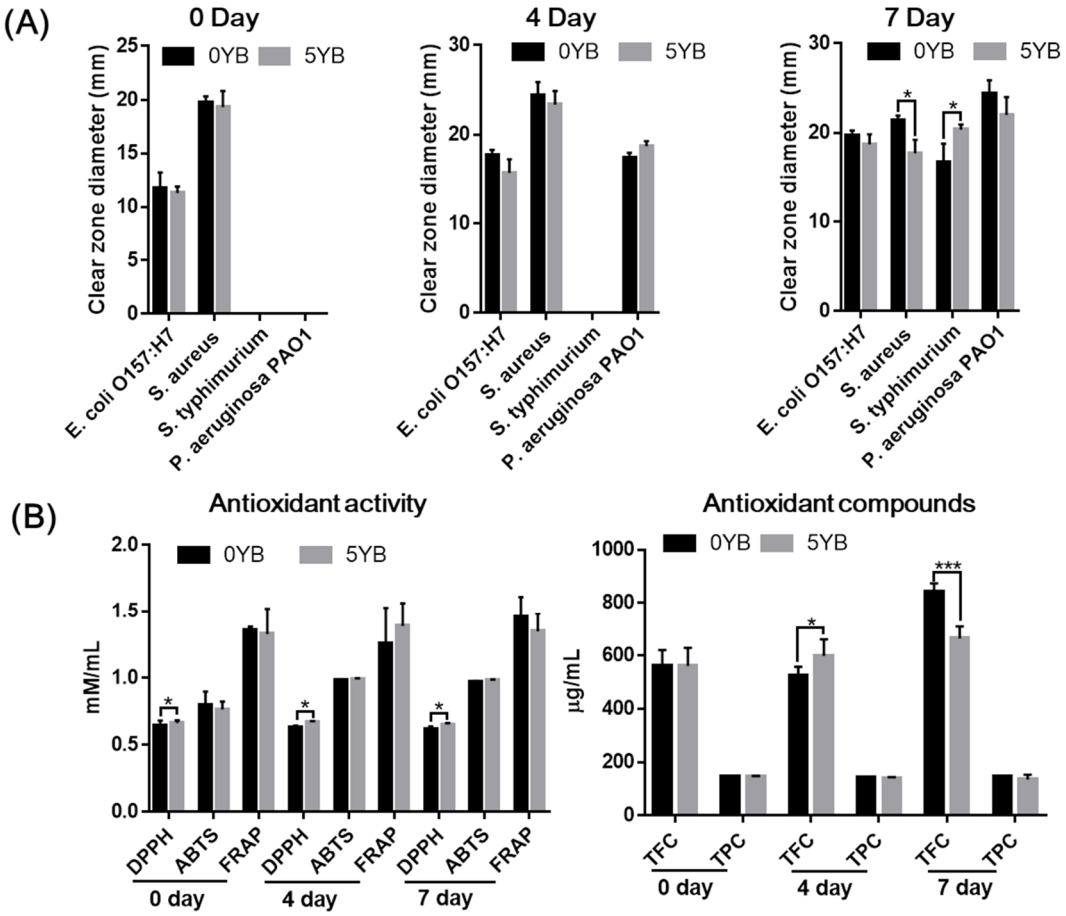

Figure 3. Antibacterial and antioxidant properties of the sterile solid-state Lp-fermented *B. papyrifera* (FBP) with (5YB) or without (0YB) 5% (w/w) yeast by-product supplementation. (**A**) Antibacterial activity of the FBP collected at 0 d, 4 d and 7 d; (**B**) Total antioxidant activity and antioxidant compound contents of the FBP collected at 0 d, 4 d and 7 d. * indicates significant difference between the two groups at $p < 0.05$. *** indicates significant difference between the two groups at $p < 0.001$. DPPH: 2,2-Diphenyl-1-picrylhydrazyl, ABTS: 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), and FRAP: ferric-reducing antioxidant power, TFC: total flavonoid content, TPC: total polyphenol content.

3.3. Characterization of the L. plantarum-Fermented B. papyrifera

The general, chemical, and functional properties of the FBP under unsterile conditions are presented in Table 1. After fermentation, pH, reducing sugar content, CF and DPPH values were significantly reduced, while the microbial cell count, free protein content, CP, antioxidant capacity in terms of ABTS, FRAP TFC, and TPC as well as the antibacterial activities were notably increased. All the samples showed good coverage of bacterial sequences (>0.99), indicating adequate sequencing depth was conducted. Five α-diversity indexes were used, and among these, ACE, Chao1, and Observed_species are abundance-based estimators of species richness, while Shannon and Simpson are species richness and evenness estimators. Overall, a total of 194 to 560 OTUs were detected, and α-diversity tended to decrease after fermentation process (Figure S2). The 16S rDNA sequencing data revealed that Cyanobacteria, Firmicutes and Proteobacteria phyla with *Cyanobacteria*, *Lactobacillus*, and *Sphigomonas* genera were the most predominant in BP materials. At the phylum level, fermentation significantly increased the relative abundance of Firmicutes and Proteobacteria phyla, while it decreased the Cyanobacteria abundance (Figure 4A). At the genus level, *Lactobacillus*, *Sphigomonas*, *Methylobacterium*, *Rhizobiaceae*, *Aureimonas*, *Cronobacter*, *Acinetobacter*, and *Massilia* were enriched accompanying with the decreased abundance of

Cyanobacteria within the FBP as compared with those within the CBP (Figure 4B). The Venn diagram analysis showed 97 core species in all samples, more differentially clustered bacterial species were presented in the CBP (Figure 4C). Based on the PCoA plot, the microbial communities were relatively clustered between the FBP and CBP, and the largest variation was observed in the bacterial diversity of the FBP (Figure 4D). The metabolites were varied in response to the change in bacterial community during BP fermentation. The Venn diagram revealed a total of 861 identified compounds including 726 shared compounds, 32 and 103 unique compounds in the CBP and FBP samples, respectively (Figure 5A). The volcano plot displayed 202 differentially changed compounds during fermentation, comprising 175 up-regulated and 27 down-regulated compounds in the FBP compared to those in the CBP (Figure 5B). Among the changed compounds, the top 24 dominant metabolites are listed in the high-to-low concentration order, based on the mzCloud > 60 (Table 2). The polyphenol compounds including apigenin, luteolin, diosmetin, caffeic acid, quercetin, and taxifolin, were increased in the FBP.

Table 1. Characterization of the raw (CBP) and the Lp-fermented *B. papyrifera* product (FBP) under practical condition.

Characterization	Items [1]	CBP	FBP
General properties	pH	6.7 ± 0.0 [a]	4.6 ± 0.0 [b]
	LAB cell count, log10 CFU/g	5.6 ± 0.1 [b]	6.9 ± 0.7 [b]
	Reducing sugar, mg/g	1.5 ± 0.1 [a]	0.71 ± 0.0 [b]
	Protein concentration, mg/g	3.1 ± 0.4 [a]	8.5 ± 1.0 [b]
Proximate composition	DM, %	47.9 ± 0.8	45.7 ± 4.9
	NDF, %DM	42.2 ± 1.4	39.8 ± 5.8
	CP, %DM	18.4 ± 0.1 [a]	18.8 ± 0.2 [b]
	CF, %DM	9.8 ± 0.6 [a]	8.7 ± 0.5 [b]
	Ash, %DM	13.8 ± 0.3	14.2 ± 0.3
	TE, kJ	16.1 ± 0.5	16.7 ± 0.2
Total antioxidant activity	DPPH assay, mmol/L AAE/BPME mL	0.71 ± 0.02 [a]	0.67 ± 0.01 [b]
	ABTS assay, mmol/L TE/BPME mL	0.84 ± 0.04 [a]	0.95 ± 0.01 [b]
	FRAP assay, mmol/L $Fe2^{+}$/BPME mL	0.83 ± 0.22 [a]	1.34 ± 0.05 [b]
Antioxidant compound contents	TFC, QE μg/mL BPME	363.8 ± 46.2 [a]	703.8 ± 68.6 [b]
	TPC, GAE μg/mL BPME	139.1 ± 2.7 [a]	144.4 ± 1.2 [b]
Antibacterial activity (clear zone diameter: mm)	*E. coli* O157:H7	0.0 ± 0.0 [a]	21.0 ± 1.0 [b]
	P. aeruginosa PAO1	0.0 ± 0.0 [a]	20.0 ± 0.0 [b]
	S. aureus	19.0 ± 1.0 [a]	27.3 ± 0.6 [b]
	S. typhimurium	0.0 ± 0.0 [a]	21.7 ± 0.6 [b]

[1] Items: DPPH = 2,2-diphenyl-1-picrylhydrazyl; FRAP = ferric-reducing activity power; ABTS = 2, 2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AAE = ascorbic acid equivalent; TE = trolox equivalent; BPME = *B. papyrifera* methanolic equivalent; TFC = total flavonoid content; TPC = total polyphenol content; QE = quercetin equivalent; GAE = gallic acid equivalent. The data within a row with superscript letters mean significant difference ($p < 0.05$).

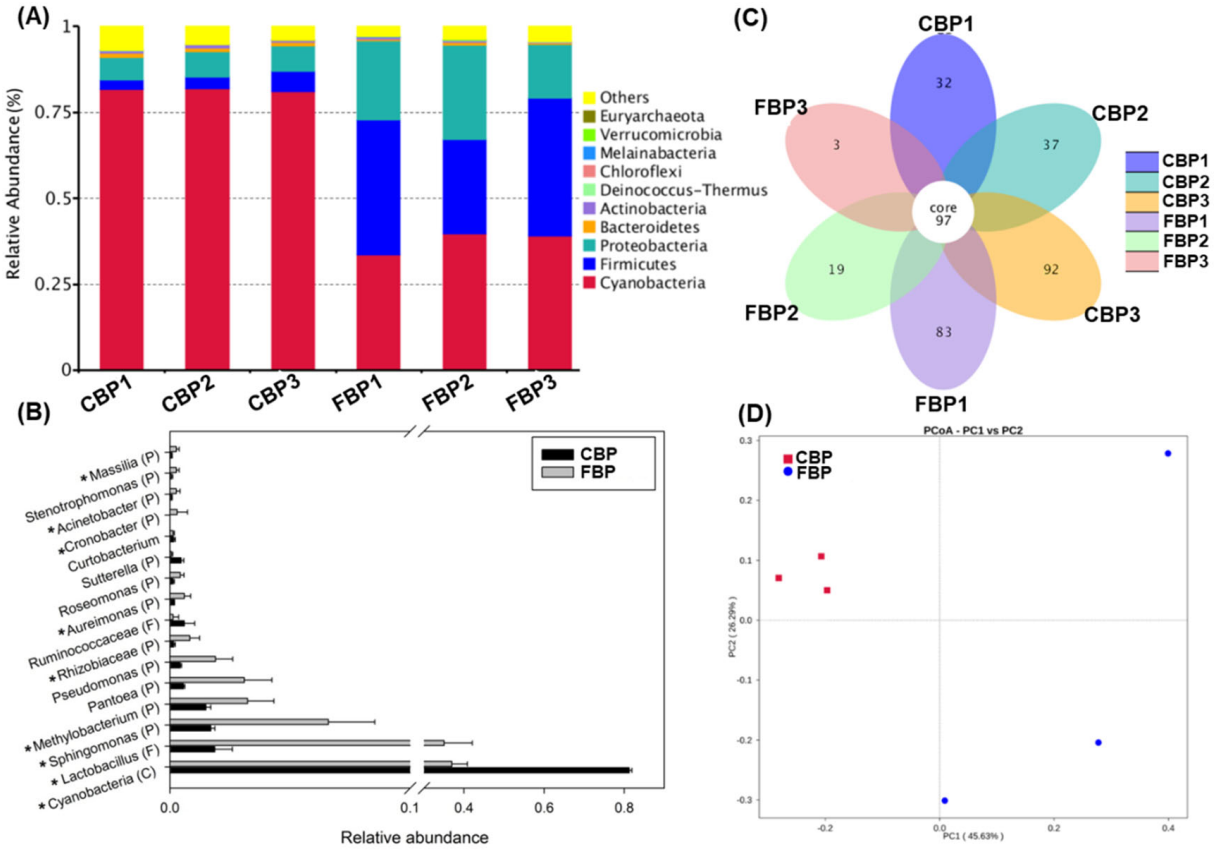

Figure 4. Bacterial community structure and composition in the raw *B. papyrifera* (CBP) and the Lp−fermented *B. papyrifera* product (FBP) under practical condition. (**A**) Relative abundance at phylum level, (**B**) flower diagram, (**C**) dominant genus, and (**D**) PCA plot. * indicates significant difference between the two groups at $p < 0.05$.

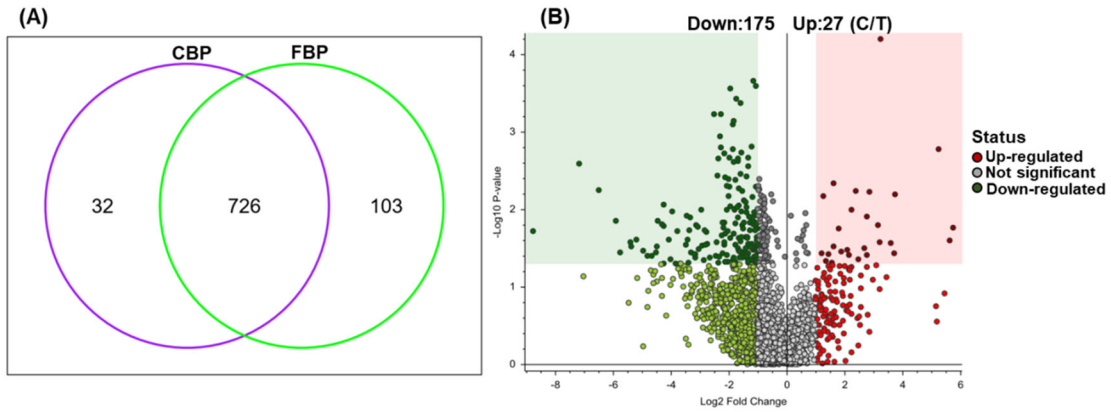

Figure 5. Metabolite contents of the raw *B. papyrifera* (CBP) and the Lp−fermented *B. papyrifera* product (FBP) under practical condition. (**A**) Venn diagram and (**B**) volcano plots (CBP vs. FBP).

Table 2. Twenty-four significantly discriminant metabolites of the Lp-fermented *B. papyrifera* product (FBP) under practical condition based on mzCloud > 60.

No.	Tentatively Identified Metabolites	MW	RT [min]	FC [T/C]	Class of Compounds
1	Apigenin	270	11.77	3.34	Flavone
2	Luteolin	286	10.95	4.26	Flavonoid
3	Diosmetin	300	11.95	3.5	Flavonoid
4	16-Hydroxyhexadecanoic acid	272	18.98	3.17	Fatty acid
5	Acetylcholine	145	1.24	7.17	Vitamin
6	Caffeic acid	180	7.86	6.49	Phenolic acid
7	Quercetin	302	10.99	3.14	Flavonol
8	Leucylproline	228	5.45	5.36	Amino acid
9	4-Hydroxybenzaldehyde	122	12.30	2.04	Aldehyde
10	5-Hydroxymethyl-2-furaldehyde	126	1.47	-1.46	Aldehyde
11	Alternariolmethylether	272	11.45	2.77	Ether
12	Taxifolin	304	9.31	2.75	Flavonoid
13	3-Methyl-5-(5,5,8a-trimethyl-2-methylene-7-oxodecahydro-1-naphthalenyl)pentyl acetate	348	20.05	2.35	Ester
14	DL-Arginine	174	1.18	2.27	Amino acid
15	L-Ascorbic acid 2-sulfate	256	2.63	4.46	Vitamin
16	1-Linoleoyl glycerol	354	15.20	−3.74	Coumestan
17	Cytosine	111	1.22	2.91	Nucleotide
18	3-Amino-2-naphthoic acid	187	8.50	3.45	Fatty acid
20	Emodin	270	10.60	2.86	Anthraquinone
21	10-Propoxydecanoic acid	230	15.66	1.06	Fatty acid
22	DL-Lysine	146	1.08	2.16	Amino acid
23	N8-Acetylspermidine	187	1.16	2.82	Amine
24	Docosahexaenoic acid (DHA)	328	16.55	1.14	Fatty acid

BP: *B. papyrifera*; Lp: *L. plantarum*; YB: yeast by-product powder; MW: molecular weight; RT: room temperature; FC: fold change.

3.4. Dietary Effects of Fermented B. papyrifera on Intestinal Health of Laying Hens

The effect of dietary FBP supplementation on laying hens' serum antioxidant capacity is shown in Figure 6. Dietary supplementation of FBP significantly enhanced serum SOD activity and produced the highest values recorded in laying hens fed an FBP5 diet. Dietary treatment, however, had no significant effect on serum GSH-Px and CAT activities and T-AOC, as compared to those values recorded in the control group. Dietary FBP supplementation had no remarkable impact on intestinal morphology (Figure S3). Similarly, the supplementation of FBP did not alter the α-diversity (Figure 7A) and PCoA-based β-diversity (Figure 7B). A total of 643 shared OTU were detected in the three groups, in which the FBP1 group has the highest unique OTU 259 (Figure 7C). At the phylum level, Bacteroidota and Firmicutes were the most dominant, and no significant changes were detected among the three groups (Figure 7D). At the genus level, *Bacteroides barnesiae* was the most dominant and was significantly reduced with supplementation of the FBP1 diet (Figure 7E). The effects of dietary FBP supplementation on the relative expression of the antioxidant, inflammation, and barrier function-related genes in the ileum are shown in Figure 8. Dietary FBP supplementation significantly lowered the gene expression of AhR, mucin2, and ZO-2, and occludin. However, the relative expression of the antioxidant and immune-related genes, including Nrf2, IgA, TNF-α, and IL-6, were not affected by the dietary treatment.

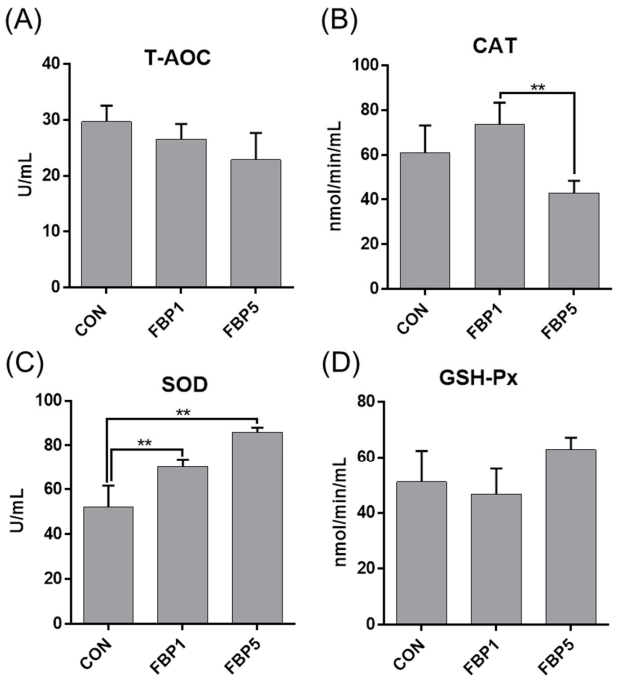

Figure 6. Dietary effects of the Lp-fermented *B. papyrifera* product (FBP) under practical condition on serum antioxidant of laying hens. (**A**) Total antioxidant capacity (T-AOC), (**B**) catalase activity, (**C**) superoxide oxidase (SOD), and (**D**) glutathione peroxidase (GSH-Px). Con: the hens fed with basal diet; FBP1, the hens fed with 1% (wt/wt) FBP product in basal diet; FBP5, the hens fed with 5% (wt/wt) FBP product in basal diet. ** indicates significant difference among the treatments at $p < 0.01$ level.

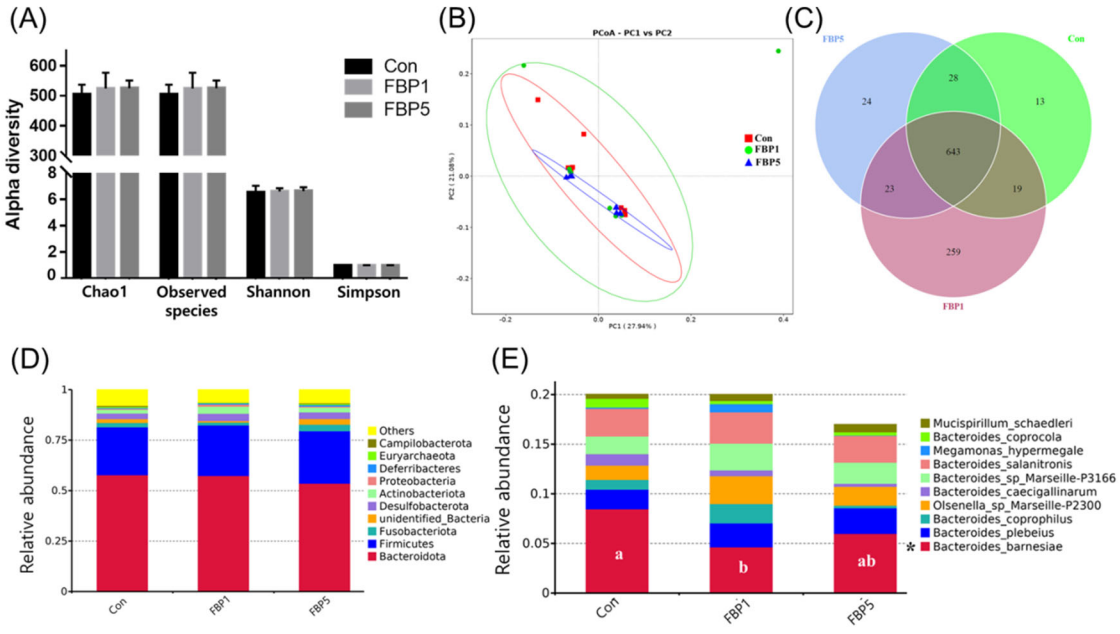

Figure 7. Dietary effects of the Lp−fermented *B. papyrifera* product (FBP) under practical condition on gut microbiota of laying hens. (**A**) Alpha diversity, (**B**) PcoA plot, (**C**) Venn diagram, (**D**) dominant phylum, (**E**) top 10 dominant species. Con: the hens fed with basal diet; FBP1, the hens fed with 1% (wt/wt) FBP product in basal diet; FBP5, the hens fed with 5% (wt/wt) FBP product in basal diet. * and letters indicate significant difference among the treatments at $p < 0.05$ level.

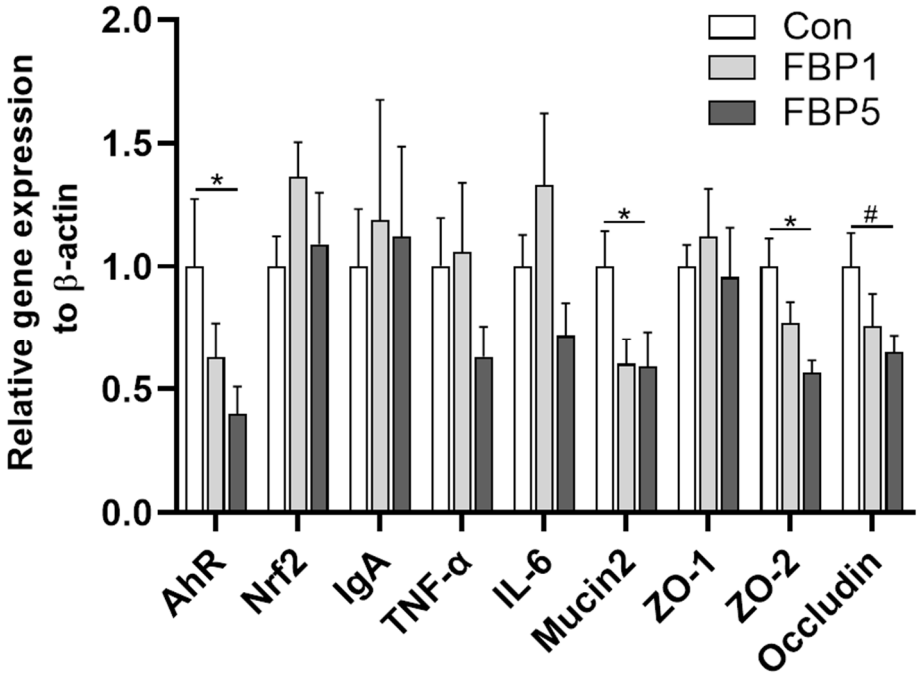

Figure 8. Dietary effects of the Lp-fermented *B. papyrifera* product (FBP) under practical condition on the antioxidant, barrier function, and immune related gene expression in ileum of laying hens. Con: the hens fed with basal diet; FBP1, the hens fed with 1% (wt/wt) FBP product in basal diet; FBP5, the hens fed with 5% (wt/wt) FBP product in basal diet. * indicates significant difference among the treatments at $p < 0.05$ level; # indicates significant difference among the treatments at $0.05 < p < 0.10$ level.

4. Discussion

There are several factors that can affect the quality of fermented products and, subsequently, its impact on farm animals when use as a dietary ingredient. *Lactobacillus*, *Bacillus*, and yeast species are the most commonly used inocula to improve the quality of fermented products. In the present study, three different probiotic strains, including Lp, Sc, and Bv, were evaluated for their ability to ferment BP. BP is a good source of protein (18% to 23%) with a balanced amino acid profile, minerals, and WSC (8% to 19%) [5,27], making it a good candidate for commercial silage production. Indeed, the protein and WSC contents of the BP appears to satisfy the nutrient requirements of all the three tested probiotic strains, and they exhibited acceptable growth using BP as the primary source of nutrient. However, judging by the number of viable cells and drop in pH value, Lp was selected as the suitable fermentative agent for BP fermentation. BP is usually compared with alfalfa (*Medicago sativa*), a high-quality forage crop for ruminants, and shows a similar or even higher nutritional value [28]. Alfalfa is used to ensile for making silage using fresh materials. An earlier study has reported nutritive characters in 28 days *L. plantarum*-fermented alfalfa silage: pH 4.94, dry matter content, 36%; crude protein, 17% [29]. These results are relatively lower when compared to the corresponding values in the present study. Nutritional additives, such as grains and molasses, are widely used to promote the fermentation quality in fermented products by partially providing fermentable substrates and/or absorbing excessive moisture to direct the course of fermentation [30]. High nutritional content, good water adsorption ability, low cost, as well as marketing facilities, easy transportation and storage, are among the chief advantages possessed by YB, making it a promising candidate as a nutritional additive for fermented products. Moreover, YB is a rich source of polysaccharides, proteins, lignosulfonate, mannitol, calcium sulfate, propylene glycol, sodium alginate, and humic substances. Dietary supplementation of humic acid in poultry feed has been shown to have a positive impact on egg weight and production of laying hens [31]. The results of the present study indicated that 5% YB supplementation could improve the quality of the fermented BP.

The BP was also found to be a rich source of polysaccharides, exhibiting antibacterial activity against *E. coli*, *S. aureus*, and *P. aeruginosa* [14]. The papyriflavonol A, a flavonoid found in BP, shows a broad-spectrum antimicrobial activity against *Candida albicans*, *E. coli*, *S. typhimurium*, and *S. aureus* [32]. Different levels of total polyphenol and flavonoid were detected in the ethanolic extract of the different part of BP plant, with the root extract displaying the strong quenching activity on DPPH and ABTS radicals [16]. The polysaccharide extracts from BP fruits also presented DPPH scavenging and ferric-reducing activities [14]. The hydrolysis and synthesis reactions are suggested to be closely related to the dynamic change in the content of antioxidant compounds and antioxidant capacities detected in plants [33]. Indeed, a wide range of metabolites were produced during fermentation, including lactic acid, organic acid, peptide, polyphenols, flavonoids, which could be attributed to the improved antimicrobial activities in fermented plant products. Metagenomic genome-based analysis in *L. plantarum*-fermented alfalfa showed up-regulated gene abundance and diversity coding for carbohydrate-active enzymes, promoted growth of beneficial lactic acid bacteria and inhibited undesirable microbes which exert the improved quality of the ultimate silage [34]. Overall, we found that the FBP had better functional activities than the raw BP, which could mainly be ascribed to the enriched active compounds after fermentation.

Fermented silage products are usually produced in often open and typically unsterile environments. The external additives and fermentation process can largely alter the original microbial composition of the raw fermentable ingredients, which in turn could impact the quality of the final silage product. A sharp decrease in microbial diversity of BP was observed throughout the time of fermentation [5]. The results of the present study are in accordance with Yang et al., who reported that the fermentation of the alfalfa with Lp reduced the bacterial diversity of the resultant silage product [35]. The lower diversity was closely related to the decreased pH caused by the production of organic acids during fermentation, which inhibited the growth of some microbes [36]. During silage fermentation, the relative abundance of Cyanobacteria significantly decreased with a concomitant increase in Firmicutes and Proteobacteria abundance, as has been previously reported [11]. In an earlier study, Cyanobacteria was the most abundant phylum in the typical woody forages including paper mulberry (*Broussonetia papyrifera*) and mulberry (*Morus alba*), with *Sphingomonas*, *Pseudomonas*, *Pantoea*, and *Acinetobacter* being the dominant genera in mulberry materials [11,27]. Cyanobacteria can produce microcystin which has a potential to inhibit certain key regulatory enzymes, causing an overwhelming antioxidant activity, which in turn could induce cell death [37]. Therefore, the observed decrease in Cyanobacteria abundance, during fermentation, may imply that the resultant FBP has little or limited adverse physiological and/or health consequences when used as a dietary supplement in farmed animal feed. *Sphingomonas* is a growth-promoting endophyte bacterium that aids the plants to resist salinity stress [38]. *Methylobacterium* is aerobic, neutrophilic, and common endosymbiotic bacteria in plants, which is positively correlated with silage pH [39]. It is likely that *Sphingomonas* and *Methylobacterium* are autochthonous bacteria in BP materials. The slightly enriched *Acinetobacter* may contribute to the increase in the acetate content of the FBP, which is consistent with a previous study [40]. Raw BP has a wide range of polyphenols such as caffeic acid, quercetin, coumaric acid, and broussoflavonol A and B, endowing its antioxidant, antibacterial, and anti-inflammatory activities [16,19,41]. *Lactobacillus* spp. are known to produce diverse phenolic hydrolyzing enzymes such as glycosyl hydrolase, cinnamoyl esterase, tannin acyl hydrolase, decarboxylase, phenolic acid decarboxylase, and reductase, which are able to break complex ingredients into small molecules, thereby improving their functional properties [42]. The fermentation process enriched the 16-hydroxyhexadecanoic, docosahexaenoic, 10-propoxydecanoic, and 3-amino-2-naphthoic fatty acids contents with a decreased level of 1-linoleoyl glycerol in the fermented BP. As an explanation for this phenomenon is elusive, herein, we speculate that the glycerol components were converted to fatty acids during fermentation in response to the reduction in crude fat. In addition, lysine, as the first essential amino acid, was also increased. The

enrichment of the active metabolites in the FBP is closely related to the alteration of the bacterial community during the fermentation process. Fermentation is likely to enrich active compounds in BP, which might be closely related to the enhanced functionality.

As a roughage resource, BP has already been applied in ruminants, and the BP silage enhanced the immunity and antioxidant capacity of dairy cows [2]. Apart from the positive aspects, it is also very important to assess the health risks when first introducing a new functional feed stuff into animal diets. Until now, very few studies have reported the supplementary effects of FBP on the intestinal health of laying hens. In the present study, FBP supplementation improved serum SOD activity of the hens. Similar results have been reported with increased serum CAT, SOD, and TAC when feeding 15% (w/w) *B. papyrifera* silage to dairy cattle [2]. Oxidative stress caused by environmental and nutritional factors threatens the health of laying hens in the commercial pens. The SOD, as the first antioxidant defense line, catalyzes the conversion of the superoxide radical to the hydrogen peroxide. The gastrointestinal tract (GI) is a major organ involved in digestion, absorption, and overall health of the host. Our results showed that the dietary supplementation of FBP5 had no negative effect on intestinal structure of the laying hens, suggesting the safe use of the FBP product as a dietary ingredient. The absence of any major alterations in the gut microbiota composition may also suggest that the dietary supplementation of the FBP may not disturb the intestinal microbiota balance of the hens. We further investigated the ileum gene expression related to antioxidant, immune, and barrier function with dietary FBP supplementation. Aryl hydrocarbon receptor (AhR) is a transcription factor that modulates xenobiotic metabolism via cytosolic ligand-binding, involving immunity, inflammation, and barrier function [43,44]. Nuclear factor erythroid 2-related factor 2 (Nrf2) is also a critical transcription factor that mediates antioxidant-reactive element (ARE), and in turn regulates the expression of antioxidant phase II detoxifying enzymes [45]. The AhR-Nrf2-dependent pathway plays important roles in regulating epithelial tight junction proteins [46]. It has been reported that quercetin and apigenin could act as indirect AhR agonists to attenuate inflammation [43]. The decrease in gene expression regarding AhR, Mucin2, ZO-2, and occluding implies that FBP may cause potential risks on the gut barrier functions of hens. A more comprehensive study will be conducted to assess the effects of dietary supplementation of FBP on laying performance, egg quality, and health status of laying hens.

5. Conclusions

In conclusion, BP is able to support the growth of different inoculants showing the potential to make multi-purpose fermented products. YB as a novel additive was firstly included in fermented BP at 5%, and it enhanced the growth of a dedicated Lp inoculant. In a practical Lp and YB-added FBP, higher cell count, protein content, antibacterial and antioxidant activities with raised levels of bioactive metabolites, coupled with the changed bacterial community, were observed. It suggests that dried BP is also a good resource to make fermented products with nutritional and functional properties by the inclusion of *L. plantarum* and yeast culture by-products. In an in vivo trial, the dietary FBP supplementation did not alter the intestinal morphology and microbiota-associated gut health of hens, but changed the gut barrier-related gene expression. As far as we know, this is the first report to systematically investigate the functionality and bacterial community of *L. plantarum*-fermented BP with a newly introduced additive, as well as its supplementary effects on the gut health of laying hens.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8100547/s1, Figure S1: Effects of incremental levels of yeast by-product supplementation on the growth and pH of the sterile solid-state Lp− (A) and Bv− (B) fermented *B. papyrifera*; Figure S2: Alpha diversity in raw (CBP) and Lp−fermented *B. papyrifera* product (FBP) under practical condition; Figure S3: Dietary effects of Lp−fermented *B. papyrifera* (FBP) on intestinal morphology of laying hens. Con, the hens fed with basal diet; FBP1, the hens fed with 1% (wt/wt) FBP product in basal diet; FBP5, the hens fed with 5% (wt/wt) FBP

product in basal diet; Figure S4: Dietary effects of Lp−fermented *B. papyrifera* (FBP) product on villus length, crypt depth and villus length-to-crypt depth ratio of laying hens. Con, the hens fed with basal diet; FBP1, the hens fed with 1% (wt/wt) FBP product in basal diet; FBP5, the hens fed with 5% (wt/wt) FBP product in basal diet; Table S1: Effects of yeast by-product supplementation on proximate composition of the sterile solid-state Lp−fermented *B. papyrifera*; Table S2: Primers used in the present study.

Author Contributions: Conceptualization, K.N. and L.C.; data curation, Y.W.; formal analysis, Y.W., Z.Z. and R.W.; funding acquisition, X.W.; investigation, J.L. (Jianping Liu), L.C. and J.L. (Jianxi Li); methodology, J.L. (Jianping Liu); project administration, X.W.; resources, L.D.; supervision, X.W.; validation, J.L. (Jianxi Li); visualization, L.D.; writing—original draft, K.N.; writing—review and editing, K.N. and S.K. All authors have read and agreed to the published version of the manuscript.

Funding: This work was financially supported by National key R&D program of China (2021YFD1301002), Jiangxi Provincial Innovation and Entrepreneurship Projects, Key research projects of Jiangxi Academy of Sciences (2018-YZD2-17), and Key Research Projects (2019-XTPH1-03) and Doctoral Fund Project (2019-YYB-03) of Jiangxi Academy of Sciences.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the first author. The data are not publicly available due to restrictions by the research group.

Conflicts of Interest: The authors declare no conflict of interest.

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