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Influence of Fermented Mulberry Leaves as an Alternative Animal Feed Source on Product Performance and Gut Microbiome in Pigs

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Abstract: Mulberry leaves are rich in nutrients but contain anti-nutrient factors that hinder their digestion and absorption. Feeding animals with mulberry leaves directly could harm their health. The microbial fermentation of mulberry leaves could reduce their anti-nutritional factors' content and improve their nutritional value. Sequencing and analyzing mulberry leaves before and after fermentation showed that fermentation increased the relative abundance of *Pediococcus*, *Bradyrhizobium*, *Hydrotalea*, and *Rhodanobacteria*, and decreased that of *Enterobacter*. Fermentation improved the quality of mulberry leaves by rebuilding the bacterial community. Finishing pigs were raised on fermented mulberry leaves (FML), and their carcass performance, meat quality, economic benefits, and gut microbiome were evaluated. FML had no negative impact on pig carcass performance, meat quality, and antioxidant capacity, and could somewhat improve the economic benefits. FML decreased the relative abundance of *Proteobacteria* in the colon and *Streptococcus* in the feces, and increased that of Actinobacteria (cecum, colon, feces) and *Prevotella* (colon). The gut core microorganisms in the FML group were mainly enriched with Actinobacteria, *Bifidobacterium*, Bifidobacteriaceae, Bifidobacteriales, and other beneficial microorganisms. Dietary FML reduced ammonia, indole, and skatole contents in the feces. In conclusion, FML reshaped the gut microbiota without negatively affecting pig product performance, produced cleaner waste, and improved environmental protection and sustainability, making it an attractive prospective feed for pigs.

Keywords: fermented mulberry leaves; pig; microbiome; odorous component

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1. Introduction

Feed shortages and food competition between humans and livestock are expected to expand with the increasing demand for animal protein. Incorporating alternative feed ingredients into animal production could be a feasible strategy to ensure an effective feed supply and reduce rearing costs. Alternative feed resources can partially replace traditional ones, such as corn and soybean meal, to help alleviate feed shortages and reduce feeding

costs and the food conflict between humans and livestock. High-value and diversified utilization of unconventional feed resources is a clean production technology conducive to realizing low-carbon livestock farming [1].

Mulberry leaves (ML) are abundant in resources and rich in crude protein, amino acids, and trace elements, making them an important unconventional feed source [2,3]. The rich active substances in ML (polyphenols, polysaccharides, alkaloids, etc.) have various functions (antioxidants, immune regulation, anti-stress, and lipid-lowering) [3]. However, ML tannin, which is unconducive to nutrient digestion and absorption, could damage animal health and negatively impact breeding and production. Fermentation utilizes microorganisms to convert plant components into microbial proteins, active peptides, and other substances, degrading anti-nutritional factors and improving the nutritional value of raw materials [4]. We previously used microbial fermentation to reduce the tannin content in ML and improve their nutritional value [5]. However, the microbial community structure of fermented mulberry leaves (FML) remained unclear. Understanding microbial communities in fermented feed is key to identifying microorganisms suitable for fermented feed production; therefore, additional attention has been paid to the bacterial communities in FML.

ML are limited as a feed protein source for pigs. ML levels can reduce the carcass performance and meat yield of finishing pigs because ML contain many anti-nutritional factors (tannin, waxiness) [6–9]. Feed fermentation can effectively reduce anti-nutrient factors, improve intestinal morphology, and maintain balanced intestinal flora [10,11]. As an unconventional feed, FML have rarely been used in finishing pigs. Our previous studies have shown that feeding FML did not alter pig growth performance but improved plasma and urine metabolite contents [5]. Feeding unconventional feed might change animal product quality. However, it is unclear whether feeding FML will change pig product performance. Therefore, we conducted carcass performance and meat quality measurements. Gut flora is tightly bound to host health and is intensively affected by diet. FML are rich in microorganisms and nutrients and could benefit the animal by helping it digest nutrients. However, little is known about how FML affect the gut microbial community structure. We hypothesized that FML could be used to feed finishing pigs without negatively affecting carcass performance and meat quality, while improving gut bacterial community structure and feces odor emissions. Carcass performance, meat quality, and bacterial community were assessed to verify this assumption.

2. Materials and Methods

2.1. Fermented Mulberry Leaves

FML were prepared as reported in our previous publication [12]. Briefly, fresh ML were cut into 1–2 cm pieces and mixed evenly with bran at a mass ratio of 9:1. The mixture was placed in breathing bags (23 cm × 30 cm, 200 g substrate per bag) for fermentation or storage. In total, 20 such bags were prepared, of which 10 bags were stored at −20 °C without fermentation or bacterial solution. A 2% bacterial solution of *Pediococcus cellicola* and *Bacillus licheniformis* was added to the remaining 10 bags, which were fermented for 4 d at 25 °C. We randomly selected 6 bags per treatment for sequencing analysis. The nutritional components (dry matter) of FML included 26.88% crude protein, 2.14% ether extract, 14.65% crude fiber, 31.43% neutral detergent fiber, and 14.95% acidic detergent fiber [12].

2.2. Experimental Design, Animals, and Diets

Animal procedures and experiments were approved by the Animal Care and Use Committee of the Guangdong Academy of Agricultural Sciences (authorization number GAASIAS-2021-0909).

In total, 18 Duroc × Landrace × Large White barrow pigs (78.2 ± 2.05 kg) were randomly assigned to 2 groups with 9 replicates per group and 1 pig per pen (4.70 m length × 1.64 m width × 0.92 m height). Pigs in the control (CON) group received a basal diet, while pigs in the FML group received the basal diet supplemented with 10% FML. The composition and nutritional levels of the diets were previously reported [5] and are shown in Table S1. Feed and water were provided ad libitum to all pigs throughout the 69-day study. The growth performance was evaluated by recording their weights at the beginning and end of experiment period. The daily feed intake was also recorded to calculate the average daily intake.

2.3. Slaughter Procedure, Sample Collection, and Processing

On the 69th day, 6 pigs were randomly selected from each group, and 200 g of stool was collected using the rectal method, by which the pig's anus was stimulated with fingers to induce defecation. The samples were collected quickly after defecation into sterile bags; 100 g of stools were stored at −80 °C pending microbiome assessment, and the remaining stools were stored at room temperature for odorous compounds' assessment.

The pigs were stunned and slaughtered following current slaughterhouse practice. About 200 g of the longissimus thoracis muscle was quickly collected and stored at −80 °C pending chemical composition and antioxidant index determination. We also collected 100 g of whole cecum and middle colon contents and stored them at −80 °C for microbiome analysis.

2.4. Determination of Product Performance

The measurement of carcass and meat quality was conducted according to Cui et al. [13]. Within 20 min of slaughter, the carcass and abdominal fat weights and back fat thickness were measured. The loin muscle area was measured by a KP-90N planimeter (Koizumi, Nagaoka-shi, Japan). Muscle pH was measured at 45 min and 24 h after slaughter using a Testo 205 pH meter (Bad Camberg, Germany). Meat color was measured using a CR-410 chromameter (Japan) after a 15 min blooming period. The drip loss was calculated as the percentage weight difference between those measured 45 min and 24 h after slaughter. The meat samples were cut into 2 cm × 3 cm × 5 cm strips, weighed, hooked inside plastic bags, and kept at 4 °C for 24 h before measuring the final weight. For shear force analysis, the meat cores were sheared perpendicular to the muscle fibers and analyzed with a C-LM3B tester (TENOVO, Beijing, China). The marbling score was measured using an NPPC 5 grading colorimeter (USA, 1999).

2.5. Chemical Composition Determination

Muscle contents of dry matter, crude protein, ether extract, inosinic acid, and fatty acids were determined according to Cui et al.'s [12,14] and Chinese standards and general protocols using an ALPHA 2-4 LSC freeze-dryer (Martin Christ GmbH, Osterode am Harz, Germany), an 8400 nitrogen analyzer (FOSS, Hillerød, Denmark), an ether extract analyzer (2055 SOXTEC, FOSS, Hillerød, Denmark), an LC-20AD high-performance liquid chromatograph (Shimazu, Kyoto, Japan), and a 6890 gas chromatograph (Agilent, Santa Clara, CA, USA), respectively.

2.6. Antioxidant Index Determination

The supernatant was collected after homogenizing 0.1 g of muscle with 0.9 mL of saline and centrifugation at 1800× *g* and 4 °C for 10 min. The total protein and malondialdehyde contents, total superoxide dismutase and glutathione peroxidase activities, and total antioxidant capacity in the supernatant were determined by Nanjing Jiengcheng Bioengineering Institute kits (A045-2-2, A003-2-2, A001-1-2, A015-1-2, and A005-1-2).

2.7. Economic Benefit Calculations

The economic benefits were calculated according to the price and proportion of feedstocks and the weight gain and feed intake of the pigs during the experiment period.

$$\text{Feed cost (CNY/pig)} = \text{Feed intake (kg/pig)} \times \text{Unit-price of diet (CNY/kg)}$$

$$\text{Weight gain income (CNY/pig)} = \text{Weight gain (kg/pig)} \times \text{Pig selling price (CNY/kg)}$$

$$\text{Gross profit (CNY/pig)} = \text{Weight gain income (CNY/pig)} - \text{Feed cost (CNY/pig)}$$

$$\text{Weight gain feed cost (CNY/kg)} = \text{Unit-price of diet (CNY/kg)} \times \text{Feed to gain ratio}$$

2.8. Gene Sequencing

ML, FML, feces, cecum, and colon microbial DNA were isolated with a DNA Kit (Omega, Bio-Tek, Norcross, GA, USA). PCR reactions were performed in triplicate 20 μL mixtures containing 4 μL of 5× FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. The 16S rRNA (V3–V4 regions) was amplified using 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) primers. The library was sequenced on an Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA) by Biozeron Biotechnology Co., Ltd. (Shanghai, China). An Uclust algorithm was used to obtain operational sequences, and operational taxonomic units (OTUs) for species classification were clustered with 97% similarity. Species annotation and statistical analysis of the species composition for each sample at different levels were conducted. The raw reads were stored in the Sequence Read Archive database (SRP443024).

2.9. Odorous Compound Determination

We placed fecal samples (100 g) in sterile bags and stored them at room temperature for 24 h. Subsequently, portable hydrogen sulfide (H2S/C-200, GRI Instrument, Changsha, China) and ammonia (NH3/CR-200) detectors (GRI Instrument, Changsha, China) were used to determine their contents in the feces.

Fecal samples (0.1 g) were processed according to Yu et al. [15] and loaded into a high-performance liquid chromatograph (Waters Alliance e2695, Milford, MA, USA) equipped with a separation column (Zorbax Eclipse XDB-C18, Agilent Technologies, Santa Clara, CA, USA) to determine phenolic and indolic compound contents.

The measurement of short-chain fatty acids (SCFAs) content was conducted according to Yu et al.'s approach [16]. Samples (0.4 g) were processed and assessed using a gas chromatograph (7890B, Agilent Technologies, Santa Clara, CA, USA). The conditions were a nitrogen flow rate of 7.68 mL/min and oven, detector, and injector port temperatures of 130, 250, and 220 °C, respectively. The SCFA content was calculated using an external standard curve.

2.10. Data Analysis

The growth performance, carcass performance, meat quality, chemical composition and antioxidant capacity of muscle, economic benefit, and odorous compounds were analyzed using independent samples *t*-tests in SPSS Statistics (Version 25.0, IBM Corp., Armonk, NY, USA). Spearman's correlation coefficient was used to analyze the association between microorganisms and blood parameters, and between microorganisms and odorous compounds. Figures were prepared using Graphpad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). The results are expressed as means and standard errors (SE). Statistical significance was set at $p < 0.05$ and $0.05 \leq p < 0.10$ was considered a trend.

The diversity indices were calculated using rarefaction analysis in Mothur v.1.21.1 [17]. Beta diversity analysis, performed using the community ecology package in UniFrac [18], compared the principal coordinate analysis (PCoA) results. We analyzed overlapping and unique operational taxonomic units (OTUs) during treatment. The linear discriminant analysis effect size (LEfSe) compared microorganism abundances before and after FML feeding and between the FML and CON groups. The phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) predicted microbiota functional differences among samples.

3. Results

3.1. Fermented Mulberry Leaves

3.1.1. Overview of the Microbial Community in FML

As shown in Table S2, the richness, Shannon, Simpson, ACE, and evenness indexes in FML were higher than in ML ($p < 0.05$). The before- and after-fermentation samples shared 1242 OTUs, while the ML and FML had 381 and 1879 specific OTUs, respectively (Figure 1A). The PCoA analysis found that the microbial communities in ML and FML differed (Figure 1B). Figure 1C shows the dominant phyla in ML and FML. After fermentation, the Proteobacteria and Cyanobacteria relative abundances decreased ($p < 0.01$), while Firmicutes, Bacteroidetes, and Actinobacteria increased ($p < 0.01$; Figure 1D). Fermentation decreased dominant genera's relative abundance (*Enterobacter*, *Cyanobacterium*, *Cronobacter*, and *Lactococcus*; $p < 0.01$) and increased that of *Pediococcus*, *Bradyrhizobium*, *Hydrotalea*, *Rhodanobacter*, and *Mycobacterium* ($p < 0.01$), making them the dominant genera in the FML group (Figure 1E,F). The ML samples were differentially enriched in 17 microorganisms, including Enterobacteriales, Enterobacteriaceae, Gammaproteobacteria, Enterobacteria, Cyanobacterium, and Proteobacteria, while the FML samples were differentially enriched in 43 microorganisms, including Lactobacillaceae, *Pediococcus*, Firmicutes, *Bacilli*, Lactobacillales, Rhizobiales, Alphaproteobacteria, Bacteroidetes, and Bradyrhizobiaceae (Figure 1G,H).

3.1.2. Spearman's Correlation Analysis

Figure 2 shows the correlation analysis outcomes between microorganisms and nutritional components in ML and FML. *Pediococcus*, *Bradyrhizobium*, *Hydrotalea*, *Rhodanobater*, *Mycobacterium*, *Methylovirgula*, and *Mesorhozobium* were negatively associated with the pH, neutral detergent fiber, and tannins, and positively correlated with crude protein, ether extract, and ash content. *Enterobacter*, *Cyanobacteria*_norank, *Cronobacter*, *Lactococcus*, *Weissella*, *Mitochondria*_norank, *Enterobacteriaceae*_Unclassifie, and *Enterococcus* were negatively correlated with crude protein, ether extract, and ash content, and positively correlated with the pH, neutral detergent fiber, and tannins.

3.1.3. Prediction of the Microbial Community Functions in FML

The main microbial community function in ML and FML was metabolism (Figure 3A). Fermentation significantly altered the microbial community functions to include metabolism, environmental information processing, cellular processes, and human diseases ($p < 0.05$; Figure 3B). Figure 3C,D show that at level 2, fermentation improved microbial community functions such as carbohydrates, amino acids, lipids, terenoids, polyketides, xenobiotics, xenobiotic biodegradation metabolism, cell growth and death, and the biosynthesis of other secondary metabolites ($p < 0.05$). ML fermentation reduced functions such as energy metabolism, membrane transport, cofactors and vitamins metabolism, signal translation, translation folding, sorting, and degradation ($p < 0.05$).

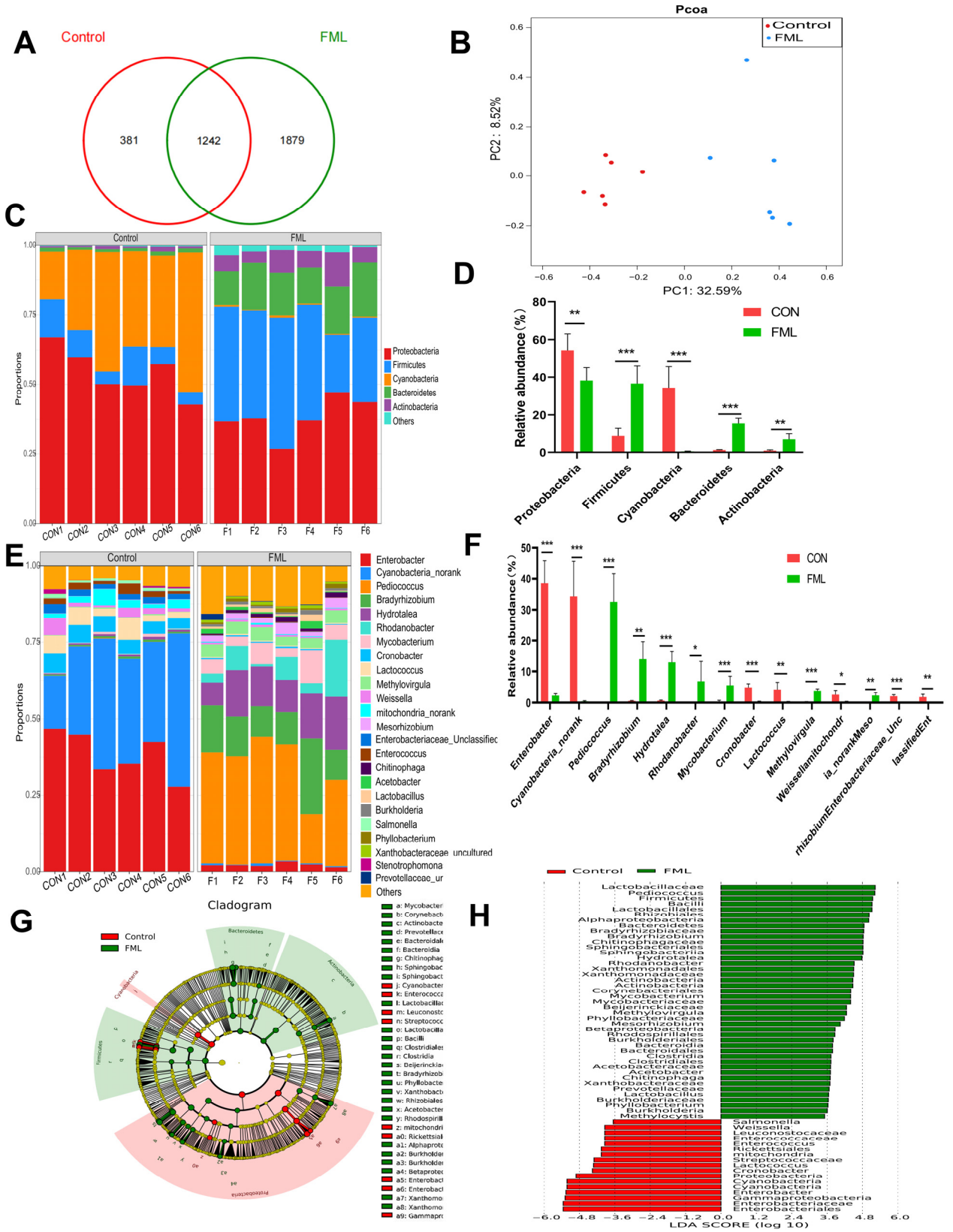

Figure 1. Overview of the microbial community in mulberry leaves (CON) and fermented mulberry leaves (FML). Venn diagram (**A**), PCoA analysis (**B**), phylum-level bacterial composition (**C**) and differential abundance (**D**), genus-level bacterial composition (**E**) and differential abundance (**F**), differential abundance (**G**,**H**). * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$.

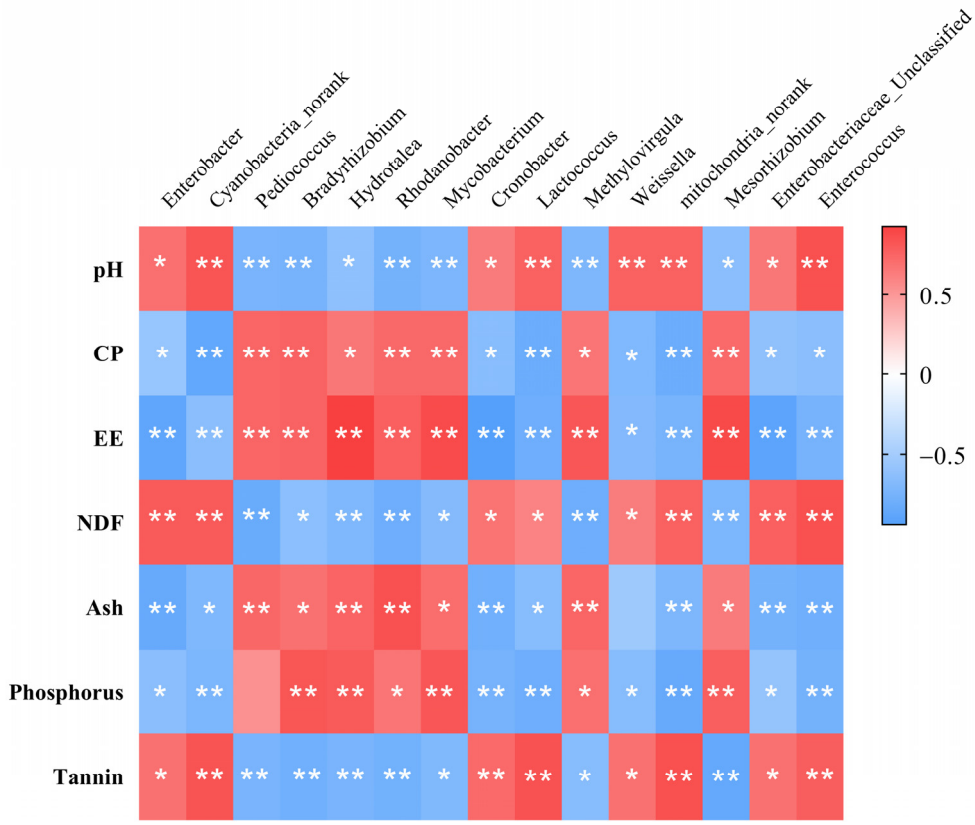

Figure 2. Spearman correlation analysis of microorganisms and nutritional components in fermented mulberry leaves. * indicates $p < 0.05$, ** indicates $p < 0.01$. CP, crude protein; EE, ether extract; NDF, acid detergent fiber.

3.2. Carcass Performance and Meat Quality

FML tended to reduce abdominal fat weight in finishing pigs ($p = 0.088$) but did not alter other carcass or meat quality indexes ($p > 0.05$; Table 1).

Table 1. Effects of fermented mulberry leaves on carcass performance and meat quality of finishing pigs.

Items	CON	FML	*p*-Value
Carcass performance			
Slaughter weight (kg)	132.73 ± 1.63	128.23 ± 13.35	0.431
Carcass weight (kg)	96.84 ± 3.14	90.82 ± 9.01	0.153
Carcass yield (%)	72.97 ± 2.63	70.86 ± 1.79	0.136
Abdominal fat weight (kg)	2.63 ± 1.05	1.71 ± 0.55	0.088
Back fat thickness (mm)	29.91 ± 6.31	27.56 ± 6.22	0.531
Loin muscle area (cm^2)	60.93 ± 2.03	63.03 ± 1.70	0.646
Meat quality			
L* 45 min	48.06 ± 1.84	48.28 ± 1.07	0.805
L* 24 h	58.56 ± 3.99	59.79 ± 1.38	0.503
a* 45 min	17.28 ± 0.85	17.30 ± 0.85	0.956
a* 24 h	17.01 ± 1.27	17.18 ± 1.53	0.833
b* 45 min	5.87 ± 0.41	6.22 ± 0.45	0.191
b* 24 h	7.48 ± 0.76	8.13 ± 1.00	0.232
pH 45 min	6.22 ± 0.17	6.16 ± 0.11	0.502
pH 24 h	5.56 ± 0.18	5.52 ± 0.11	0.677
Drip loss 24 h (%)	2.29 ± 0.12	2.21 ± 0.20	0.434
Shear force (N)	64.76 ± 14.23	60.23 ± 6.02	0.489
Marbling score	2.70 ± 0.32	2.54 ± 0.73	0.639

CON, basal diet; FML, basal diet +10% fermented mulberry leaves.

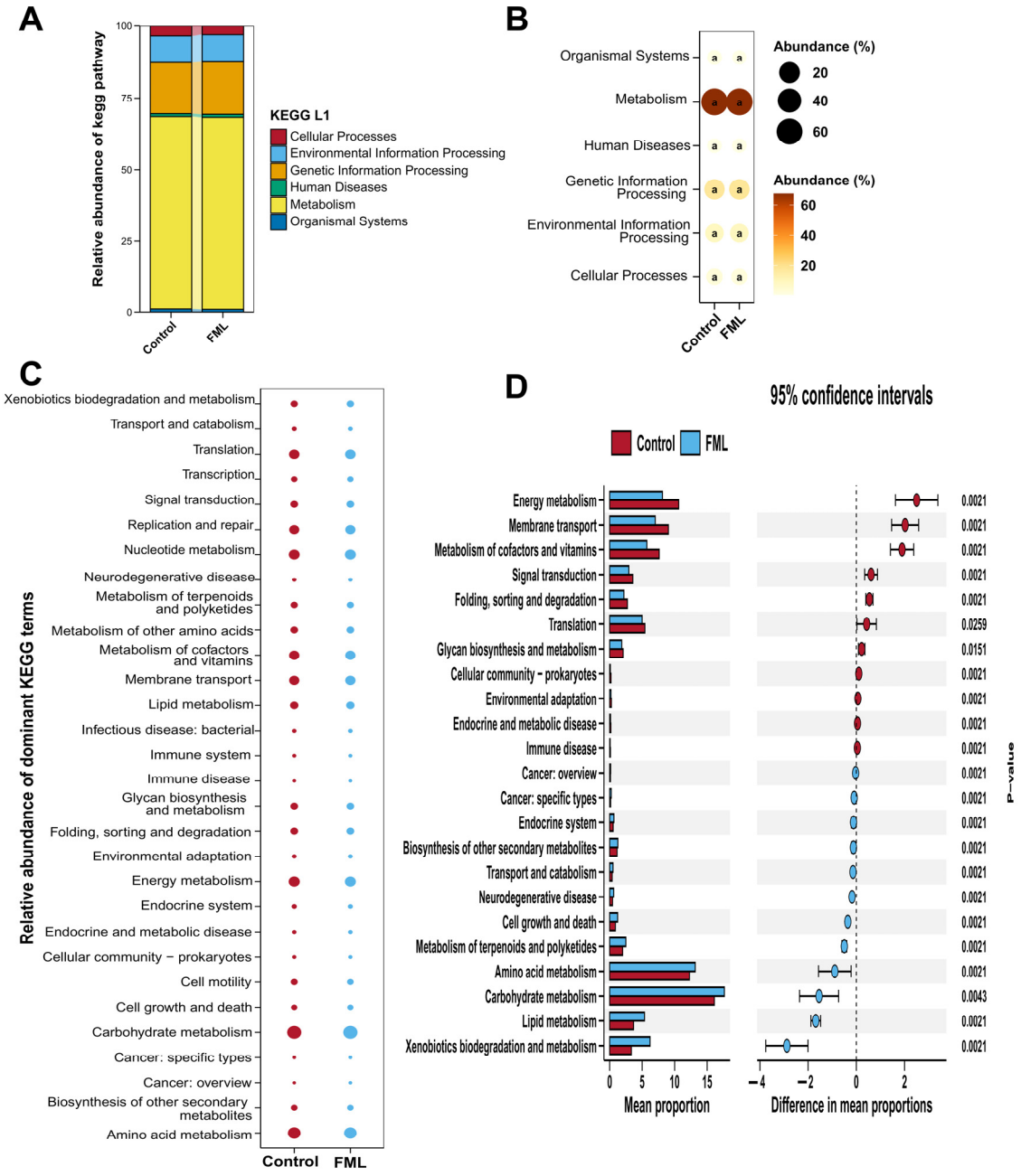

Figure 3. Prediction of microbial community function in fermented mulberry leaves. Relative abundance (**A**) and differentials (**B**) at level 1. Relative abundance (**C**) and differentials (**D**) at level 2.

3.3. Muscle Chemical Composition and Antioxidant Capacity

FML supplementation did not change the chemical composition and antioxidant capacity in pig muscle ($p > 0.05$; Table S3). Compared to the CON group, the FML group had higher levels of C17:0 and C18:3 (all-cis-9,12,15) ($p < 0.05$; Table S4).

3.4. Growth Performance and Economic Benefits

The growth performance had been previously published [5]. Table 2 shows that the feed cost ($p = 0.155$) and weight gain feed cost ($p = 0.078$) in the FML group were lower than in the CON group.

Table 2. Effects of fermented mulberry leaves on growth performance and economic benefits of finishing pigs.

Items	CON	FML	*p*-Value
Weight gain (kg)	49.20 ± 2.71	49.36 ± 3.14	0.970
Feed intake (kg)	173.97 ± 5.72	178.88 ± 5.54	0.556
Feed cost (CNY/pig)	644.69 ± 23.59	597.44 ± 18.50	0.155
Income from weight gain (CNY/pig)	1176.86 ± 64.94	1180.62 ± 75.00	0.970
Gross profit (CNY/pig)	532.17 ± 43.39	583.18 ± 58.51	0.485
Feed cost of weight gain (CNY/kg)	13.22 ± 0.30	12.26 ± 0.42	0.078

3.5. Overview of the Gut Microbial Community

Table S5 shows that the cecal ACE index ($p = 0.043$) and fecal Chao and ACE indices ($p < 0.05$) in the FML group were lower than in the CON group. The two groups had similar diversities and richness in the colon ($p > 0.05$). Figure 4A shows common and unique OTUs in the cecum, colon, and feces of the two groups. The PCoA analysis showed that pigs fed with basal and FML diets differed in their cecal, colon, and fecal microbial community structures (Figure 4B).

Firmicutes, Bacteroidetes, and Proteobacteria were the dominant phyla in the cecum, with *Lactobacillus*, *Clostridium*_sensu_stricto_1, and *Prevotella* being the dominant genera. The FML group had lower Prevotelaceae_uncultured and S24−7_ norank ($p < 0.05$) and higher Actinobacteria ($p < 0.05$; Figure 4C,D) abundances than the CON group.

In the colon, Firmicutes, Bacteroidetes, and Spirochaetae were dominant phyla, with Ruminococcaceae_uncultured, *Streptococcus*, S24−7_norank, Prevotellaceae_uncultured, and *Lactobacillus* being the dominant genera. Dietary FML decreased the relative abundance of Proteobacteria and Lachnospiraceae_uncultured ($p < 0.05$) and increased the relative abundance of Actinobacteria and *Prevotella* ($p < 0.05$; Figure 4E,F).

Firmicutes, Bacteroidetes, and Spirochaetae were the dominant phyla in the feces, with Ruminococcaceae_uncultured, S24−7_norank, and *Treponema* the dominant genera. FML supplementation increased the relative abundance of Actinobacteria ($p < 0.05$) and decreased that of *Streptococcus* and Lachnospiraceae_uncultured ($p < 0.05$; Figure 4G,H).

3.6. LEfSe Analysis

Figure S1 shows that the FML diet enriched microorganisms such as Actinobacteria, Bifidobacteriaceae, *Bifidobacterium*, and Bifidobacteriales in the cecum, colon, and feces, while the CON diet enriched microorganisms such as Spirochaetaceae and Spirochaetae in the cecum, Enterobacteriales and Enterobacteriaceae in the colon, and Streptococcaceae and *Streptococcus* in the feces.

3.7. Spearman's Correlations between Microorganisms and Blood Parameters

The blood parameter results were reported in our previous publication [5]. The spearman's correlation analysis results are shown in Figure 5. Some gut microorganisms correlated with nitrogen metabolism indicators, while other were negatively correlated with cytokines. Plasma creatinine and uric acid were negatively correlated with *Faecalibacterium* in the cecum and *Prevotella* and *Dialister* in the colon ($p < 0.05$), and positively correlated with Lachnospiraceae_uncultured in the colon and *Streptococcus* in the feces ($p < 0.05$). Serum cytokines were negatively correlated with S24-7_norank, *Streptococcus*, *Treponema*, and RC9_gut_group in the cecum, Lachnospiraceae_uncultured in the colon, and *Streptococcus* and Lachnospiraceae_uncultured in the feces ($p < 0.05$).

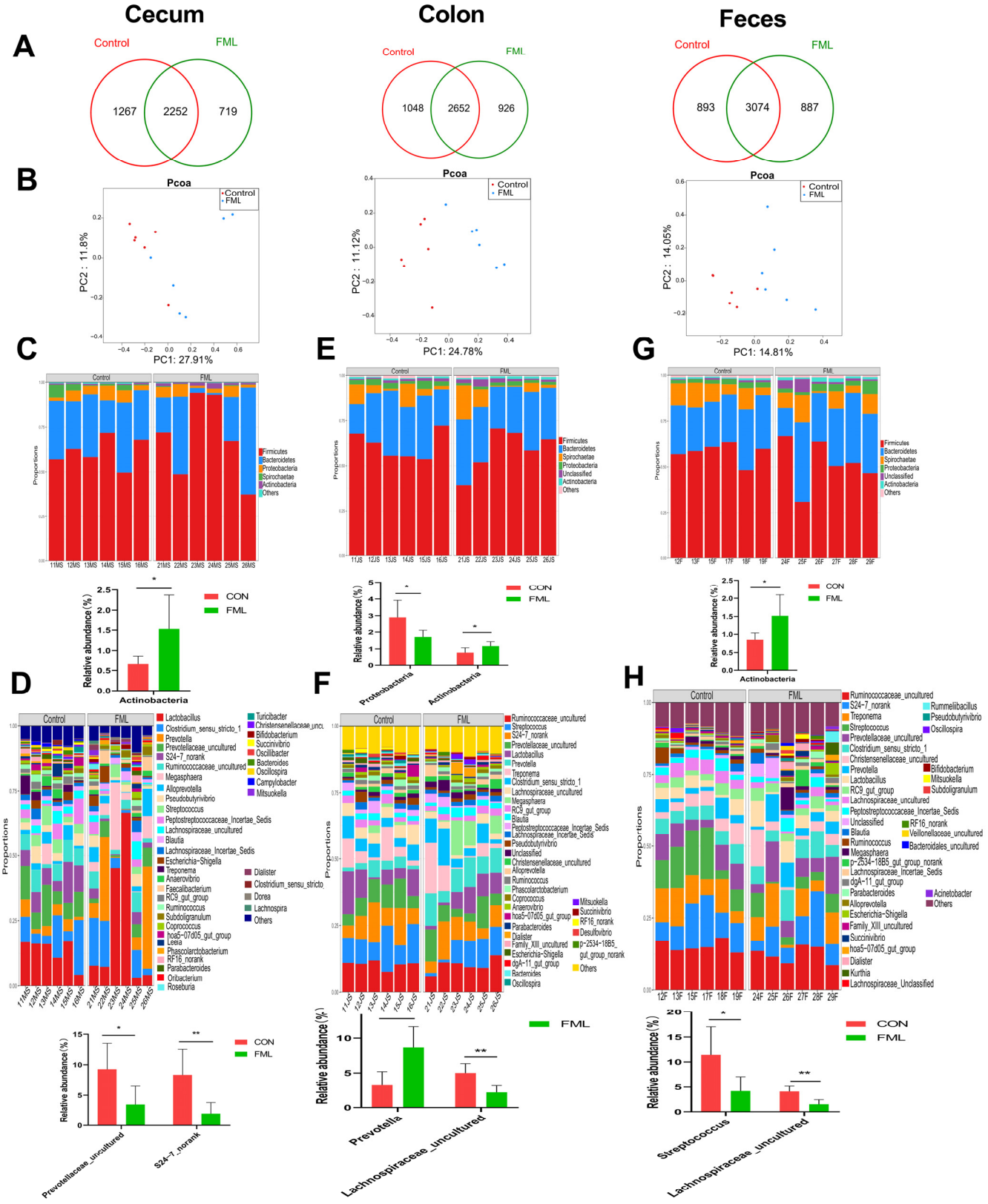

Figure 4. Overview of the effect of the dietary basal diet (CON) and fermented mulberry leaves (FML) diets on gut microbial community in pigs. Venn diagram (**A**), PCoA analysis (**B**), bacterial composition at the level of phylum (**C**,**E**,**G**) and genus (**D**,**F**,**H**). The top part of the figure shows the bacterial composition at each level, and the bottom part shows the differential bacterial abundances at each level. * indicates $p < 0.05$, ** indicates $p < 0.01$.

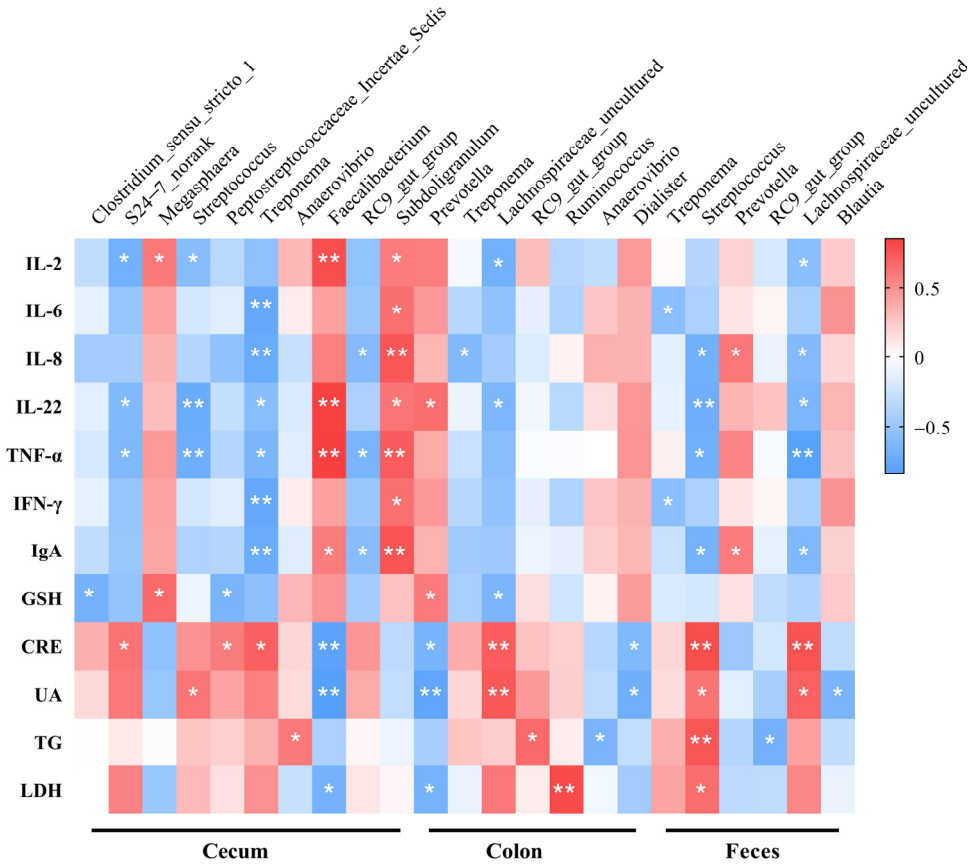

Figure 5. Spearman's correlation analysis between microorganisms and blood parameters in pigs fed on fermented mulberry leaves. IL, interleukin; TNF-α, tumor necrosis factor-α; IgA, immunoglobulin A; GSH, glutathione; CRE, creatinine; UA, uric acid; TG, triglyceride; LDH, lactic dehydrogenase.* $p < 0.05$; ** $p < 0.01$.

3.8. Prediction of Microbial Community Function in the Gut

As shown in Figure S2, the gut microbiota's main function in pigs was related to metabolism. Feeding FML did not alter this primary function ($p > 0.05$).

3.9. Odorous Compounds

Figure 6 shows the fecal odorous compound content. Compared to the CON group, the FML group showed a 26% reduction in fecal ammonia emission ($p = 0.011$) and decreased skatole and indole concentrations ($p < 0.05$). However, both groups had similar SCFA contents ($p > 0.05$; Table S6). The correlations between odor compounds and fecal microorganisms were generally weak (Figure S3).

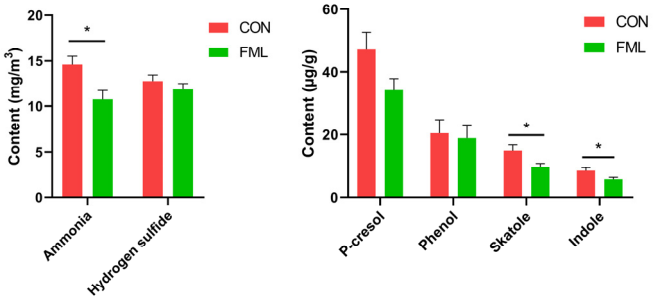

Figure 6. Odorous compounds in the feces. CON, basal diet; FML, basal diet +10% fermented mulberry leaves. * indicates $p < 0.05$.

4. Discussion

4.1. Fermented Mulberry Leaves

After the microbial fermentation of the ML, alpha diversity increased, and the community had a wide variety of species. This was related to adding microbial agents, which altered the ML colony structure. Firmicutes and Proteobacteria were dominant in ML and FML, as in previous studies [19,20]. ML fermentation increased the relative abundance of Firmicutes, Bacteroidetes and Actinobacteria. It was reported that the relative abundance of Firmicutes increased, and that of Proteus decreased in silage [21,22]. The higher relative abundance of Firmicutes in FML might be due to the low pH and anaerobic conditions at the end of fermentation [19] and the addition of *P. cellicola* and *B. licheniformis* (both Firmicutes members). The presence of Cyanobacteria before fermentation could be attributed to soil inclusions during ML collection. These were replaced by Firmicutes, Bacteroidetes, and Actinobacteria during fermentation.

Fermentation is a process of a succession of microbial colonies. *P. cellicola* was added before fermentation, so the increase in *Pediococcus* in FML could be expected. Lactic acid bacteria help control fast fermentation. Consequently, the abundance of *Enterobacter*, *Cyanobacteria*_norank, *Cronobacter*, and *Lactococcus* decreases significantly through fermentation. LEfSe analysis showed that fermentation enriched beneficial microorganisms, including Lactobacillaceae, *Pediococcus*, Firmicutes, *Bacilli*, and Lactobacillales, instead of microorganisms such as Enterobacteriales, Enterobacteriaceae, and *Enterobacter*, which are abundant in ML. Furthermore, the *P. cellicola* and *B. licheniformis* added to ML can compete with *Enterobacter* for fermentation substrate, inhibiting its growth and reducing the Enterobacteriaceae's abundance. *Bacillus* can promote feed fermentation and prevent the growth of undesired bacteria [23].

Fermentation significantly improved the microbial communities' functions, including the metabolism of carbohydrate, amino acids, and lipids, consistent with the changes in nutritional components observed in FML. Fermentation significantly changed the contents of neutral detergent fiber, carbohydrates, arginine, serine, glutamic acid, total amino acid, and ether extract in ML. In this experiment, *Pediococcus* and *Lactobacillus* were negatively correlated with pH, which is consistent with previous reports [23]. Firmicutes and Proteobacteria are important for fiber degradation in anaerobic environments [24]. Changes in neutral detergent fiber content in FML might be related to these phyla, as indicated by the analysis, which found a significant correlation between most Firmicutes and Proteobacteria species and neutral detergent fiber content. The abundance of *Pediococcus*, *Bradyrhizobium*, *Hydrotalea*, *Rhodanobacter*, and *Mycobacterium* in FML significantly increased with the decrease in pH (decomposing in the acidic environment produced by lactic acid bacteria), which is beneficial for the degradation of fiber components. Previous studies reported the degradation of tannins [25,26] and neutral detergent fiber [4] by *Pediococcus*. *Bradyrhizobium* can utilize (degrade) tannins and synthesize proteins (nitrogen fixation) and lipids [27]. These bacteria played a prominent role in the ML fermentation process, especially in degrading tannins and neutral detergent fibers, and reducing the pH.

4.2. Carcass Performance and Meat Quality

Anti-nutritional factors in ML hinder animal growth and carcass performance. Pigs' slaughter and carcass weights, as well as their dressing percentage, decreased linearly with the increase in ML supplementation [28]. A diet with 15% ML reduced the carcass weight and dressing percentage of finishing pigs [29]. In this experiment, FML did not affect pig carcass and growth performance, as in previous reports [30]. This might be because the FML dietary supplement contains fewer anti-nutritional factors and is rich in free amino acids, small peptides, and beneficial bacteria, making nutrients easier to digest and absorb and eliminating the negative effects of ML on carcass performance [30]. Meat quality is an important livestock economic trait. The effect of FML on meat quality in this study was minimal, resulting in meat color, shear force, drip loss, and marbling scores similar to those of the CON group, as previously reported [9,28,31,32]. This might be due to nitrogen and

energy balance adjustments in the feed. Overall, FML had no negative impact on pig meat quality. Feeding pigs with FML could reduce corn and soybean meal use and increase feed resources.

4.3. Muscle Chemical Composition and Antioxidant Capacity

Muscle chemical composition is an important indicator of meat quality. In this study, dietary FML had little effect on muscle chemical composition. It was reported that adding mulberry flavonoids or ML did not affect dry matter, crude protein, intramuscular fat, or inosinic acid contents in pig muscle [31,32]. One study [9] reported that ML increased eicosadienoic acid content in the dorsal muscle without altering the concentration of other fatty acids. Liu et al. [33] reported a negative association between dietary ML content and the concentrations of palmitic, palmitoleic, and myristic acids in the dorsal muscles, and a positive association with the concentrations of linoleic, α-linolenic, and arachidonic acids. The differences among these studies might be due to the sources, processing methods, and feeding times of ML. γ-linoleic acid plays an important biological role in the human body. As a substrate for arachidic acid synthesis, it is involved in the transport and oxidation of cholesterol [34]. The increase in muscle γ-linolenic acid content after feeding FML could benefit human health. Fermentation leads to changes in the structure or content of some active components (flavonoids and polyphenols) in ML, which affect the muscle fatty acid profile; the reason for this remains unknown.

The reactions between lipid oxidation products and other substances can affect the meat flavor. FML did not alter the antioxidant enzyme activity in the longissimus thoracis muscle, as reported by Fan et al. [9], who found no difference in muscle antioxidant capacity between the ML and FML groups. This result is also consistent with the effects of ML and FML on blood antioxidant enzyme activity [5]. We saw no improvement in antioxidant capacity in this experiment, possibly because the high dietary vitamin E level (150 mg/kg) masked the effect of FML. Vitamin E is a strong antioxidant that can protect pigs from oxidative stress [35].

4.4. Economic Benefits

Due to its abundance and low utilization rate, ML are cheaper than conventional feed and can act as a good unconventional feed resource. The low-cost mature FML used in this study had no negative impact on the product performance of pigs, but tended to reduce the weight gain feed cost, thereby moderately increasing the weight gain income and gross profit. This outcome indicates that feeding FML to finishing pigs might reduce feed costs and increase breeding benefits. Effective ML utilization could save costs and achieve high-value resource utilization.

4.5. Gut Microbial Community

ML (silage, sun-dried) did not alter fecal bacterial community richness and diversity in cattle and sheep [36,37], and FML did not change the ileal flora alpha diversity index in pigs [38]. However, compared to the CON group, the microbial richness in the FML group in our study was lower in the cecum and feces but similar in the colon. The gut microbiome is influenced by fiber source and type, which affect the microbial abundance and diversity in the pig gut [39,40]. Furthermore, fructooligosaccharides could reduce these indices [41]. The FML fiber content may have decreased the alpha diversity index in the cecum and feces in our study.

Firmicutes and Bacteroidetes, the dominant bacteria in the cecum, colon, and feces, are involved in carbohydrate and protein degradation [37], play a significant role in digestion, and provide essential substances for host and gut microorganisms [42,43]. Our results are similar to those of previous research that found differences in fecal microflora composition between sows fed basal or FML diets, and the main bacterial categories were Firmicutes, Bacteroides, Spirochaetes, and Proteobacteria (both groups) [43,44]. A decrease in Proteobacteria abundance in the colons of pigs fed FML could benefit them. It has

been reported that animals with inflammatory bowel disease have a higher level of Proteobacteria than healthy animals [45–47]. Similarly, dietary quercetin (mulberry) can reduce fecal Proteobacteria [48]. Finishing pigs with low Proteobacteria abundance have healthier intestinal tracts and are at a lower risk of developing pathogenic bacterial.

Prevotella is the main dietary fiber-digesting bacterium. It is associated with the metabolism of amino acids, carbohydrates, and lipids, and is important for maintaining energy balance [44]. The crude fiber level in the FML group was higher than in the CON group, possibly resulting in higher fiber content in the hindgut of the FML group. FML supplementation increased *Prevotella* abundance and promoted dietary fiber digestion in the colon. Our study was similar to that of Xu et al. [48], who reported that dietary quercetin enhanced *Prevotella copri* abundance in piglet feces. FML contain abundant flavonoids such as quercetin, which might also help increase the relative abundance of *Prevotella*. This suggests that supplementing the diet with FML could improve fiber digestibility and utilization in finishing pigs. *Prevotella* was negatively correlated with plasma creatinine and uric acid. Their association with dietary fiber digestion can reduce serum uric acid and urea nitrogen levels by weakening dietary adenine absorption [49]. The abundance of potential pathogens including *Streptococcus* and *Enterococcus* was higher in the feces of pigs lacking dietary fibers than in control [50]. After feeding pigs with FML, the fecal abundance of *Streptococcus* decreased, reflecting the beneficial effects of FML in reducing the proportion of harmful bacteria in the gut and helping maintain a healthy intestinal environment and stable microbial community. *Streptococcus* could cause diarrhea, intestinal inflammation, and other diseases in pigs [51]. Furthermore, *Streptococcus* was negatively correlated with blood cytokines (IL2, IL8, IL22, and TNF-α) and positively correlated with creatinine and uric acid, so its reduction indirectly reflects improved health in these pigs.

In the current study, FML supplementation changed the gut bacterial microbiome composition. The gut core microorganisms in the FML group were mainly enriched in Actinobacteria, *Bifidobacterium*, Bifidobacteriaceae, Bifidobacteriales, and others, resulting in an increased Actinobacteria relative abundance in the pigs' cecum, colon, and feces. Actinobacteria and Bifidobacteriales have been associated with increased animal feed utilization through extracellular enzyme production [52,53]. Bifidobacteriales enrichment is beneficial to animal health. As a dominant microbial community in normal intestines [54], Bifidobacteriales can enhance the intestinal mucosa immune barrier function by increasing goblet cells number and mucin-2 secretion [55]. FML can increase the abundance of beneficial bacteria (Actinobacteria and *Bifidobacteriaceae*), maintaining microbial structure without affecting the pig's weight [5]. One possible reason FML affect the gut microbiota composition is the decomposition of large molecules in ML into small ones during fermentation, which enhances feed palatability and digestibility. Alternatively, dietary *P. cellicola* and *B. licheniformis* may have acted as probiotics that affected the abundance and composition of the fecal microorganism community.

4.6. Odorous Compounds

Ammonia and hydrogen sulfide are the main pig manure odor components, with skatole being one of the most unpleasant odor compounds [56]. Odor compound emissions into the environment negatively impact human health and environmental safety. Adding fermented carbohydrates to the feed helps reduce foul-smelling chemical production and improve intestinal health [57]. FML have a high dietary fiber content, leading to increased fecal fiber content in pigs (unpublished data) that could help reduce odorous substance production (fecal ammonia content in the FML group was approximately 26% lower than in the CON group). Feeding high-fiber diets could increase fecal fiber content and reduce the emission of ammonia and volatile sulfur compounds [58,59]. FML altered the gut microbiota structure and reduced odorous substance synthesis. *Escherichia coli*, *Proteus*, and paracoliform bacteria can synthesize indole [60]. The pig feces in the FML group was enriched in beneficial bacteria such as Bifidobacterium, while the CON group was enriched in Enterobacteriales. The decreased number of microorganisms synthesizing indole and

skatole resulted in a decrease in fecal odor compound concentrations [56,58]. Feeding high levels of chicory roots reduced the skatole concentration [61,62].

SCFA is the main product of carbohydrate fermentation by gut bacteria. The phylum Firmicutes includes many carbohydrate-fermenting bacteria, including *Ruminococcus*, *Lactobacillus*, and *Clostridium* [63]. These bacteria's relative abundances in the two treatments were similar, explaining their similar intestinal SCFA concentrations. Similarly, fecal SCFA level was unaffected by dietary chicory root [63]. Functional FML substances such as flavonoids and small-molecule substances increased the body's protease activity and improved protein digestibility (unpublished data). These resulted in a reduced protein fermentation substrate availability for the large intestinal microorganisms, and, subsequently, fecal odor substance emission. The odor emitted after feeding FML was clean, improving the pig housing environment and animal welfare.

5. Conclusions

This study evaluated the impacts of microbial fermentation on the ML bacterial community. Adding *P. cellicola* and *B. licheniformis* to ML increased the relative abundance of *Pediococcus*, *Bradyrhizobium*, *Hydrotalea*, and *Rhodanobacteria* and decreased that of *Enterobacter*. This restructured bacterial community improved FML quality.

The fermentation of industrial excess/surplus ML could produce clean fermented products to feed pigs without reducing their carcass performance and meat quality. FML supplementation could reshape the pig gut microbiota and produce cleaner waste by reducing ammonia emission and fecal odor compound concentrations, resulting in improved environmental protection and sustainability. Feeding FML to animals is a feasible ecological breeding and clean production strategy. FML have prospects of being used as a pig feed.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation10040215/s1, Figure S1: Linear discriminant analysis effect size (LEfSe) analysis ($p < 0.05$, LDA > 2.0) of the gut and fecal microbiota. Control, basal diet; FML, basal diet +10% fermented mulberry leaves; Figure S2: Prediction of the microbial community function in the gut and feces of finishing pigs; Figure. S3. Spearman's correlation analysis between microorganisms and odorous compounds in the feces; Table S1: Composition and nutrient levels of basal diets (%, as air-dry basis); Table S2: Alpha diversity analysis of unfermented (CON) and fermented mulberry leaves (FML); Table S3: Effects of fermented mulberry leaves on chemical composition (fresh meat) and antioxidant capacity of longissimus thoracis in finishing pigs; Table S4: Effect of fermented mulberry leaves on fatty acid profile of longissimus thoracis in finishing pigs (% fatty acid); Table S5: Alpha diversity analysis of gut and feces.

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