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The Fermentation Quality, Antioxidant Activity, and Bacterial Community of Mulberry Leaf Silage with *Pediococcus*, *Bacillus*, and Wheat Bran

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Abstract: This study was conducted to investigate the effects of different strains and wheat bran on the fermentation quality, antioxidant activity, and bacterial community of mulberry leaf silage. Mulberry leaves were ensiled with *Pediococcus acidilactici* and *Pediococcus pentosaceus* (A), *Bacillus subtilis* and *Bacillus licheniformi* (DK), and *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Bacillus subtilis*, and *Bacillus licheniformi* (AK). Each treatment was supplemented with 10% wheat bran (fresh matter basis), and the strains were added in equal proportions for 7 days. The results indicated that the DK and AK groups exhibited higher dry matter (DM) content compared to the A group ($p < 0.05$). The A group (37.25 mg/g DM) and AK group (34.47 mg/g DM) demonstrated higher lactic acid content and lower pH (<4.40). Furthermore, the DK group had a significantly higher acetic acid content compared to the AK group ($p < 0.05$). Additionally, both the A and AK groups exhibited lower levels of ammonia-N content than the DK group ($p < 0.05$). The number of yeasts, molds, and coliform bacteria were low in mulberry leaf silage. Moreover, the antioxidant activity in the fermentation groups increased, with higher relative abundance of beneficial bacteria, *Lactococcus* and *Lactobacillus*, in the AK group. In summary, the AK group was observed to enhance fermentation quality and antioxidant capacity, leading to the establishment of a favorable microbial community composition.

Keywords: mulberry leaf; fermentation quality; antioxidant activity; bacterial community

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1. Introduction

The exponential growth in animal populations in China has resulted in a critical scarcity of feed resources, posing a hindrance to the advancement of animal husbandry [1,2]. Thus, there is an urgent necessity to explore new unconventional feed resources such as mulberry (*Morus alba* L.), which holds enormous potential.

Mulberry trees belong to the *Morus* perennial woody plant of the *Moraceae* family, with mulberry leaves (*Morus alba*) being its primary product. Originating from China, mulberry has been utilized in the sericulture industry for over 5000 years [3]. Mulberry trees exhibit adaptability to diverse environments and thrive in most temperate, tropical, and subtropical regions [4]. It has been noted that the planting area of mulberry cultivation exceeds 10^6 hectares, and the biomass yield of fresh mulberry leaves in China ranges from 25 to 30 tons per hectare annually [5]. The nutrient content of mulberry leaves is comprehensive and balanced, including crude protein (15~35%), crude fat (3.5~5.57%), vitamins, minerals (2.42~4.71% Ca, 0.23~0.97% P), and amino acids, surpassing that of feed crops such as alfalfa (13.0–28% CP and 1.3–3.0% EE) [6,7]. Furthermore, numerous studies have emphasized the presence of natural bioactive compounds in mulberry leaves, including flavonoids, alkaloids, polysaccharides, and polyphenols, which exhibit hypoglycemic, lipid-lowering, antibacterial, antioxidant, and anti-inflammatory effects [8–11].

Consequently, mulberry leaves are extensively employed as a high-quality protein feed material in animal production, catering to animals including pigs, chickens, ruminants, and fish [12–15].

Although fresh mulberry leaves offer several benefits as animal feed, certain challenges persist. The high moisture and protein content of mulberry leaves make them unsuitable for long-term storage, especially considering the increased accumulation during the short rainy season in southern China, as reported [16]. Silage presents an effective method of preserving fresh mulberry leaves owing to its prolonged storage period, favorable palatability, and high nutritional value [17]. It is widely acknowledged that adequate quantities of lactic acid bacteria, a specific amount of water-soluble carbohydrates (WSC), and nitrogen sources are pivotal for successful silage [18]. Nevertheless, inadequacy in epiphytic lactic acid bacteria and nitrogen sources can lead to an increase in harmful bacteria such as clostridia [19]. Recent research has underscored that the supplementation of inoculants and sufficient fermentation substrates enhances silage fermentation quality [20,21]. With the widespread application of inoculants, the addition of *Lactobacillus* and fungi has become common practice to improve silage quality [17,22]. A prior study has demonstrated that rice bran contains soluble sugars, providing an adequate substrate for fermentation, thereby promoting the success of the fermentation process [23].

Numerous studies have substantiated the potential for lactic acid bacteria to improve the nutrient content and preservation rate of silage mulberry leaf feed [20,21]. Additionally, attention has been given to the degradation of the crude fiber content of mulberry leaves through the addition of cellulase [24]. However, there are few reports on the direct addition of *Bacillus* to mulberry leaves for fermentation. Our study aimed to investigate the effects of adding two strains of *Pediococcus* and two strains of *Bacillus*, either separately or in combination, on fermentation quality, antioxidant activity, and bacterial community composition during the ensiling of mulberry leaves. The anticipated result of our research is to establish a theoretical basis and provide practical technical support for the application of mulberry leaves in husbandry.

2. Materials and Methods

2.1. Strain Activation

Laboratory-preserved *Pediococcus acidilactici* (A6), *Pediococcus pentosaceus* (A2), *Bacillus subtilis* (DB), and *Bacillus licheniformi* (KL) were employed for silage. Four strains were isolated and screened from livestock and poultry digesta. *Pediococcus acidilactici* (A6) and *Pediococcus pentosaceus* (A2) were cultured on de Man, Rogosa, and Sharpe agar (MRS, Beijing Land Bridge Technology Co., Ltd., Beijing, China), and the seed liquid was incubated for 12 h at 37 °C prior to fermentation. *Bacillus subtilis* (DB) and *Bacillus licheniformi* (KL) were cultured on beef peptone yeast medium, (BPY, Beijing Land Bridge Technology Co., Ltd., Beijing, China), with the seed liquid incubated at 37 °C and 180 rpm for 15 h prior to fermentation.

2.2. Silage Preparation

Mulberry leaves (*Qiangsang*) were manually harvested from mulberry leaf plantations in Huzhen Town, Jinyun County, Zhejiang Province, China. Fresh mulberry leaves were chopped to 1–2 cm. A total of 180 g of fresh mulberry leaves was mixed with 10% (*w*/*w*) wheat bran (on a fresh matter (FM) basis) and an 8% mixed bacteria solution containing over 10^8 cfu/g of fresh matter. The silage treatments comprised the following: (1) fresh mulberry leaves (CON); (2) *Pediococcus acidilactici* and *Pediococcus pentosaceus* in a 1:1 inoculation ratio, complemented by 10% wheat bran (fresh matter basis) (A); (3) *Bacillus subtilis* and *Bacillus licheniformi* in a 1:1 inoculation ratio, complemented by 10% wheat bran (fresh matter basis) (DK); (4) *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Bacillus subtilis,* and *Bacillus licheniformi* in a 1:1:1:1 inoculation ratio, complemented by 10% wheat bran (fresh matter basis) (AK).

Following thorough mixing, approximately 200 g of sample are packed into feed fermentation bags (23 cm × 30 cm), sealed with a sealing machine, and stored at 37 °C for ensiling. After seven days, three loads from each treatment were analyzed for fermentation characteristics, chemical composition, antioxidant activity, and bacterial community.

2.3. Determination of Chemical Composition

The dry matter (DM) content of pre-ensiled fresh and silage mulberry leaves was determined by oven drying at 65 °C for 48 h, followed by crushing the dried sample through a 60-mesh sieve for subsequent analysis using a high-speed grinder. The crude protein (CP) content was analyzed using the Kjeldahl nitrogen analyzer (Kjeltec^{TM} 3400 Auto-Analyzer, FOSS Analytical Instruments Inc., Hillerød, Denmark). The levels of neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured following the methods outlined by the Association of Official Analytical Chemists [25]. Additionally, the content of water-soluble carbohydrates (WSC) was determined via anthrone colorimetry [26].

2.4. Fermentation Parameters Detection and Analysis

For fermentation parameter determination, 10 g of each silage sample were mixed with 90 mL of distilled water and refrigerated at 4 °C for 24 h. The mixture was then filtered using four layers of gauze and qualitative filter paper. The filtrates were utilized to measure pH, ammonium nitrogen (NH_3-N), and organic acids. The pH was immediately assessed using a precision pH meter (FiveEasyPlus, METTLER TOLEDO Instruments Co., Ltd., Shanghai, China). The NH_3-N concentration was determined via the phenol–sodium hypochlorite method [27]. The lactic acid content was analyzed using high-performance liquid chromatography (HPLC) (column, Waters SymmetryR C18 (4.6 mm × 250 mm, 5 μm) (Shimadzu, Tokyo, Japan); ultraviolet detector (210 nm); flow rate, 1.0 mL/min; injection volume, 10 μL; oven temperature, 35 °C). Additionally, the contents of acetic acid, propionic acid, and butyric acid were quantified using gas chromatography (GC) (column, DB-FFAP (30.0 m × 0.32 mm × 0.25 μm) (Shimadzu, Tokyo, Japan); total flow rate, 33.8 mL/min; column flow rate, 1.47 mL/min; injection volume, 10 μL; detector temperature, 270 °C; vaporizing chamber temperature, 250 °C).

2.5. Microbiological Evaluation

10 g of silage samples were homogenized with 90 mL of sterile 0.9% NaCl saline solution and shaken at 180 rpm and 37 °C for 1 h to obtain a bacterial suspension. Subsequently, the bacterial suspension was then diluted from 10^{-1} to 10^{-7} using sterile saline solution (0.9% NaCl). The lactic acid bacteria (LAB) population was counted on MRS medium agar, while coliform bacteria were counted in eosin–methylene blue agar medium. The samples were then incubated at 37 °C for 48 h. Yeast and mold counts were determined by incubating the samples on rose bengal agar after 48 h incubation at 30 °C. The counts were then converted to log10 (cfu/g) for quantitative analysis of microorganisms.

2.6. Determination of Active Substance and Antioxidant Activity

After drying to constant weight, the samples were crushed and passed through a 60-mesh sieve. Approximately 1 g of the sample was then weighed, and 10 mL of 70% anhydrous ethanol were added for ultrasonic extraction. The extraction process involves applying an initial ultrasonic power of 300 W at 60 °C for 30 min, followed by centrifugation at 12,000 rpm and 25 °C for 10 min.

The contents of flavonoids and polyphenols were quantified using assay kits (kit No. BC1335, BC1345; Beijing Solarbio Technology Co., Ltd., Beijing, China) following the manufacturer's instructions. The polysaccharide content was determined using the phenol–sulfuric acid method [28,29].

The scavenging activity of 2,2-diphenyl-1-pyridinyl-pyrazolyl (DPPH), the scavenging activity of 2,2-diphenyl-3-ethylbenzene-thiazolium-6-ammonium sulfonate (ABTS) radical cation, hydroxyl radical scavenging activity (HAS), and iron-reducing antioxidant capacity (FRAP) were assessed using the supernatant solution [30–33].

2.7. Analysis of Bacterial Community

Fresh or silage samples (5 g) were added to a sterile tube, and 25 mL 0.1 M potassium phosphate buffer (pH = 8.0) were included. The samples underwent ultrasonic washing for 1 min and swirling for 10 s, repeated twice. Subsequently, the samples were filtered with sterilized four-layer gauze, combined with the filtrate, and centrifuged at 13,000 rpm for 10 min at 4 °C to collect microbial precipitates for DNA extraction. Total genomic DNA from microbial communities was extracted following the manufacturer's instructions of the E.Z.N.A.® soil DNA kit (Omega Bio-tek, Norcross, GA, USA), and its quality was evaluated through 1% agarose gel electrophoresis. Subsequently, the DNA concentration and purity were determined using NanoDrop2000 (Thermo Scientific, Waltham, MA, USA). The DNA extracted was used as a template for PCR amplification of the V3-V4 variable region of the 16S rRNA gene, using the upstream primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and the downstream primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′), which contain barcode sequences. The amplification procedure is as follows: pre-denaturation at 95 °C for 3 min, 27 cycles (denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s), followed by a final extension at 72 °C for 10 min and storage at 4°C (PCR machine: ABI GeneAmp® 9700, Foster, CA, USA) [17]. Each sample underwent triplicate runs. Subsequently, the PCR products from the same sample were pooled, recovered using 2% agarose gel, purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA), subjected to 2% agarose gel electrophoresis, and quantified using the Quantus™ Fluorometer (Promega, Madison, WI, USA). Illumina's Miseq PE300 platform (Shanghai Meiji Biomedical Technology Co., Ltd., Shanghai, China) was employed for sequencing, and data analysis was performed on the Meiji Bio Cloud platform (https://cloud.majorbio.com) (accessed on 1 July 2024).

2.8. Statistical Analysis

The data were analyzed with IBM SPSS statistics for windows, version 26.0 software (IBM Corp., Armonk, NY, USA) SPSS 26.0 software, employing the one-way ANOVA and Duncan's method to determine statistical significance at the $p < 0.05$ level. The test data are expressed as average values.

3. Results

3.1. The Chemical Composition and Microbial Population of Mulberry Leaves before Ensiling

The characteristics of mulberry leaves before ensiling are shown in Table 1. The contents of dry matter, crude protein, crude fat, neutral detergent fiber, acid detergent fiber, and water-soluble carbohydrate were 28.70%, 27.60%, 4.55%, 36.45%, 15.69%, and 7.47%, respectively. Besides the presence of a low level of lactic acid bacteria in fresh mulberry leaves, yeast and the pathogenic bacterium *E. coli* were also found.

Table 1. Chemical composition and microbial population of fresh mulberry leaves (mean ± SEM, n = 3).

Item	Contents
Chemical composition	
Dry matter (%)	28.70 ± 0.09
Crude protein (% DM)	27.46 ± 0.14
Crude fat (% DM)	4.55 ± 0.06
Neutral detergent fiber (% DM)	36.45 ± 0.60

Table 1. *Cont.*

Item	Contents
Acid detergent fiber (% DM)	15.69 ± 0.14
Water-soluble carbohydrate (% DM)	7.47 ± 0.13
Microbial population	
Lactic acid bacteria (log cfu/g FM)	4.32 ± 0.24
Yeasts (log cfu/g FM)	4.37 ± 0.04
Mold (log cfu/g FM)	<2.00
Coliform bacteria (log cfu/g FM)	3.66 ± 0.06

DM: dry matter; FM: fresh matter; cfu CFU: colony forming unit; SEM: standard error of means.

3.2. Chemical Composition, Fermentation Quality and Microbial Population of Mulberry Leaf Silage

The chemical composition, fermentation quality, and microbial population of mulberry leaf (ML) silages are shown in Table 2. Notably, the A group exhibited lower DM content compared to the other two groups ($p < 0.05$), whereas no difference in DM content was observed between the DK group and AK group. The acetic acid content in the AK group was significantly lower than that in the DK group ($p < 0.05$). Additionally, both the A and AK groups demonstrated lower ammonia-N content than the DK group ($p < 0.05$). Furthermore, propionic acid and butyric acid were undetected, and the counts of yeasts, molds, and coliform bacteria were all low in all three groups.

Table 2. Effects of combined strains on chemical composition, fermentation quality, and microbial population of mulberry leaf silage.

Item	Treatments			SEM	*p*-Value
	A	DK	AK		
Chemical composition					
Dry matter (%)	28.50 ^{b}	28.99 ^{a}	29.03 ^{a}	0.13	0.01
Crude protein (% DM)	27.08	28.00	28.06	0.51	0.18
Crude fat (% DM)	4.83	4.98	4.60	0.26	0.39
Neutral detergent fiber (% DM)	25.96	25.25	24.38	1.01	0.36
Acid detergent fiber (% DM)	11.75	11.63	11.89	0.44	0.84
Water soluble carbohydrate (% DM)	1.99	3.48	2.94	0.46	0.05
Fermentation quality					
pH	4.27	4.67	4.31	0.14	0.06
Lactic acid (mg/g FM)	37.25	26.94	34.47	3.35	0.05
Acetic acid (mg/g FM)	3.08 ^{ab}	3.78 ^{a}	2.60 ^{b}	0.34	0.04
Propionic acid (mg/g FM)	ND	ND	ND	-	-
Butyric acid (mg/g FM)	ND	ND	ND	-	-
Ammonia-N (%DM)	0.02 ^{b}	0.03 ^{a}	0.02 ^{b}	0.00	<0.01
Microbial population					
Lactic acid bacteria (log cfu/g FM)	7.97	5.57	6.65	1.29	0.26
Yeasts (log cfu/g FM)	<2.00	<2.00	<2.00	-	-
Mold (log cfu/g FM)	<2.00	<2.00	<2.00	-	-
Coliform bacteria (log cfu/g FM)	<2.00	<2.00	<2.00	-	-

A: A6 + A2; DK: DB + KL; AK: A6 + A2 + DB + KL; DM: dry matter; FM: fresh matter; CP: crude protein; WSC: water-soluble carbohydrates; NDF: neutral detergent fiber; ADF: acid detergent fiber; SEM: standard error of means; ND: not detected. Different lowercase letters $^{(a, b)}$ in the same row indicate significant differences at $p < 0.05$, while the same letters or absence of markings indicate no significant differences $p > 0.05$.

3.3. Active Components and Antioxidant Activity of Fresh Mulberry Leaves and Mulberry Leaf Silage

The biological substances and antioxidant activity in fresh ML and ML silages are shown in Table 3. The levels of polyphenols in the A and DK groups were significantly higher than in the CON and AK group ($p < 0.05$). Furthermore, the content of polysaccharides in the CON group was significantly higher than in the experimental groups ($p < 0.05$). The hydroxyl radical scavenging activity (HAS) in ML silages was significantly higher compared to the CON group ($p < 0.05$).

Table 3. Bioactive components and antioxidant activity of fresh mulberry leaves and mulberry leaf silage.

Item	Treatments				SEM	*p*-Value
	CON	A	DK	AK		
Bioactive components						
Flavonoid (mg/g DM)	6.72	7.41	6.06	6.58	0.54	0.17
Polyphenol (mg/g DM)	13.60 ^{b}	19.07 ^{a}	17.89 ^{a}	13.99 ^{b}	1.17	<0.01
Polysaccharides (mg/g DM)	27.83 ^{a}	13.02 ^{b}	12.93 ^{b}	12.00 ^{b}	1.32	<0.01
Antioxidant activity						
DPPH (%)	91.18	92.29	93.51	95.16	1.25	0.06
ABTS (%)	95.45	98.53	97.46	98.03	1.43	0.23
HAS (%)	42.72 ^{b}	75.57 ^{a}	74.30 ^{a}	77.36 ^{a}	8.32	<0.01
FRAP (%)	81.90	91.51	90.98	91.04	4.02	0.12

CON: fresh mulberry leaves; A: A6 + A2; DK: DB + KL; AK: A6 + A2 + DB + KL; DM: dry matter; SEM: standard error of means; DPPH: free radical DPPH scavenging activity; ABTS: radical ABTS+ scavenging activity; HAS: hydroxyl radical scavenging activity; FRAP: ferric-reducing antioxidant power. Different lowercase letters $^{(a, b)}$ in the same row indicate significant differences at $p < 0.05$, while the same letters or absence of markings indicate no significant differences $p > 0.05$.

3.4. Bacterial Diversity of Fresh Mulberry Leaves and Mulberry Leaf Silage

The alpha diversity of the bacterial community of fresh ML and ML silages are shown in Table 4. Specifically, the Sobs, Chao1, and Shannon indexes in the CON group were significantly higher than those in the fermentation groups ($p < 0.05$). Moreover, the Shannon index of the AK group showed a notably higher value compared to the A and DK groups ($p < 0.05$). Notably, the coverage index for all groups was 1.00, indicating that the samples in all groups effectively represent the general bacterial community situation.

Table 4. Alpha diversity of bacterial community for fresh mulberry leaves and mulberry leaf silage.

Treatments	Sobs	Chao1	Ace	Shannon	Coverage
CON	106.33 ^{a}	114.16 ^{a}	112.82	2.35 ^{a}	1.00
A	33.67 ^{b}	43.83 ^{b}	43.07	0.21 ^{c}	1.00
DK	33.33 ^{b}	43.01 ^{b}	43.1	0.21 ^{c}	1.00
AK	35.67 ^{b}	42.75 ^{b}	50.8	0.75 ^{b}	1.00
SEM	22.62	22.29	23.65	0.22	-
p-value	0.03	0.03	0.05	<0.01	-

CON: fresh mulberry leaves; A: A6 + A2; DK: DB + KL; AK: A6 + A2 + DB + KL; SEM: standard error of means. Different lowercase letters $^{(a–c)}$ in the same column indicate significant differences at $p < 0.05$, while the same letters or absence of markings indicate no significant differences $p > 0.05$.

In addition, the β diversity of the bacterial community was further analyzed (Figure 1). The PCoA results indicated a distinct separation of the bacterial community between the CON group and the fermentation groups.

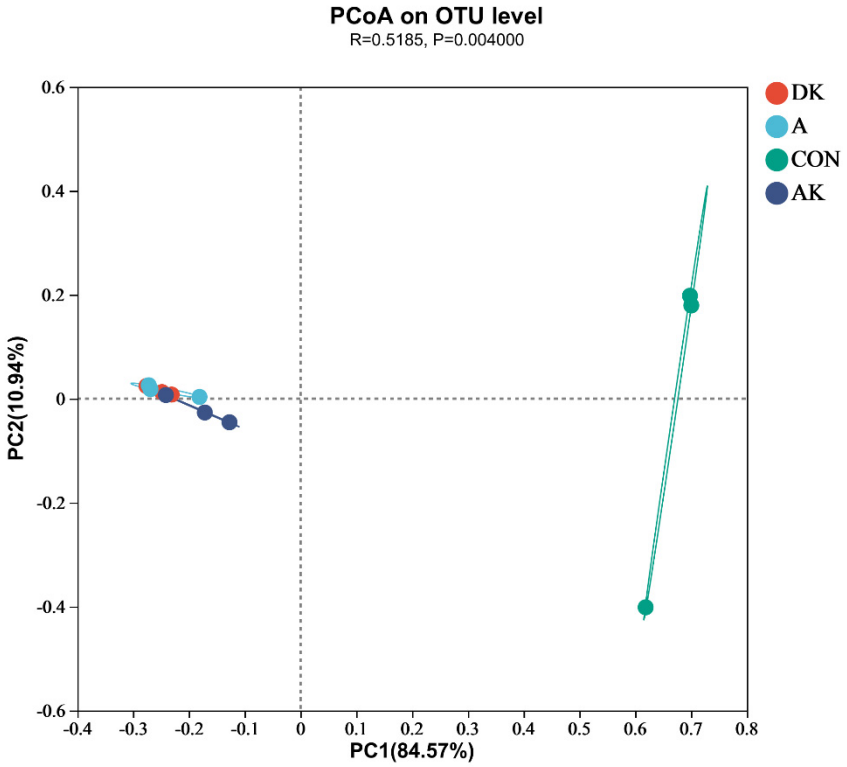

Figure 1. PCoA (Principal co-ordinates analysis) on the OTU level in fresh ML and ML silages.

3.5. Bacterial Abundance of Fresh Mulberry Leaves and Mulberry Leaf Silage

The abundances of bacterial communities of fresh ML and ML silages at the phylum level is presented in Figure 2. Firmicutes, comprising over 85% of the composition, represented the predominant phylum in the experimental groups, followed by Proteobacteria. Notably, the relative abundance of Proteobacteria was greater in the CON group.

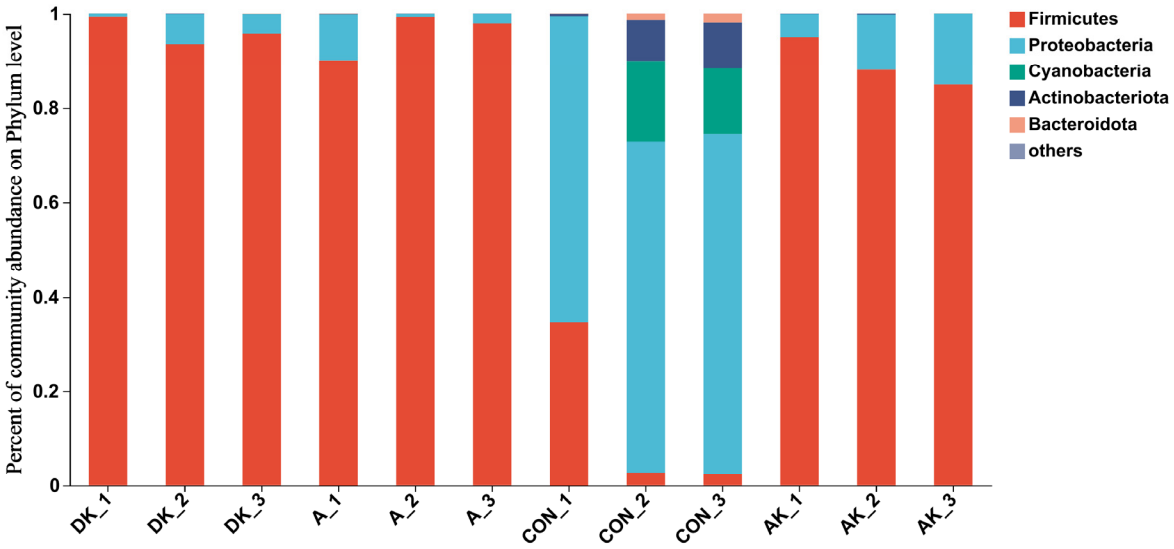

Figure 2. Microbial composition at the phylum level in fresh ML and ML silages.

The abundances of bacterial communities of fresh ML and ML silages at the genus level is presented in Figure 3. The relative abundance of *Pantoea*, *Enterobacteriaceae*, *Acinetobacter*, and *Chloroplast* was higher in the CON group. *Pediococcus* stood out as the dominant genus in the fermentation groups.

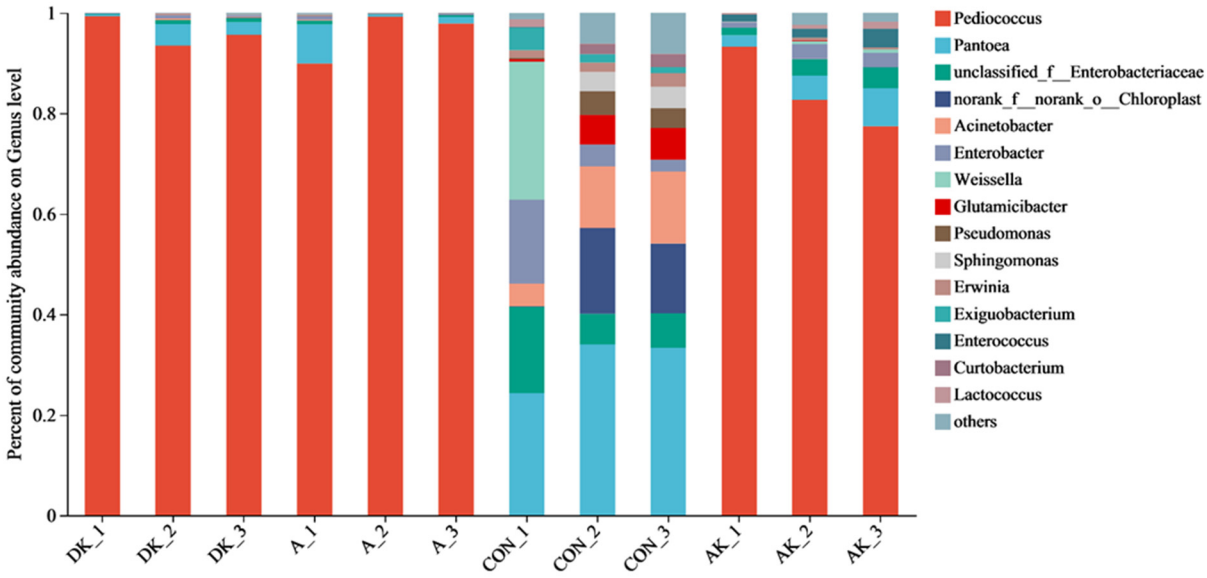

Figure 3. Microbial composition at the genus level in fresh ML and ML silages.

The LEfSe analysis of microbial composition differences between fresh mulberry leaves and mulberry leaf silage is presented in Figure 4. The LDA score indicated a significant enrichment of harmful bacteria, including *Enterobacteriaceae*, *Pseudomonas*, *Enterobacter*, and *Brachybacterium* in the CON group. By contrast, the DK group exhibited an enriched relative abundance of *Lactobacillaceae* and *Pediococcus*, while the AK group showed higher levels of beneficial bacteria such as *Lactococcus* and *Lactobacillus*.

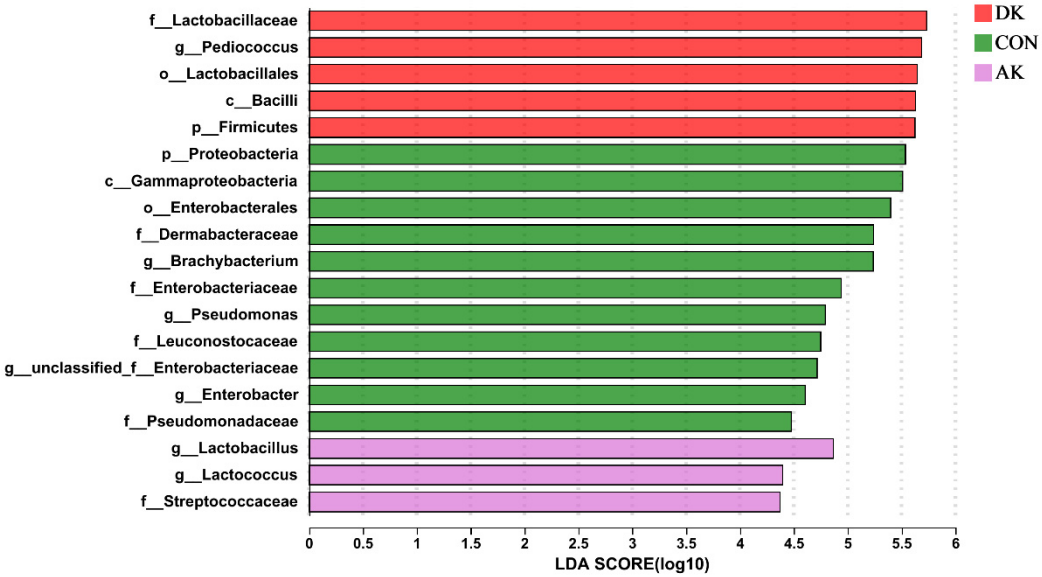

Figure 4. LDA in fresh ML and ML silages. LEfSe, linear discriminant analysis effect size; LDA, linear discriminant analysis. $p < 0.05$ and LDA score > 4 are displayed.

4. Discussion

In this study, the DM content in ML was significantly lower than that of the ideal DM content for high-quality silage (typically within the range of 30–35%) [34]. The CP content in ML measured 27.60% DM, indicating a higher level than previously reported [21]. This disparity in protein content may be attributed to the diverse varieties of mulberry leaves and their varying growth durations. Notably, the high protein content in mulberry leaf is comparable to alfalfa, suggesting that it can serve as an alternative to soybean meal and

other protein feeds. The WSC content, an essential factor for assessing fermentation quality, was found to be at a sufficient concentration of 7.47% DM in ML for ensuring adequate fermentation during ensiling [34]. In this regard, the WSC concentration in ML (7.47% DM) was sufficient for adequate fermentation during ensiling in terms of extent and rate. The epiphytic microbial community was considered to be a significant factor when predicting the adequacy of silage fermentation and determining whether or not to add inoculants to silage materials [35]. Moreover, fermented substrates with a lactic acid bacteria count exceeding 10^5 cfu/g FM were found to effectively improve silage quality [36]. However, in the present study, the LAB counts in mulberry leaves were measured at 4.32 log cfu/g FM, while undesirable microorganisms, including yeasts and coliform bacteria, exhibited counts of 4.37 and 3.66 log cfu/g FM, respectively. These low levels of LAB and water-soluble carbohydrates may lead to reduced lactic acid accumulation and elevated pH values, thereby promoting the growth of harmful microorganisms during the early stage of ensiling. Therefore, supplementation of sugar or inoculation of LAB is necessary to achieve high-quality silage.

Additionally, compared with fresh mulberry leaves, the fermentation groups showed an increase in crude protein content. This could be attributable to the fact that, on the one hand, microorganisms will consume part of the organic matter in mulberry leaves through respiration, resulting in a reduction in the total amount of product and a protein "concentration effect" of carbon mineralization (CO_2). On the other hand, protein sources include bacterial inoculum and ammonia in the culture medium [37]. The neutral detergent fiber and acid detergent fiber of silage mulberry leaves were found to be reduced, likely due to the action of cellulase produced by *Bacillus*, which degrades complexes such as cellulose into carbohydrates [24,38,39]. The pH of fermented feed plays a crucial role in its quality, with a pH of 4.2 generally considered the benchmark for good fermentation, especially for fermented feeds with a high moisture content, and a lower pH ensuring adequate fermentation and long-term preservation [40]. pH decreases during fermentation due to the accumulation of organic acids. In the present study, the pH of the A and AK groups reached a relatively low value. Furthermore, compared with the DK group, the lactic acid content of the A and AK groups was higher. Lactic acid (pKa 3.86) is usually produced mainly by homotypic fermented lactic acid bacteria using carbohydrate conversion, and it greatly contributes to the rapid reduction in the pH in fermented feeds because it is about 10–12 times more acidic than other major organic acids (pKa 4.75 for acetic acid and 4.87 pKa for propionate) [18,41]. The content of ammonia nitrogen in silage usually reflects the hydrolysis of proteins, which is another important indicator when evaluating the quality of fermentation [34]. The results of this study are similar to those of Wang et al. [21]. Ammonia nitrogen levels were also low in the A and AK groups, indicating the inhibition of clostridia growth due to the production of acid by lactic acid bacteria, thereby forming a low-pH environment. In this study, the addition of lactic acid bacteria strains A6 and A2 was suggested as a potential factor in homolactic acid fermentation, contributing to the predominant generation of lactic acid from the fermentation of glucose. The low lactic acid content in the DK group was due to the fact that lactic acid bacteria were not added. The dominant bacteria were lactic acid bacteria in the present study, which could explain the inhibition of harmful bacteria such as coliform bacteria [35].

Compared with fresh mulberry leaves, the polyphenol content and the antioxidant capacity of DPPH, ABTS, HAS, and FRAP in the fermentation groups were enhanced. Similarly, the study performed by He et al. [24] showed that the addition of cellulase and *Lactobacilli* increased the antioxidant capacity, which could be attributed to the role of polyphenols. Correlation analysis showed that the antioxidant activity of white and black mulberry extracts was significantly positively correlated with phenolic compounds [42].

In this study, lower Sobs, Chao1, and Shannon index values were exhibited in mulberry leaf silage relative to the fresh material, indicating an increase in the bacterial community diversity and richness in the fresh mulberry leaves. A similar result was also reported by He et al. [35]. It was inferred that the acids produced by the inoculated strains in

silage lead to a decrease in pH, and the acidic environment restricts the growth of some microorganisms [43]. Other similar results have been found by Wang et al. [21], who fermented mulberry leaves with *Lactobacillus casei* and molasses, and the lactic acid bacteria proliferated to become the dominant bacteria during the fermentation process, forming an acidic environment that was not conducive to the growth of other microorganisms, which, in turn, led to a decrease in the relative abundance and diversity of bacterial communities. The results of principal coordinates analysis (PCoA) revealed the alteration of the microbial community composition in mulberry leaf silage following the addition of *Pediococcus* and *Bacillus*. Similar results have been reported by Chi et al. [38], who found that the microbial community structure of the silage supplemented with mulberry was changed compared to P100. In this study, Firmicutes and Proteobacteria were the predominant phylum for 7-day silage. Similar results were also found by Wang et al. [21]. In general, Firmicutes and Proteobacteria are the most abundant phylum in silage at any time point in silage; this can be mainly attributed to the low pH and anaerobic conditions during silage, which favor the growth of Firmicutes and Proteobacteria [44]. In this study, the fermentation time of mulberry leaf silage was 7 days, and it was observed that *Pediococcus* was the predominant strain in the fermentation groups, which is consistent with the results of another study [45]. Typically, *Pediococcus* serves as the dominant LAB species, initiating lactic acid fermentation in the early stages of silage. However, as the pH drops, *Lactobacillus* will flourish and become the new dominant species [44,46]. Therefore, the number of *Enterobacteriaceae* in silage mulberry leaves is reduced compared to fresh mulberry leaves.

5. Conclusions

Overall, the study demonstrated that the addition of *Pediococcus* and *Bacillus* could improve the fermentation quality of mulberry leaf silage, and mulberry leaves treated with *Pediococcus acidilactici*, *Pediococcus pentosaceus*, *Bacillus subtilis*, and *Bacillus licheniformi* had better fermentation quality than other treatments. The AK group exhibited a higher content of crude protein, lower neutral detergent fiber, improved silage quality, enhanced antioxidant activity, increased abundance of desirable *Lactobacillus*, and reduced abundance of harmful bacteria such as *Enterobacter*.

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References

1. Li, Y.; Wang, J.Q.; Mei, J.; Huang, L.X.; Liu, H.Y. Effects of Mulberry Branch and Leaves Silage on Microbial Community, Rumen Fermentation Characteristics, and Milk Yield in Lactating Dairy Cows. *Fermentation* **2022**, *8*, 86. [CrossRef]
2. Cheng, Q.M.; Li, M.Y.; Fan, X.Y.; Chen, Y.L.; Sun, H.; Xie, Y.X.; Zheng, Y.L.; Chen, C.; Li, P. Effects of epiphytic and exogenous lactic acid bacteria on fermentation quality and microbial community compositions of paper mulberry silage. *Front. Microbiol.* **2022**, *13*, 973500. [CrossRef] [PubMed]
3. Cai, M.; Mu, L.; Wang, Z.L.; Liu, J.Y.; Liu, T.L.; Wanapat, M.; Huang, B.Z. Assessment of mulberry leaf as a potential feed supplement for animal feeding in PR China. *Asian-Australas. J. Anim. Sci.* **2019**, *32*, 1145–1152. [CrossRef] [PubMed]
4. Kandylis, K.; Hadjigeorgiou, I.; Harizanis, P. The nutritive value of mulberry leaves (*Morus alba*) as a feed supplement for sheep. *Trop. Anim. Health Prod.* **2009**, *41*, 17–24. [CrossRef] [PubMed]
5. Wang, Y.Y.; Shen, Q.M.; Zhong, S.; Chen, Y.L.; Yang, Y.X. Comparison of Rumen Microbiota and Serum Biochemical Indices in White Cashmere Goats Fed Ensiled or Sun-Dried Mulberry Leaves. *Microorganisms* **2020**, *8*, 981. [CrossRef] [PubMed]
6. Ouyang, J.L.; Wang, M.Z.; Hou, Q.R.; Feng, D.; Pi, Y.; Zhao, W.G. Effects of Dietary Mulberry Leaf Powder in Concentrate on the Rumen Fermentation and Ruminal Epithelium in Fattening Hu Sheep. *Animals* **2019**, *9*, 218. [CrossRef]
7. Al-kirshi, R.; Alimon, A.R.; Zulkifli, I.; Sazili, A.; Zahari, M.W.; Ivan, M. Utilization of mulberry leaf meal (*Morus alba*) as protein supplement in diets for laying hens. *Ital. J. Anim. Sci.* **2010**, *9*, 265–267. [CrossRef]
8. Chen, C.; Razali, U.H.M.; Saikim, F.H.; Mahyudin, A.; Noor, N. *Morus alba* L. Plant: Bioactive Compounds and Potential as a Functional Food Ingredient. *Foods* **2021**, *10*, 689. [CrossRef]
9. Thabti, I.; Elfalleh, W.; Tlili, N.; Ziadi, M.; Campos, M.G.; Ferchichi, A. Phenols, Flavonoids, and Antioxidant and Antibacterial Activity of Leaves and Stem Bark of *Morus* Species. *Int. J. Food Prop.* **2014**, *17*, 842–854. [CrossRef]
10. Katsube, T.; Imawaka, N.; Kawano, Y.; Yamazaki, Y.; Shiwaku, K.; Yamane, Y. Antioxidant flavonol glycosides in mulberry (*Morus alba* L.) leaves isolated based on LDL antioxidant activity. *Food Chem.* **2006**, *97*, 25–31. [CrossRef]
11. Park, E.; Lee, S.M.; Lee, J.E.; Kim, J.H. Anti-inflammatory activity of mulberry leaf extract through inhibition of NF-κB. *J. Funct. Foods* **2013**, *5*, 178–186. [CrossRef]
12. Ma, J.Y.; Ma, H.; Liu, S.J.; Wang, J.; Wang, H.L.; Zang, J.J.; Long, S.F.; Piao, X.S. Effect of Mulberry Leaf Powder of Varying Levels on Growth Performance, Immuno-Antioxidant Status, Meat Quality and Intestinal Health in Finishing Pigs. *Antioxidants* **2022**, *11*, 2243. [CrossRef] [PubMed]
13. Lin, W.C.; Lee, M.T.; Chang, S.C.; Chang, Y.L.; Shih, C.H.; Yu, B.; Lee, T.T. Effects of mulberry leaves on production performance and the potential modulation of antioxidative status in laying hens. *Poult. Sci.* **2017**, *96*, 1191–1203. [CrossRef] [PubMed]
14. Long, Y.; Han, Y.; Zhao, Y.F.; Chen, D.Q.; Wang, D.F.; Yang, Y.; Su, C.Z.; Shen, X.Y. Effect of Mulberry Leaf TMR on Growth Performance, Meat Quality and Expression of Meat Quality Master Genes (ADSL, H-FABP) in Crossbred Black Goats. *Foods* **2022**, *11*, 4032. [CrossRef] [PubMed]
15. Xv, Z.C.; He, G.L.; Wang, X.L.; Shun, H.; Chen, Y.J.; Lin, S.M. Mulberry leaf powder ameliorate high starch-induced hepatic oxidative stress and inflammation in fish model. *Anim. Feed. Sci. Technol.* **2021**, *278*, 115012. [CrossRef]
16. Zhang, Y.C.; Li, D.X.; Wang, X.K.; Lin, Y.L.; Zhang, Q.; Chen, X.Y.; Yang, F.Y. Fermentation quality and aerobic stability of mulberry silage prepared with lactic acid bacteria and propionic acid. *Anim. Sci. J.* **2019**, *90*, 513–522. [CrossRef] [PubMed]
17. Cui, X.P.; Yang, Y.X.; Zhang, M.J.; Jiao, F.; Gan, T.T.; Lin, Z.W.; Huang, Y.Z.; Wang, H.X.; Liu, S.; Bao, L.J.; et al. Optimized Ensiling Conditions and Microbial Community in Mulberry Leaves Silage With Inoculants. *Front. Microbiol.* **2022**, *13*, 813363. [CrossRef] [PubMed]
18. Zhang, Q.; Yu, Z.; Wang, X.G. Isolating and evaluating lactic acid bacteria strains with or without sucrose for effectiveness of silage fermentation. *Grassl. Sci.* **2015**, *61*, 167–176. [CrossRef]
19. Kung, L.M.; Shaver, R.D.; Grant, R.J.; Schmidt, R.J. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. *J. Dairy Sci.* **2018**, *101*, 4020–4033. [CrossRef]
20. Trabi, E.B.; Yuan, X.J.; Li, J.F.; Dong, Z.H.; Shah, A.A.; Shao, T. Effect of Glucose and Lactic Acid Bacteria on the Fermentation Quality, Chemical Compositions and in vitro Digestibility of Mulberry (*Morns alba*) Leaf Silage. *Pak. J. Zool.* **2017**, *49*, 2271–2277. [CrossRef]
21. Wang, Y.; Chen, X.Y.; Wang, C.; He, L.W.; Zhou, W.; Yang, F.Y.; Zhang, Q. The bacterial community and fermentation quality of mulberry (*Morus alba*) leaf silage with or without Lactobacillus casei and sucrose. *Bioresour. Technol.* **2019**, *293*, 122059. [CrossRef] [PubMed]
22. Zhao, X.L.; Wang, F.; Fang, Y.; Zhou, D.W.; Wang, S.P.; Wu, D.Q.; Wang, L.X.; Zhong, R.Z. High-potency white-rot fungal strains and duration of fermentation to optimize corn straw as ruminant feed. *Bioresour. Technol.* **2020**, *312*, 123512. [CrossRef] [PubMed]
23. You, J.T.; Zhang, H.; Zhu, H.F.; Xue, Y.L.; Cai, Y.M.; Zhang, G.J. Microbial Community, Fermentation Quality, and in vitro Degradability of Ensiling *Caragana* With Lactic Acid Bacteria and Rice Bran. *Front. Microbiol.* **2022**, *13*, 804429. [CrossRef] [PubMed]
24. He, L.W.; Zhou, W.; Wang, C.; Yang, F.Y.; Chen, X.Y.; Zhang, Q. Effect of cellulase and *Lactobacillus casei* on ensiling characteristics, chemical composition, antioxidant activity, and digestibility of mulberry leaf silage. *J. Dairy Sci.* **2019**, *102*, 9919–9931. [CrossRef] [PubMed]
25. Van Soest, P.J.; Robertson, J.B.; Lewis, B.A. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597. [CrossRef] [PubMed]

26. Murphy, R.P. A method for the extraction of plant samples and the determination of total soluble carbohydrates. *J. Sci. Food Agric.* **1958**, *9*, 714–717. [CrossRef]
27. Broderick, G.A.; Kang, A.J.H. Automated Simultaneous Determination of Ammonia and Total Amino Acids in Ruminal Fluid and In Vitro Media. *J. Dairy Sci.* **1980**, *63*, 64–75. [CrossRef] [PubMed]
28. Ma, Q.Q.; Santhanam, R.K.; Xue, Z.H.; Guo, Q.W.; Gao, X.D.; Chen, H.X. Effect of different drying methods on the physicochemical properties and antioxidant activities of mulberry leaves polysaccharides. *Int. J. Biol. Macromol.* **2018**, *119*, 1137–1143. [CrossRef]
29. Cuesta, G.; Suarez, N.; Bessio, M.I.; Ferreira, F.; Massaldi, H. Quantitative determination of pneumococcal capsular polysaccharide serotype 14 using a modification of phenol-sulfuric acid method. *J. Microbiol. Methods* **2003**, *52*, 69–73. [CrossRef]
30. Jiang, X.; Liu, X.; Xu, H.J.; Sun, Y.K.; Zhang, Y.G.; Wang, Y. Improvement of the nutritional, antioxidant and bioavailability properties of corn gluten-wheat bran mixture fermented with lactic acid bacteria and acid protease. *Lwt-Food Sci. Technol.* **2021**, *144*, 111161. [CrossRef]
31. Liao, B.Y.; Zhu, D.Y.; Thakur, K.; Li, L.; Zhang, J.G.; Wei, Z.J. Thermal and Antioxidant Properties of Polysaccharides Sequentially Extracted from Mulberry Leaves (*Morus alba* L.). *Molecules* **2017**, *22*, 2271. [CrossRef] [PubMed]
32. Yuan, Q.X.; Xie, Y.F.; Wang, W.; Yan, Y.H.; Ye, H.; Jabbar, S.; Zeng, X.X. Extraction optimization, characterization and antioxidant activity in vitro of polysaccharides from mulberry (*Morus alba* L.) leaves. *Carbohydr. Polym.* **2015**, *128*, 52–62. [CrossRef] [PubMed]
33. Zou, Y.X.; Liao, S.T.; Shen, W.Z.; Liu, F.; Tang, C.M.; Chen, C.Y.O.; Sun, Y.M. Phenolics and Antioxidant Activity of Mulberry Leaves Depend on Cultivar and Harvest Month in Southern China. *Int. J. Mol. Sci.* **2012**, *13*, 16544–16553. [CrossRef] [PubMed]
34. He, L.W.; Lv, H.J.; Chen, N.; Wang, C.; Zhou, W.; Chen, X.Y.; Zhang, Q. Improving fermentation, protein preservation and antioxidant activity of Moringa oleifera leaves silage with gallic acid and tannin acid. *Bioresour. Technol.* **2020**, *297*, 122390. [CrossRef] [PubMed]
35. He, L.W.; Wang, C.; Xing, Y.Q.; Zhou, W.; Pian, R.Q.; Yang, F.Y.; Chen, X.Y.; Zhang, Q. Dynamics of proteolysis, protease activity and bacterial community of *Neolamarckia cadamba* leaves silage and the effects of formic acid and *Lactobacillus farciminis*. *Bioresour. Technol.* **2019**, *294*, 122127. [CrossRef]
36. Cai, Y.M.; Benno, Y.; Ogawa, M.; Ohmomo, S.; Kumai, S.; Nakase, T. Influence of *Lactobacillus* spp. from an inoculant and of *Weissella* and *Leuconostoc* spp. from forage crops on silage fermentation. *Appl. Environ. Microbiol.* **1998**, *64*, 2982–2987. [CrossRef] [PubMed]
37. Opazo, R.; Ortúzar, F.; Navarrete, P.; Espejo, R.; Romero, J. Reduction of Soybean Meal Non-Starch Polysaccharides and α-Galactosides by Solid-State Fermentation Using Cellulolytic Bacteria Obtained from Different Environments. *PLoS ONE* **2012**, *7*, e44783. [CrossRef]
38. Chi, Z.; Deng, M.; Tian, H.C.; Liu, D.W.; Li, Y.K.; Liu, G.B.; Sun, B.L.; Guo, Y.Q. Effects of Mulberry Leaves and Pennisetum Hybrid Mix-Silage on Fermentation Parameters and Bacterial Community. *Fermentation* **2022**, *8*, 197. [CrossRef]
39. Jain, J.; Kumar, A.; Singh, D.; Singh, B. Purification and kinetics of a protease-resistant, neutral, and thermostable phytase from *Bacillus subtilis* subsp. subtilis JJBS250 ameliorating food nutrition. *Prep. Biochem. Biotechnol.* **2018**, *48*, 718–724. [CrossRef]
40. He, L.W.; Chen, N.; Lv, H.J.; Wang, C.; Zhou, W.; Chen, X.Y.; Zhang, Q. Gallic acid influencing fermentation quality, nitrogen distribution and bacterial community of high-moisture mulberry leaves and stylo silage. *Bioresour. Technol.* **2020**, *295*, 122255. [CrossRef]
41. Ni, K.K.; Wang, F.F.; Zhu, B.G.; Yang, J.X.; Zhou, G.A.; Pan, Y.; Tao, Y.; Zhong, J. Effects of lactic acid bacteria and molasses additives on the microbial community and fermentation quality of soybean silage. *Bioresour. Technol.* **2017**, *238*, 706–715. [CrossRef] [PubMed]
42. Polumackanycz, M.; Wesolowski, M.; Viapiana, A. *Morus alba* L. and *Morus nigra* L. Leaves as a Promising Food Source of Phenolic Compounds with Antioxidant Activity. *Plant Food Hum. Nutr.* **2021**, *76*, 458–465. [CrossRef] [PubMed]
43. Méndez-García, C.; Peláez, A.I.; Mesa, V.; Sánchez, J.; Golyshina, O.V.; Ferrer, M. Microbial diversity and metabolic networks in acid mine drainage habitats. *Front. Microbiol.* **2015**, *6*, 475.
44. Liu, B.Y.; Huan, H.L.; Gu, H.R.; Xu, N.X.; Shen, Q.; Ding, C.L. Dynamics of a microbial community during ensiling and upon aerobic exposure in lactic acid bacteria inoculation-treated and untreated barley silages. *Bioresour. Technol.* **2019**, *273*, 212–219. [CrossRef]
45. Wang, Y.; He, L.W.; Xing, Y.Q.; Zhou, W.; Pian, R.Q.; Yang, F.Y.; Chen, X.Y.; Zhang, Q. Bacterial diversity and fermentation quality of *Moringa oleifera* leaves silage prepared with lactic acid bacteria inoculants and stored at different temperatures. *Bioresour. Technol.* **2019**, *284*, 349–358. [CrossRef]
46. Zhou, Y.; Drouin, P.; Lafrenière, C. Effect of temperature (5–25 °C) on epiphytic lactic acid bacteria populations and fermentation of whole-plant corn silage. *J. Appl. Microbiol.* **2016**, *121*, 657–671. [CrossRef]