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Effects of Mulberry Leaves and *Pennisetum* Hybrid Mix-Silage on Fermentation Parameters and Bacterial Community

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Abstract: The silage quality and bacterial community of hybrid *Pennisetum* (*P. hydridum* × *P. americanum*) with or without 30% and 50% mulberry leaves for 3, 7, 14, and 30 days were investigated. Results showed that compared with the 100% hybrid *Pennisetum* group, more lactic acid (40.71 vs. 80.81 g/kg dry matter (DM)), acetic acid (10.99 vs. 31.84 g/kg DM), lactic acid bacteria (8.46 vs. 8.51 $\log_{10}$ cfu/g fresh matter), water-soluble carbohydrates (2.41 vs. 4.41 g/100 g DM), crude protein (4.97 vs. 10.84 g/100 g DM), and true protein (3.91 vs. 8.52 g/100 g DM) content as well as less neutral detergent fiber (67.30 vs. 47.26 g/100 g DM), acid detergent fiber (33.85 vs. 25.38 g/100 g DM), and yeast counts (4.78 vs. 2.39 $\log_{10}$ cfu/g fresh matter) and an appropriate pH (3.77 vs. 4.06) were found in silages added with 50% mulberry leaves at 30 days of ensiling. Moreover, the addition of mulberry leaves also influenced the relative abundance of the bacterial community. The relative abundance of *Firmicutes* increased and *Proteobacteria* decreased when mulberry leaves were added. *Weissella* and *Lactobacillus* abundance also increased. To sum up the above, mixing with 50% mulberry leaves yielded the greatest fermentation quality in this study. In conclusion, mixing with mulberry leaves could be a reasonable way to improve the quality of hybrid *Pennisetum* silage.

Keywords: *Pennisetum* hybrid; mulberry leaves; fermentation parameters; bacterial community

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1. Introduction

The need for livestock and poultry production, as well as traditional feed resources, is growing as populations grow and people's living standards rise. Feedstuff scarcity has grown more visible, and the price of traditional feedstuffs with high consumption has progressively risen [1,2]. One of the effective measures for solving the shortage of forage is the development of new crude feeds for local conditions.

Hybrid *Pennisetum* (*P. hydridum* × *P. americanum*) can be found in several Chinese provinces, and it is a prolific and renewable herbaceous plant that has attracted increasing research due to its various advantages, such as salinity and drought resistance, rapid growth, adaptability, and high biological yield [3,4]. Hybrid *Pennisetum* is widely utilized as a bioenergy grass and may also be used as livestock feed. Hybrid *Pennisetum* is also a popular tropical grass that is also one of the most prolific. In addition, it is a resourceful plant that can thrive in a variety of situations, including wet or dry regions, and may be grown by small farmers or large-scale agribusiness. In addition, it is an essential fodder that is widely used in the tropics and subtropics [5].

Mulberry (*Morus alba*) leaf, a Moraceous plant, is widely utilized as Chinese traditional medicine for humans and the primary feeding source for silkworms [6]. It has high protein contents with antioxidant and antibacterial effects, which can be used as green additives to replace antibiotics [7]. As an excellent protein supplement, mulberry leaves are widely used in the diets of terrestrial farm animals such as pigs and sheep, which can promote the growth of animals [8,9]. At the same time, it can adapt to many environmental conditions,

from harsh cold regions to tropical regions. Therefore, mulberry leaves are biologically important feedstuff with important biological activities.

However, mulberry leaves have relatively high moisture content and high buffering energy, making them difficult to store for a long time. It is known that gramineous forage is better preserved than legumes due to a higher content of soluble carbohydrates, but its relatively lower nutritional value and higher fiber content make it difficult to promote animal production. In contrast, leguminous plants have less fermentable carbohydrates but have greater protein and mineral content [10]. Therefore, mixed silage of a certain proportion of mulberry leaves and hybrid *Pennisetum* could increase the success rate of silage and contain complementarity of nutrients.

The present study was conducted to examine the addition of mulberry leaves to the fermentation characteristics and bacterial community composition of hybrid *Pennisetum* and mulberry leaves mixed silage. We aim to broaden the choice of forage and provide a basis for the further utilization of hybrid *Pennisetum* and mulberry leaves mixed silage.

2. Materials and Methods

2.1. Silage Preparation

At the Yuyuan Agriculture and Animal Husbandry Base in November 2020 (Meizhou, China, 115°95′ E, 24°64′ N), stems and leaves of hybrid *Pennisetum* (*P. hydridum* × *P. americanum*) and mulberry leaves were harvested and chopped to 1–2 cm. It is considered that grass forages are better mixed with leguminous forages for silage when the leguminous forage content is less than 60% [10]. Therefore, the silages were then separated into three mixing ratios of hybrid *Pennisetum* (P) and mulberry leaves (M) based on raw material fresh weight: 100:0 (P100), 70:30 (PM73), and 50:50 (PM55). Hybrid *Pennisetum* was cut at a height of 2 m with a stubble of 20–30 cm. Mulberry leaves were chopped at a height of 1.5 m with a 20–30 cm stubble. A total of 36 bags, each weighing 200 g, were used (3 mixing ratios, 4 silage days, and 3 repeats). After each group of raw materials had been thoroughly mixed according to three treatments, the silages were placed in a polyethylene bag and vacuumed to imitate wrapping silage with a vacuum packing machine (Deli 14886, Guangdong, China). On days 3, 7, 14, and 30, samples were opened for examination of fermentation characteristics and bacterial community.

2.2. Chemical Composition and Fermentation Quality of Silage

Ten grams of individual silage samples were homogenized with distilled water (90 mL) in an orbital shaker at room temperature, and the supernatants were then serially diluted from 10^{-1} to 10^{-6}. Yeast and mold counts were incubated and counted using Rose Bengal agar (Sigma, St. Louis, MO, USA) at 28 °C for 48 h. Lactic acid bacteria (LAB) were cultured, respectively, on de Man, Rogosa, Sharpe (MRS) agar (Oxoid, London, UK) at 30 °C for 48 h [11]. Another sample (10 g) from each silage bag was diluted in sterile distilled water (90 mL) and frozen at 4 °C for 18 h before being filtered. Then, the filtrate was used to measure pH value with a pH meter (Sartorius, PB-10, Gottingen, Germany). According to Josefa et al. [12], lactic acid (LA) was detected by a colorimetric method. According to Erwin et al. [13], acetic acid (AA), propionic acid (PA), and butyric acid (BA) were detected by the high-efficiency gas chromatograph (Agilent 7890B). The ammonia-N (NH_3-N) content was detected by the phenol-hypochlorite colorimetric method [14]. The surplus silages were oven-dried for calculating dry matter (DM) and ground for chemical analysis. Those chemical compounds were analyzed in triplicate and expressed on DM basis. Crude protein (CP) and true protein (TP) were analyzed by the Kjeldahl nitrogen analyzer according to the methods of the Association of Official Analytical Chemists [15]. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed according to the method of Van Soest et al. [16]. The content of water-soluble carbohydrates (WSC) was detected by the anthrone method [17].

2.3. Bacterial Community Analysis

Microbial DNA was extracted using the HiPure Soil DNA Kits (or HiPure Stool DNA Kits) (Magen, Guangzhou, China). The 16S rDNA target region of the ribosomal RNA gene was amplified by PCR (95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 7 min [18]. The V3–V4 regions of 16S rDNA were amplified using the primers 515F (5′-GTGCCAGCMGCCGCGGTAA -3′) and 806R (5′-GGACTACHVGGGTATCTAAT-3′) [18]. PCR reactions were performed in triplicate 50 µL mixture containing 10 µL of 5× Q5@ Reaction Buffer, 10 µL of 5× Q5@ High GC Enhancer, 1.5 µL of 2.5 mM dNTPs, 1.5 µL of each primer (10 µM), 0.2 µL of Q5@ High-Fidelity DNA Polymerase, and 50 ng of template DNA. Related PCR reagents were from New England Biolabs (NEB), Ipswich, CA, USA. 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 and quantified using ABI StepOnePlus Real-Time PCR System (Life Technologies, Foster City, CA, USA). Purified amplicons were pooled in equimolar concentrations and paired-end sequenced (PE250) on an Illumina platform according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database. The paired-end sequenced (PE250) was performed on the Illumina Novaseq 6000 platform. The raw reads were assembled according to the procedure set by Liu et al. [19]. Alpha diversity was analyzed in QIIME (version 1.9.1). Beta diversity was analyzed in the R Project Vegan package (version 2.5.3). The functions of microflora were predicted based on the PICRUSt (version 2.1.4) database.

2.4. Statistical Analyses

The effects of mixing rate and ensiling days on the fermentation quality and chemical characteristics of mulberry leaves and hybrid *Pennisetum* mix-silage were analyzed with IBM SPSS 22.0 for Windows statistical software package. The results were evaluated using two-way analysis of variance (ANOVA), with Duncan's multiple range tests. Statistical significance was determined at the $p < 0.05$ level [20].

3. Results and Discussion

3.1. Characteristics of Pre-Ensiled Mulberry Leaves and Hybrid Pennisetum Material

The characteristics of silage raw materials are shown in Table 1. The CP content of hybrid *Pennisetum* (6.40% DM) and mulberry leaves (13.92% DM) was relatively lower than the results reported by Ogunsakin et al. [21]. The difference might be because the forage quality could be influenced by many factors, such as climate [22], fertilization, and harvest time [23]. Among the two raw materials, hybrid *Pennisetum*'s crude protein (6.40%) is much lower than mulberry leaves (13.92%), while DM (35.35%), WSC (10.27%), NDF (72.60%), and ADF (38.32%) were higher than mulberry leaves' DM (32.95%), WSC (7.34%), NDF (33.93%), and ADF (22.03%). The relatively high CP content and low fiber content of mulberry leaves indicate that mulberry leaves can potentially be used as a high-quality protein source for animals. The WSC content of mulberry leaves and hybrid *Pennisetum* is greater than 6% DM, so it is sufficient to ensure fermentation quality during silage [24].

Table 1. The chemical composition of pre-ensiled mulberry leaves and hybrid *Pennisetum*.

Items	DM (%)	WSC (% DM)	NDF (% DM)	ADF (% DM)	CP (% DM)
Mulberry leaves (± SEM)	32.95 ± 1.54	7.34 ± 0.52	33.93 ± 1.27	22.03 ± 1.05	13.92 ± 0.05
Hybrid *Pennisetum* (±SEM)	35.35 ± 0.38	10.27 ± 0.44	72.60 ± 1.76	38.32 ± 0.15	6.40 ± 0.01

3.2. Fermentation Quality of Mulberry Leaves and Hybrid Pennisetum Mix-Silage

3.2.1. The Chemical Composition of Mulberry Leaves and Hybrid *Pennisetum* Mix-Silage

The chemical composition of mixed silages is shown in Table 2. Factor analysis revealed that ensiling days (D), mixing rate (T), and the interaction of days and rate had significant effects on DM and WSC content. Ensiling days and mixing rate had a significant effect on NDF and ADF. The DM content of each group remained relatively stable. At 3, 7, and 30 days of ensiling, DM content in the PM73 group was significantly higher ($p < 0.01$) than in the P100 and PM55 groups. In this study, the DM content of three treatments was between 34–37%, which was slightly higher than the ideal content of 30–35% DM [25]. The DM content was not excessively high enough to lead to a restricted fermentation, as evidenced by the good levels of total acids and pH [26].

Table 2. The chemical composition of mulberry leaves and hybrid *Pennisetum* mix-silage.

Item	Treatment	Ensiling Days				SEM	*p* Value		
		3	7	14	30		D	T	D × T
DM (%)	P100	34.73 ^{b}	35.09 ^{b}	34.52 ^{b}	35.10 ^{b}	0.046	<0.01	<0.01	0.001
	PM73	35.33 ^{aB}	36.62 ^{aA}	36.53 ^{aA}	36.64 ^{aA}				
	PM55	34.36 ^{bB}	35.33 ^{bA}	35.73 ^{aA}	36.03 ^{bA}				
WSC (% DM)	P100	6.44 ^{A}	4.56 ^{bB}	3.02 ^{bC}	2.41 ^{bC}	0.042	<0.01	<0.01	<0.01
	PM73	6.18 ^{A}	5.54 ^{aB}	4.71 ^{aC}	4.35 ^{aD}				
	PM55	5.93 ^{A}	5.25 ^{aB}	4.88 ^{aB}	4.41 ^{aC}				
NDF (% DM)	P100	70.53 ^{aA}	69.01 ^{aA}	68.41 ^{aA}	67.30 ^{aB}	0.17	<0.01	<0.01	0.81
	PM73	60.58 ^{bA}	58.54 ^{bA}	58.76 ^{bA}	57.70 ^{bB}				
	PM55	50.48 ^{cA}	49.89 ^{cA}	48.65 ^{cB}	47.26 ^{cC}				
ADF (% DM)	P100	36.93 ^{aA}	35.37 ^{aB}	34.31 ^{aC}	33.85 ^{aD}	0.078	<0.01	<0.01	0.26
	PM73	32.73 ^{bA}	31.96 ^{bA}	31.48 ^{bA}	30.32 ^{bB}				
	PM55	27.59 ^{cA}	27.01 ^{cA}	26.10 ^{cA}	25.38 ^{cB}				

D, ensiling days; T, treatments; D × T, the interaction of ensiling days and treatments; DM, dry matter; P100, 100% hybrid *Pennisetum* silage; PM73, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 7:3; PM55, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 1:1. Different capital letters (A–D) in the same row indicate significant differences between different days of the same treatment at $p < 0.05$; different lowercase letters (a–c) in the same column indicate significant differences between different treatments on the same day at $p < 0.05$.

The WSC content significantly decreased ($p < 0.01$) with the increase in ensiling days and decreased rapidly at the beginning of ensiling. The WSC content decreased with mulberry leaves addition at 3 days of ensiling. However, after 3 days of the ensiling, a reverse phenomenon was observed. Compared with P100, the PM73 and PM55 groups were significantly higher ($p < 0.01$). Additionally, at 30 days of ensiling, WSC content in PM55 was the highest, which can be better preserved and is conducive to generating more lactic acid, improving silage quality, which in turn may help to obtain good fermentation quality.

Compared with group P100, with the increase in mulberry leaves, the contents of NDF and ADF were significantly reduced ($p < 0.01$), and the PM55 group possessed the lowest amounts (47.26%, 25.38%). After 30 days of ensiling, the PM55 treatment was more conducive to the degradation of structural carbohydrates, which is related to the lower fiber raw material characteristics of mulberry leaves.

3.2.2. The Protein Fraction of Mulberry Leaves and Hybrid *Pennisetum* Mix-Silage

The dynamics of the protein fractions of mulberry leaves and hybrid *Pennisetum* mixed silage are shown in Table 3. Factor analysis revealed that ensiling days (D), mixing rate (T), and the interaction of days and rate had significant effects on crude protein, true protein content, and ammonia-N content. Compare with P100, the treatment adding mulberry leaves showed significantly higher ($p < 0.01$) CP and TP content in the same fermentation period, and PM55 had the highest content. Except for 30 days of ensiling, the TP/CP rate gradually increased after mixing with mulberry leaves and the PM55 group was significantly higher ($p < 0.01$) than the P100 group, implying that the proteins could be

preserved better and more stably after mixing with 50% mulberry leaves in the early ensiling time. Moreover, when the ensiling time was prolonged, NH_3-N content gradually increased. NH_3-N content usually reflects protein decomposition, which is another important indicator for evaluating fermentation quality [27]. After 30 days of ensiling, the content of NH_3-N increased ($p < 0.01$) significantly with the increase in mulberry leaves, and PM55 possessed the highest content, mainly because of the higher protein content of mulberry leaves than hybrid *Pennisetum*, resulting in increasing the protein decomposition. Santana et al. [10] found a similar phenomenon that when the proportion of *L. leucocephala* increased, the NH_3-N content of *Pennisetum purpureum* and *L. leucocephala* mixed silage increased.

Table 3. The protein fraction of mulberry leaves and hybrid *Pennisetum* mix-silage.

Item	Treatment	Ensiling Days				SEM	*p* Value		
		3	7	14	30		D	T	D × T
CP (% DM)	P100	5.32 ^{cA}	5.04 ^{cB}	4.96 ^{cB}	4.97 ^{cB}	0.016	0.001	<0.01	0.01
	PM73	8.31 ^{bA}	8.18 ^{bAB}	8.07 ^{bB}	8.27 ^{bA}				
	PM55	10.76 ^{aA}	10.65 ^{aB}	10.69 ^{aA}	10.84 ^{aA}				
TP (% DM)	P100	3.96 ^{cA}	3.76 ^{cB}	3.86 ^{cAB}	3.91 ^{cA}	0.014	0.023	<0.01	0.009
	PM73	6.18 ^{bAB}	6.21 ^{bAB}	6.32 ^{bA}	6.13 ^{bB}				
	PM55	8.76 ^{aA}	8.74 ^{aA}	8.71 ^{aA}	8.52 ^{aB}				
TP/CP	P100	0.750 ^{bA}	0.760 ^{bA}	0.779 ^{bAB}	0.787 ^{aAB}	0.003	0.031	<0.01	0.017
	PM73	0.749 ^{bB}	0.761 ^{bAB}	0.783 ^{bA}	0.741 ^{bAB}				
	PM55	0.814 ^{aA}	0.821 ^{aA}	0.815 ^{aA}	0.785 ^{aB}				
NH_3-N (g/kg DM)	P100	0.25 ^{cC}	0.30 ^{cB}	0.37 ^{cA}	0.39 ^{cA}	0.003	<0.01	<0.01	<0.01
	PM73	0.34 ^{bC}	0.41 ^{bB}	0.42 ^{bB}	0.44 ^{bA}				
	PM55	0.40 ^{aD}	0.48 ^{aC}	0.52 ^{aB}	0.65 ^{aA}				

D, ensiling days; T, treatments; D × T, the interaction of ensiling days and treatments; DM, dry matter; P100, 100% hybrid *Pennisetum* silage; PM73, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 7:3; PM55, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 1:1. Different capital letters (A–D) in the same row indicate significant differences between different days of the same treatment at $p < 0.05$; different lowercase letters (a–c) in the same column indicate significant differences between different treatments on the same day at $p < 0.05$.

3.2.3. Organic Acid Contents, pH of Mulberry Leaves and Hybrid *Pennisetum* Mixed Silage

The organic acids and pH of mixed silages are shown in Table 4. The factor analysis revealed that ensiling time, mixing proportion, and interaction of time and proportion had a significant effect ($p < 0.01$) on lactic acid, acetic acid, and pH.

Values of pH constitute an important index of silage fermentation. The purpose of ensiling is to reduce pH below 4.2 as soon as possible to produce stable silage [28]. The results in Table 4 show that the pH of silage increased with the increase in mulberry leaves. Chen et al. [29] found that the high pH of mulberry leaves silage may be caused by the high buffer capacity of mulberry leaves, so the pH increase after mulberry leaves were added may be due to the high buffer capacity of mulberry leaves [30]. The pH of each treatment reached the lowest at 30 days of silage, and all reached the good silage pH range (<4.2).

During the ensiling days, the lactic acid content of the treatments with added mulberry leaves was significantly increased. With the extension of ensiling time, lactic acid content increased. At 30 days of ensiling, the lactic acid content of each treatment reached the highest amount, and the PM55 treatment had the highest lactic acid content. This result may be attributed to the rapid accumulation of lactic acid caused by sufficient WSC content [27], which indicates that mixing hybrid *Pennisetum* and mulberry leaves can obtain high-quality silage. When the proportion of mulberry leaves is increased, the fermentation mediated by lactic acid bacteria proceeds rapidly while inhibiting the growth of harmful acetogenic bacteria and preventing the occurrence of acetic acid fermentation. This indicates that the mixed silage of mulberry leaves and hybrid *Pennisetum* may reduce the loss of dry matter and energy.

Table 4. Organic acid contents, pH of mulberry leaves and hybrid *Pennisetum* mixed silage.

Item	Treatment	Ensiling Days				SEM	*p* Value		
		3	7	14	30		D	T	D × T
Lactic acid (g/kg DM)	P100	31.56 ^{bB}	36.37 ^{bA}	36.54 ^{cA}	40.71 ^{cA}	0.52	<0.01	<0.01	<0.01
	PM73	55.62 ^{aB}	59.18 ^{aB}	68.02 ^{bA}	72.16 ^{bA}				
	PM55	57.88 ^{aC}	58.42 ^{aC}	74.37 ^{aB}	80.81 ^{aA}				
Acetic acid (g/kg DM)	P100	4.44 ^{bD}	6.00 ^{cC}	9.01 ^{cB}	10.99 ^{cA}	0.14	<0.01	<0.01	<0.01
	PM73	6.76 ^{aC}	21.78 ^{aB}	22.15 ^{bB}	24.72 ^{bA}				
	PM55	7.96 ^{aC}	18.84 ^{bB}	33.80 ^{aA}	31.84 ^{aA}				
Propanoic acid (g/kg DM)	P100	0	0	0	0	–	–	–	–
	PM73	2	0	0	0				
	PM55	1.96	0.75	1.31	0				
Butyric acid (g/kg DM)	P100	0	0	0	0	–	–	–	–
	PM73	2.38	0	0	0				
	PM55	0	0	0	0				
pH	P100	4.06 ^{bA}	4.01 ^{bB}	3.95 ^{cC}	3.77 ^{cD}	0.006	<0.01	<0.01	<0.01
	PM73	4.40 ^{aA}	4.20 ^{aB}	4.23 ^{aB}	3.99 ^{bC}				
	PM55	4.46 ^{aA}	4.24 ^{aB}	4.18 ^{bC}	4.06 ^{aD}				

D, ensiling days; T, treatments; D × T, the interaction of ensiling days and treatments; DM, dry matter; P100, 100% hybrid *Pennisetum* silage; PM73, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 7:3; PM55, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 1:1. Different capital letters (A–D) in the same row indicate significant differences between different days of the same treatment at $p < 0.05$; different lowercase letters (a–c) in the same column indicate significant differences between different treatments on the same day at $p < 0.05$.

Moreover, with the extension of ensiling time, the acetic acid content gradually increased. At the same ensiling time, with the proportion of mulberry leaves increased, the acetic acid content increased significantly ($p < 0.01$) in the late silage period (14–30 days). It may be because the addition of mulberry leaves allows some species of lactobacillus to metabolize lactic acid to acetic acid under conditions of sugar deficiency, thereby increasing the acetic acid content [31]. In addition, butyric acid and propanoic acid were only detected in very few groups, probably due to the rapid growth of lactic acid bacteria and the rapid decrease in pH value; clostridia were inhibited during the silage process, and the growth of butyric acid was inhibited. Similarly, most microorganisms that cause spoilage are usually inhibited at pH < 4.5 which proves the above deduction [31]. Overall, a certain proportioned addition of mulberry leaves may promote the accumulation of lactic acid and improve the quality of mixed silage.

3.3. Bacterial Community of Mulberry Leaves and Hybrid Pennisetum Silage

3.3.1. The Microbial Population of Mulberry Leaves and Hybrid *Pennisetum*

The dynamics of lactic acid bacteria, yeasts, and molds counts are shown in Table 5. Factor analysis revealed that ensiling days (D), mixing rate (T), and the interaction of days and rate had significant effects ($p < 0.01$) on lactic acid bacteria and yeasts counts. Large numbers of lactic acid bacteria can reduce pH rapidly and limit the multiplication of dangerous microbes, enhancing silage fermentation quality and lowering losses [32]. During the ensiling process, with the extension of silage time, lactic acid bacteria increased rapidly and decreased slowly. This may be because some lactic acid bacteria such as *Leuconostocs*, *Pediococcus*, *Lactococci*, and Enterococci are inhibited due to their low tolerance to low pH [33]. The lactic acid bacteria counts in the PM73 and PM55 treatment groups were significantly higher ($p < 0.01$) than that in the P100 group, which is consistent with the higher content of lactic acid. Yeast is the initiator of aerobic degradation of silage [34]. Avila et al. [35] reported that during silage fermentation, the growth and metabolism of yeast always led to the loss of DM. As the silage time increased, the yeast population in each treatment first increased rapidly and then decreased, with the peak occurring on day 14. At 3, 7, and 14 days of silage, the yeast content of the PM55 treatment was significantly lower than that of the PM73 treatment, and the content of the PM73 treatment was significantly

lower than that of the P100 treatment. At 30 days of silage, the content of the PM55 treatment was significantly lower than the PM73 and P100 treatments, and the PM73 and P100 treatments had no significant effects. In the same silage period, the number of yeasts was lower in silages added with mulberry leaves and lowest in the PM55 group. This may be explained by the presence of water-soluble substances in mulberry leaves that have a broad spectrum of antimicrobial activity [36]. Hybrid *Pennisetum* and the water-soluble substances of mulberry leaves might have synergistic effects that inhibit the growth of yeasts, and the lactic acid bacteria may not be influenced. The antibacterial properties of mulberry leaves still require more research. During the whole fermentation process, molds are below the detected range. All in all, when mulberry leaves and hybrid *Pennisetum* were mixed in a ratio of 1:1, the silage had a better inhibition effect on undesirable bacteria, such as yeasts and molds, and lactic acid bacteria dominated the silage, resulting in good fermentation quality.

Table 5. The microbial population of mulberry leaves and hybrid *Pennisetum*.

Item	Treatment	Ensiling Days				SEM	*p* Value		
		3	7	14	30		D	T	D × T
Lactic acid bacteria (log_{10} cfu/g FM)	P100	7.94 ^{cC}	8.70 ^{bA}	8.61 ^{cA}	8.46 ^{B}	0.02	<0.01	<0.01	0.01
	PM73	8.31 ^{aD}	9.21 ^{aA}	9.05 ^{aB}	8.49 ^{C}				
	PM55	8.22 ^{bC}	9.24 ^{aA}	9.00 ^{bA}	8.51 ^{B}				
Yeasts (log_{10} cfu/g FM)	P100	5.46 ^{aC}	5.65 ^{aB}	5.69 ^{aA}	4.78 ^{aD}	0.013	<0.01	<0.01	<0.01
	PM73	5.02 ^{bA}	4.70 ^{bB}	4.69 ^{bB}	4.77 ^{aB}				
	PM55	4.95 ^{cA}	4.60 ^{cB}	4.26 ^{cC}	2.39 ^{bD}				
Molds (log_{10} cfu/g FM)	P100	<2.00	<2.00	<2.00	<2.00	–	-	-	-
	PM73	<2.00	<2.00	<2.00	<2.00				
	PM55	<2.00	<2.00	<2.00	<2.00				

D, ensiling days; T, treatments; D × T, the interaction of ensiling days and treatments; DM, dry matter; P100, 100% hybrid *Pennisetum* silage; PM73, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 7:3; PM55, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 1:1. Different capital letters (A–D) in the same row indicate significant differences between different days of the same treatment at $p < 0.05$; different lowercase letters (a–c) in the same column indicate significant differences between different treatments on the same day at $p < 0.05$.

3.3.2. The Changes in the Dynamics of Relative Abundance among Bacterial Communities

The dynamics of relative abundance among bacterial communities by phylum are shown in Figure 1. During the entire silage process, the dominant phylum in three different treatments was all *Firmicutes*; similar results were observed by Tian et al. [37].

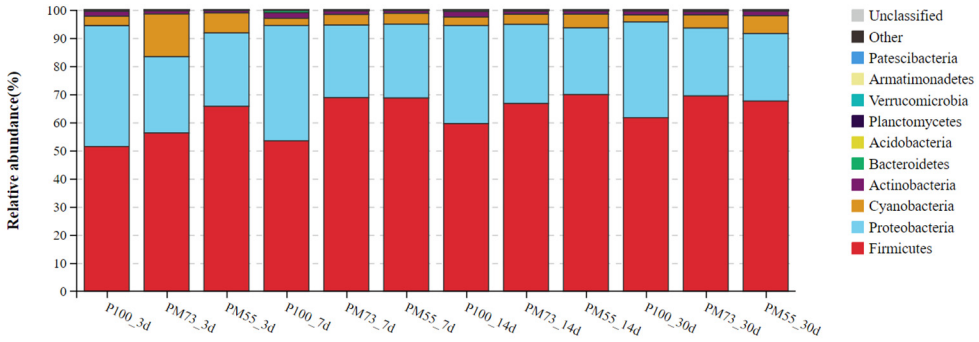

Figure 1. Bacterial community and relative abundance by phylum for mulberry leaf silages (P100, 100% hybrid *Pennisetum* silage; PM73, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 7:3; PM55, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 1:1; 3d, 3 days of ensiling; 7d, 7 days of ensiling; 14d, 14 days of ensiling; 30d, 30 days of ensiling).

With the extension of ensiling time, the relative abundance of *Proteobacteria* in the P100 treatment gradually decreased, and *Firmicutes* gradually increased, and the relative

abundance of *Bacteroides* first increased and then decreased. In the PM73 group, the relative abundance of *Cyanobacteria* first decreased and then increased. The relative abundance of *Proteobacteria* reached 28.12% at 14 days, and *Firmicutes* reached its peak at 30 days. In addition, in the PM55 group, the relative abundance of *Firmicutes* reached its peak (69.90%) at 14 days. *Firmicutes* are important to the degradation of fibrin in an anaerobic environment and can secrete various cellulases, lipases, and proteases [37]. The abundance of *Firmicutes* and *Cyanobacteria* in the group added with mulberry leaves was higher than that in the pure hybrid *Pennisetum* group, and the PM55 group had the highest, which was consistent with the lower NDF and ADF content in the PM73 and PM55 groups.

At 3 and 14 days of ensiling, as the proportion of mulberry leaves increased, the bacterial richness of *Firmicutes* increased, while *Proteobacteria* decreased. Except for the 3 days of ensiling, the abundance of *Cyanobacteria* increased with the increase in mulberry leaves. *Cyanobacteria* are a photosynthesizing phylum of bacteria [38], and they may promote protein synthesis. This may explain the better protein preservation in the mulberry-leaf-added treatment group. After 30 days of ensiling, the abundance of bacteria such as *Bacteroides*, *Acidobacteria,* and *Planctomycetes* was always less than 1%. *Proteobacteria* plays a negative role in the silage process and slows pH reduction while competing with lactic acid bacteria for water-soluble carbohydrates [39]. Compared with the P100 group, the relative abundance of *Firmicutes* in the PM73 and PM55 groups was higher, but there was lower *Proteobacteria* abundance in the whole ensiling time, indicating that mixing with mulberry leaves was beneficial for the growth of *Firmicutes*, enhances the permeability of the outer membrane of Gram-negative bacteria, and inhibits the growth of *Proteobacteria* [29].

The dynamics of relative abundance among bacterial communities by genus are shown in Figure 2. The mixed silage of hybrid *Pennisetum* and mulberry leaves is mainly attached to *Lactobacillus*, *Weissella, Pantoea, Leuconostoc* bacteria during the entire ensiling period. *Weissella* is considered to be an early settler in fermentation progress that is replaced by acid-resistant *Lactobacilli* with a reduced pH [40]. The relative abundance of *Weissella* gradually decreased and *Lactobacillus* gradually increased with the extension of ensiling days. After 14 days of ensiling, the most dominant genus was *Lactobacillus*, accounting for P100 (27.67%), PM73 (28.93%), and PM55 (25.53%), which is similar to the results of Ni et al. [41]. Moreover, the abundance of *Weissella* was the highest in the PM55 group. Dellaglio et al. [42] found that *Weissella* plays an important role in the early stage of fermentation. Tian et al. [37] found a similar phenomenon in *Moringa oleifera* leaves. After 30 days of ensiling, the relative abundance of *Lactobacillus* in the PM73 group increased to 28.93% (increasing rate 82.47%), which was the highest among the three treatments. *Lactobacillus* is the main bacteria with ideal functions [43]. Tohno et al. [44] posited that *Lactobacillus* could produce lactic acid and reduce the pH of silage to inhibit the proliferation of molds and other harmful miscellaneous bacteria. Li et al. [45] found that *Pantoea* acts similarly to *Enterobacter* in silage and will compete with lactic acid bacteria for the substrate. In this study, the relative abundance of *Pantoea* in the P100 group was higher than in the PM73 and PM55 groups, which partially explains the positive effects of mulberry leaves. Furthermore, *Leuconostoc* is a hetero-fermenting lactic acid bacteria with relatively low abundance, but it is beneficial to the aerobic stability of fermentation. In the same ensiling period, the relative abundance of *Leuconostoc* in the PM73 and PM55 groups was higher than that in the P100 group. The addition of mulberry leaves made the silage process dominated by heterolactic fermentation, which may be the reason for the higher acetic acid content in mulberry leaves treatment. Therefore, major desirable lactic acid bacteria after ensiling, *Leuconostoc*, *Pediococcus*, and *Weissella*, became more abundant in hybrid *Pennisetum* with mulberry leaves. This is consistent with the increase in the lactic acid bacteria count, which might explain the better fermentation quality of hybrid *Pennisetum* silage mixed with mulberry leaves.

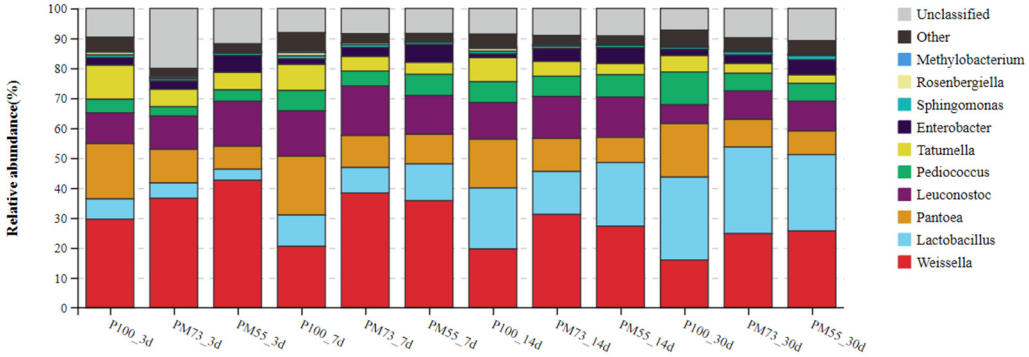

Figure 2. Bacterial community and relative abundance by genus for mulberry leaf silages (P100, 100% hybrid *Pennisetum* silage; PM73, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 7:3; PM55, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 1:1; 3d, 3 days of ensiling; 7d, 7 days of ensiling; 14d, 14 days of ensiling; 30d, 30 days of ensiling).

3.3.3. Effect of Different Proportions of Hybrid *Pennisetum* and Mulberry Leaves Mixed Silage on Alpha Diversity of Bacteria

The alpha diversity of the mixed silage bacterial community is shown in Table 6. It showed that the good coverage values of all treatments are above 0.99, indicating that the sampling data are sufficient to represent most bacterial communities in different samples. Alpha diversity is adopted to measure the richness, diversity, and evenness of species in bacterial communities. From the Chao1 and ACE index of three treatments, the bacterial species richness of three groups decreased with the increase in mulberry leaves, except for 30 days of ensiling. With prolonged ensiling time, the Sobs, Ace, and Chao1 indices reduce after an increase in the P100 group, which indicated that the bacterial richness climbs up and then declines. Compared with the P100 group, the silage with added mulberry leaves generally showed a lower Shannon index at the same stage of ensiling, which indicated that mulberry leaves decreased the diversity of the bacterial community of mixed silage. Polley et al. [46] reported that when the advantageous bacteria are abundant, the diversity of microbial communities is reduced. This may be caused by antibacterial activity, which affects the competition between different bacteria and accelerates the growth of beneficial bacteria such as lactic acid bacteria in this study.

Table 6. Alpha diversity of the bacterial community for hybrid *Pennisetum* and mulberry leaves silages.

Group	Sobs	Shannon	Simpson	Chao	Ace	Goods_Coverage
P100_3d	311.00	3.62	0.85	381.94	390.62	0.9992
PM73_3d	270.00	3.21	0.79	347.86	353.47	0.9991
PM55_3d	251.00	3.23	0.79	318.48	314.61	0.9993
P100_7d	340.67	3.84	0.88	398.86	395.82	0.9992
PM73_7d	281.67	3.34	0.81	349.45	351.21	0.9992
PM55_7d	247.67	3.37	0.83	319.03	315.84	0.9992
P100_14d	282.33	3.64	0.87	336.38	342.68	0.9993
PM73_14d	262.00	3.44	0.84	311.24	314.29	0.9994
PM55_14d	274.00	3.47	0.85	317.89	322.27	0.9993
P100_30d	272.33	3.51	0.85	311.91	306.31	0.9995
PM73_30d	322.00	3.48	0.83	358.63	363.33	0.9994
PM55_30d	263.33	3.51	0.85	298.34	298.25	0.9995

P100, 100% hybrid *Pennisetum* silage; PM73, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 7:3; PM55, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 1:1; 3d, 3 days of ensiling; 7d, 7 days of ensiling; 14d, 14 days of ensiling; 30d, 30 days of ensiling.

3.3.4. Effect of Different Proportions of Hybrid *Pennisetum* and Mulberry Leaves Mixed Silage on β-Diversity of Bacteria

The dynamic variation of the bacterial community was illustrated by principal component analysis (Figure 3). After 7 days of ensiling, it was obvious that the silages with added mulberry leaves were separated from the P100, which suggests that the microbial community changed during the ensiling process. The distinctiveness of the bacterial communities among all treatments might account for the better fermentation that occurred in the mixed silages with added mulberry leaves, which contributes to the better fermentation quality of the PM55 group. Similar results have been reported by Ni et al. [41], who found the 100% forage soybean samples were separated from the other samples (30% forage soybean + 70% crop corn/sorghum and 50% forage soybean + 50% crop corn/sorghum) by the β-diversity analysis, which suggested that mixed ensiling had an impact on the microbial community. When the ensiling time was prolonged, each group was significantly separated from the 30 days of ensiling, and the microbial community of each treatment changed during the silage process. The uniqueness of the bacterial communities in all treatments may explain the better fermentation in the silage treated with mulberry leaves.

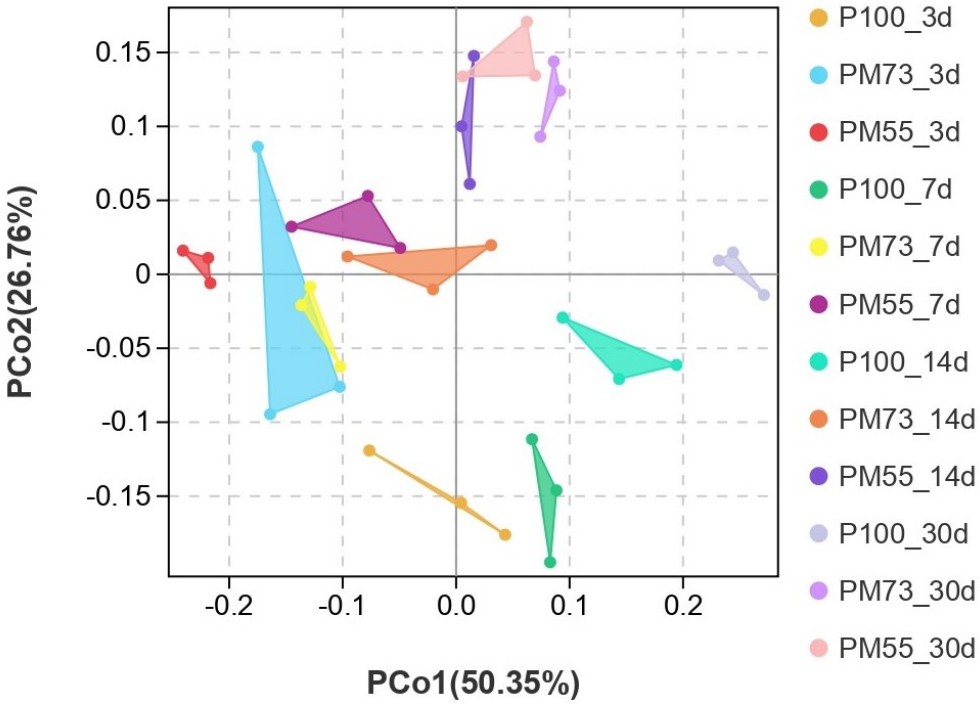

Figure 3. Principal component analysis of the bacterial community for hybrid *Pennisetum* and mulberry leaves mixed silage (P100, 100% hybrid *Pennisetum* silage; PM73, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 7:3; PM55, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 1:1; 3d, 3 days of ensiling; 7d, 7 days of ensiling; 14d, 14 days of ensiling; 30d, 30 days of ensiling).

3.3.5. 16S rDNA Gene-Predicted Functional Profiles

The 16S rDNA gene-predicted functional profiles are shown in Figure 4. Based on reference genomic databases and marker gene data, PICRUSt is a calculational method for predicting the function and pathway location of metagenomes [47]. The discrepancy of bacterial composition and abundance in respective treatment groups might be the key reason for differences in gene-predicted functions. Mulberry leaves inhibited the metabolism of other amino acids, carbohydrates, folding, sorting and degradation, membrane transport, lipid metabolism, energy metabolism, signal transduction, cell motility, biosynthesis of other secondary metabolites, and so on, which might be attributed to the antibacterial properties of mulberry leaves and directly inhibit microbial activity. What is more, the

addition of mulberry leaves may induce the abundant variation of some functional bacteria. However, the mechanisms remain unclear. Although further research is needed, the results of the predicted functions indicate that mixing with mulberry leaves had a positive effect on the fermentation quality and silage quality of hybrid *Pennisetum* and mulberry leaves mixed silage.

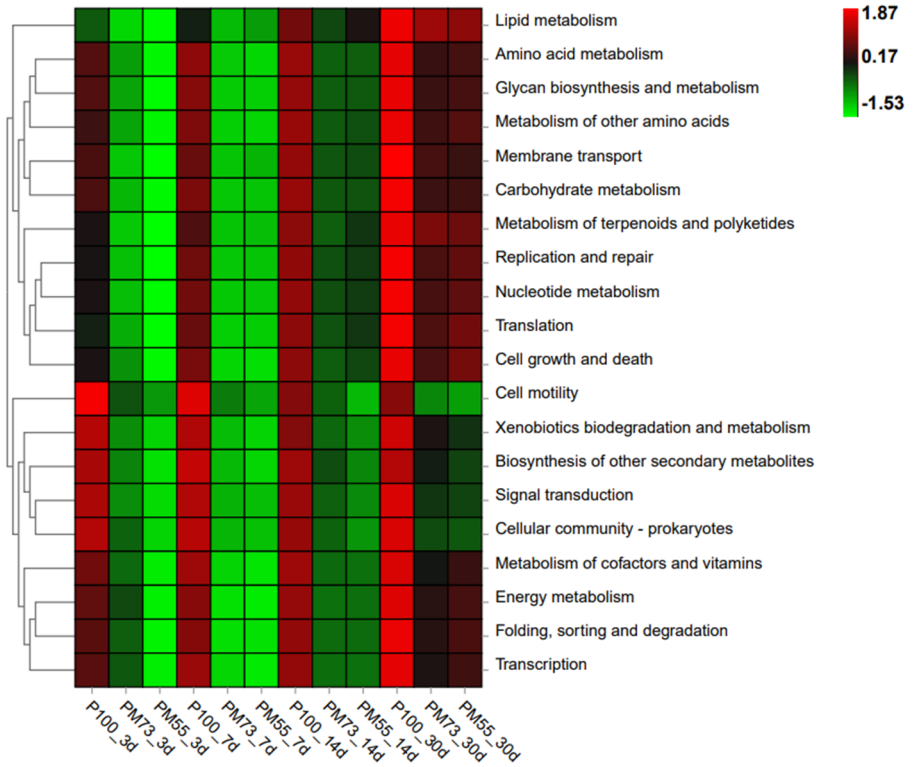

Figure 4. Heatmap of 16S rDNA gene-predicted functional profiles obtained with PICRUSt2 (P100, 100% hybrid *Pennisetum* silage; PM73, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 7:3; PM55, hybrid *Pennisetum* and mulberry leaves mix-silage in a ratio of 1:1; 3d, 3 days of ensiling; 7d, 7 days of ensiling; 14d, 14 days of ensiling; 30d, 30 days of ensiling).

4. Conclusions

This study revealed that mixing with mulberry leaves could be an alternative approach to improve the quality of hybrid *Pennisetum* silage. The content of NDF, ADF, and yeast counts decreased, whereas lactic acid, CP, and TP increased after mixing with mulberry leaves. The abundance of *Pantoea* decreased, whereas *Lactobacillus*, *Weissella*, and *Leuconostoc* abundance increased when mulberry leaves were added. Therefore, the addition of a certain percentage of mulberry leaves could effectively preserve the quality of mulberry leaves and hybrid *Pennisetum* mix-silage, and the combination of 50% mulberry leaves was the most effective.

Author Contributions: Conceptualization, Y.G.; methodology, H.T.; software, Z.C.; validation, Z.C.; formal analysis, Z.C.; investigation, M.D.; resources, M.D.; data curation, Z.C.; writing— original draft preparation, Z.C. and Y.L.; writing—review and editing, B.S. and D.L.; supervision, G.L.; project administration, Y.G. All authors have read and agreed to the published version of the manuscript.

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

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