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Effect of Sucrose and Lactic Acid Bacteria Additives on Fermentation Quality, Chemical Composition and Protein Fractions of Two Typical Woody Forage Silages

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Citation: Wang, X.; Liu, H.; Xie, Y.; Zhang, Y.; Lin, Y.; Zheng, Y.-L.; Yang, X.; Wang, N.; Ni, K.; Yang, F. Effect of Sucrose and Lactic Acid Bacteria Additives on Fermentation Quality, Chemical Composition and Protein Fractions of Two Typical Woody Forage Silages. *Agriculture* **2021**, *11*, 256. <https://doi.org/10.3390/agriculture11030256>

Academic Editors: David Parsons and Vito Laudadio

Received: 3 February 2021

Accepted: 15 March 2021

Published: 17 March 2021

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Abstract: Paper mulberry (PM) and mulberry (MU) have been considered potential substitutes for traditional forages in response to the increasing demand for high-protein feed for livestock. To improve the utility of these two typical woody forages, our study investigated the effects of sucrose and lactic acid bacteria (LAB) additives on the fermentation quality, nutritive value, and protein fractions of their leaf silages. Collected leaves were separately subjected to ensiling treatments, either with or without sucrose (S), in combination with *Lactobacillus plantarum* (LP), or *Lactobacillus casei* (LC). The silage was sampled and analyzed for fermentation parameters, carbohydrates, and protein fractions after ensiling for 60 days. The pH value of paper mulberry silages with S was 19% lower than that without S, while LAB-treated mulberry silages showed decreased ammonia nitrogen (by 71%) and fraction A in crude protein (by 15%) compared with no LAB additives. In summary, adding S improved the fermentation quality, with no positive effect on protein fractions, in PM silage, whereas LAB additives improved the potential utilization of protein in MU silage.

Keywords: woody forage; mulberry; paper mulberry; silage additive; CNCPS

1. Introduction

A regional lack of green high-protein forage resources is a key reason to develop animal husbandry in the equatorial region. This shortage is caused by climatic and environmental factors [1,2]. In southern China, focus has been placed on the technology of processing and feeding of tropical and subtropical woody bioresources recently [3]. There are two typical woody forages with potential development value: paper mulberry (PM, *Broussonetia papyrifera* L.) and mulberry (MU, *Morus alba* L.), of which preliminary utilization by livestock has been reported.

Both PM and MU are multipurpose trees, belonging to two genera of Moraceae. The CP content of the leaves of PM can reach up to 24% DM, while MU has 22% DM, with a higher water-soluble carbohydrates (WSC) content [4]. Compared with MU, with its large amount of annual fresh aboveground biomass, cultivated PM is slightly lower in yield but could be used for more than ten years periodically. The shorter regeneration time of PM is one of the advantages for feeding; the growth rate from stubble to the appropriate height for the next harvest season (1.2–1.5 m) is around 35–45 days [5]. Another advantage of PM, and a reason for preference by livestock as well, is the soft texture of the leaves, with scarce

foliar villi by hybridization [6,7]. In contrast, MU is favored in plains areas because of inclined branches when planted in high density, which is more convenient for mechanized harvesting [8].

Due to the adaptability of woody forages to continuous growth and periodic harvesting in high temperature and rainy seasons, ensiling is the main approach to woody forage production when other processing technologies, such as hay-making, are costly or risky [3]. Studies on spontaneous fermentation dynamics and diversity of bacterial communities of woody forage silages have reported that most of the crude protein content could be properly preserved with effective lactic acid fermentation [4]. It has been also proven that lactic acid bacteria (LAB) and the nutrient substrates of the forage are crucial factors to improve the fermentation quality and nutritional value of high-protein forage silages in the early stage of ensiling [9]. Belonging to one category of directly fermented water soluble carbohydrates (WSCs), sucrose is often used in research to assist LAB to improve the quality of silages [10]. However, to our knowledge, few publications have focused on the characteristics, and especially protein fractions, of processed woody forage silages. Therefore, the goal of our study was to investigate the effects of *Lactobacillus plantarum* (LP) and *Lactobacillus casei* (LC), with or without sucrose, on PM and MU leaf silage.

2. Materials and Methods

2.1. Silage Preparation

Woody forages were harvested during the vegetative growth period after about three months of growth at the Zengcheng experimental field of South China Agricultural University (23.14 N, 113.32 E, elevation 250 m, annual mean temperature 15 °C, average annual precipitation 603 mm; Guangzhou, Guangdong Province, China). Both kinds of leaves were manually chopped into 1–2 cm pieces immediately after collection. The *Lactobacillus plantarum* (LP) and *Lactobacillus casei* (LC) strains were isolated and purified from silages studied earlier, and the additives for silage preparation were made via lyophilization according to the reported procedures [11]. Raw materials were separately subjected to ensiling treatments based on a 2 × 3 factorial arrangement in a completely randomized design, either with or without sucrose (20 g/kg on a fresh matter basis), dissolved in 10 mL sterilized deionized water containing nothing or cultured LP or LC (1.0×10^5 colony forming unit/g on a fresh matter basis). Six groups of treatments were labeled with a combination of S0 and S2 with CK, LC, and LP, respectively. The given solvent was sprayed with a disposable tiny sprayer onto minced leaves for every treatment. After mixing the ingredients thoroughly, four replicates (one for backup) of 200 g of each treated batch were packed into laboratory polyethylene bags (18 cm × 26 cm) and sealed with a vacuum sealing machine at a density of approximately 642 kg fresh weight (FW)/m³ (DZ-280/2SE, Furuide Machinery Co., Ltd., Shandong, China). The silages were stored at ambient temperature conditions (25–28 °C), and opened after 60 days of ensiling for analysis.

2.2. Silage Fermentation

Silage samples were divided into samples of 20 g by the quartering method and then mixed in a blender with 180 mL sterilized distilled water for 1 min, and filtered through three layers of qualitative filter paper. The filtrate was collected for measuring the pH value, ammonium nitrogen (NH₃-N), lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA). Specifically, the pH value was measured using a glass electrode pH meter (FiveEasy 20K; Mettler-Toledo International Inc., Greifensee, Switzerland). The NH₃-N concentration was determined with the phenol-sodium hypochlorite method [12], and the above four organic acids were analyzed using a high-performance liquid chromatography method, as previously described, with some adjusted operations (column, Shodex RS Pak KC-811; Showa Denko K.K., Kawasaki, Japan; detector, DAD, 210 nm, SPD-20A; Shimadzu Co., Ltd., Kyoto, Japan; eluent, 3 mmol L⁻¹ HClO₄; flow speed, 1.0 mL min⁻¹; column oven temperature, 50 °C) [13].

2.3. Chemical Composition

Dry matter (DM) of woody forages and silages was measured after drying in a forced-air oven at 65 °C for 48 h, and then samples were ground in a hammer mill to pass through a 1 mm screen. The DM concentration was corrected for the loss of volatile compounds according to Porter and Murray [14], and the variables after ensiling were presented on the basis of corrected DM. Neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) were analyzed according to the method of Van Soest et al. [15], using an ANKOM A2000 fiber analyzer. Furthermore, the contents of hemicellulose (HC) and cellulose (CE) were calculated using the difference between NDF and ADF, and the difference between ADF and ADL, respectively. The WSC content was determined using the improved anthrone colorimetric assay [16]. Crude protein (CP) was measured according to the Association of Official Analytical Chemists (AOAC) International (2000) procedures [17]. In addition, the buffering capacity (BC) of woody forage raw materials was analyzed by titration with lactic acid (0.1 mol L⁻¹) [18]. Specifically, 1 g of sample powder was suspended in 100 mL of distilled water for 30 min. The volume for lactic acid titration during the pH value of suspension down to 4.00 was recorded.

2.4. Protein Fraction

The protein fraction was calculated by the CNCPS (Cornell Net Carbohydrate and Protein System), and divided into three fractions: (1) non-protein nitrogen (NPN; fraction A, FA), (2) true protein (fraction B, FB), and (3) bound true protein (fraction C, FC). Based on the intrinsic rates of ruminal degradation, FB was further partitioned into three subsections, including the FB1, FB2, and FB3 fractions, representing rapidly degraded protein, intermediately degraded protein, and slowly degraded protein in the proper order. The NPN, soluble protein (SOLP), neutral detergent-insoluble protein (NDIP), and acid detergent-insoluble protein (ADIP) of the silages were determined as described by Licitra et al. [19]. The protein fraction was calculated according to Sniffen et al. [20].

$$FA(\%CP) = NPN(\%SOLP) \times 0.01 \times SOLP(\%CP) \quad (1)$$

$$FB1(\%CP) = SOLP(\%CP) - FA(\%CP) \quad (2)$$

$$FB2(\%CP) = 100 - FA(\%CP) - FB1(\%CP) - FB3(\%CP) - FC(\%CP) \quad (3)$$

$$FB3(\%CP) = NDIP(\%CP) - ADIP(\%CP) \quad (4)$$

$$FC(\%CP) = ADIP(\%CP) \quad (5)$$

2.5. Statistical Analysis

Data were analyzed using the software program JMP 14 (SAS Institute). The effects of treatment on the protein fractions were separately determined for each kind of woody forage silage by one-way analyses of variance (ANOVA). The fermentation quality and chemical composition parameters were determined according to the model for a factorial treatment design:

$$Y_{ij} = \mu + I_i + T_j + (I + T)_{ij} + e_{ij} \quad (6)$$

where Y_{ij} is the observed value; μ is the mean; I_i is the effect of adding sucrose (S); T_j is the effect of LAB additives (Ad); $(I + T)_{ij}$ is the effect of interaction between S and Ad; and e_{ij} is the residual error. Tukey's test was used for multiple comparisons, with differences declared significant at $p \leq 0.05$.

3. Results

3.1. Raw Material Characteristics Before Ensiling

Table 1 shows the characteristics of the two forages. The BC values of PM and MU were almost equal (83.54 g lactic acid⁻¹ kg DM and 83.55 g lactic acid⁻¹ kg DM). Significant differences ($p < 0.01$) were found in DM (28.74 % FW and 38.67 % FW). As for nutritional

content, PM leaves were significantly ($p < 0.01$) higher than MU leaves in CP (25.97% DM and 19.38% DM), NDF (34.24% DM and 18.43% DM), ADF (23.63% DM and 12.77% DM), ADL (10.28% DM and 2.62% DM), hemicellulose (10.60% DM and 5.65% DM), and cellulose (11.64% DM and 8.53% DM). In addition, the WSC content of PM leaves (3.12% DM) was significantly ($p < 0.01$) lower than that of MU leaves (10.72% DM).

Table 1. Characteristics of woody forage raw materials.

Items	PM	MU	SEM	<i>p</i> -Value
BC (g LA ⁻¹ kg DM)	83.54	83.55	0.00	NS
DM (%FW)	28.74 ^b	38.67 ^a	4.97	**
CP (%DM)	25.97 ^a	19.38 ^b	3.29	**
NDF (%DM)	34.24 ^a	18.43 ^b	7.90	**
ADF (%DM)	23.63 ^a	12.77 ^b	5.43	**
ADL (%DM)	10.28 ^a	2.62 ^b	3.83	**
HC (%DM)	10.60 ^a	5.65 ^b	2.47	**
CE (%DM)	11.64 ^a	8.53 ^b	1.55	**
WSC (%DM)	3.12 ^b	10.72 ^a	3.80	**

PM, paper mulberry; MU, mulberry; BC, buffering capacity; DM, dry matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; HC, hemicellulose; CE, cellulose; WSC, water-soluble carbohydrates; SEM, standard error of the mean; NS, not significant; **, Significant at 0.01; means in the row (a–b) with different superscript letters differ significantly from each other ($p < 0.05$).

3.2. Fermentation Quality of Silages

After ensiling for 60 days, all fermentation indicators, including pH value, LA, AA, PA, BA, and NH₃-N, were affected to varying degrees by different treatments (Table 2). Both S and Ad caused significant differences ($p < 0.01$) in the pH of the two woody forage silages, while significant interactions occurred only in MU silage ($p < 0.01$). Specifically, S2 resulted in a lower pH value in PM silage, and the lowest grade occurred in the S2+LP and S2+LC samples. Regardless of adding sucrose, silages inoculated with LP and LC had lower pH values for both PM and MU. LP caused a greater pH reduction than LC in PM, while the opposite was found in MU. Furthermore, the mean of the pH value in the PM group was higher than for MU. For the four organic acids, BA was not detected in the two kinds of woody forage silages, but there were significant differences in contents of LA, AA, and PA. The mean value of PM was lower than that of MU in LA and higher in AA and PA. Only S2-treated silages had lactic acid in PM, while the lactic acid content in the S2 group was higher than that in the S0 group of MU (except S2+LC). In PM samples, the S2+LC-treated silages contained the most LA, and S2+LP-treated silages had lowest AA and PA concentrations. For the MU samples, LP and LC-treated samples did not contain PA, and the AA concentration of S2+LP-treated silages was strikingly higher than that of S2+CK.

Compared with MU silages, the NH₃-N concentration of PM silages was significantly higher ($p < 0.01$). S reduced the NH₃-N concentration of PM and MU silages significantly ($p < 0.01$), while Ad exerted a significant effect only in the MU samples ($p < 0.01$). The production of NH₃-N in LP and LC-treated PM samples with sucrose was inhibited compared to those without sucrose. LC resulted in a greater decrease of the NH₃-N concentration than LP in S0-treated silages of MU. However, the reduction caused by various LAB additives did not show differences in the S2-treated silages of MU and all samples of PM.

3.3. Chemical Nutrition of the Silages

Additional sucrose in the PM samples resulted in differences in DM, CP, ADF, ($p < 0.05$) and CE ($p < 0.01$), and adding sucrose or additives affected ($p < 0.01$) the contents of CP, NDF, ADF and WSC of MU silages, respectively (Table 3). Silages of PM were higher ($p < 0.05$) in CP, NDF, ADF, HC, and CE compared with MU silages (Table 3). Meanwhile, no difference ($p > 0.05$) was found in the ADL and WSC from two woody forage silages. Sucrose application increased the DM content of PM silage inoculated with LP, while

the DM content of S2+LC-treated MU samples was higher than the other treatments. Inoculation of LP and LC with sucrose reduced the CP content of MU silage, but LC led to a lower loss than LP. Interestingly, there was no significant difference between each treatment in CP content in PM silages ($p > 0.05$). S2+CK-treated silage had lower contents of NDF, ADF, ADL, HC, CE, and WSC than S0+CK in PM. LP or LC-treated MU silages also showed lower contents of NDF, ADF, ADL, and HC than CK. It is worth emphasizing that the combination of LC or LP with S2 led to a lower WSC content than S0 in PM silages, while the opposite occurred in MU silages.

Table 2. The effect of sucrose and additives on fermentation quality of woody forage silages.

Items	Species	S0			S2			Mean	SEM	p-Value		
		CK	LP	LC	CK	LP	LC			S	Ad	S × Ad
pH value	PM	6.89 ^a	6.46 ^c	6.71 ^b	5.71 ^d	5.47 ^e	5.50 ^e	6.13 ^A	0.25	**	**	NS
	MU	5.06 ^a	4.33 ^c	4.15 ^d	4.73 ^b	4.14 ^d	3.99 ^e	4.40 ^B	0.17	**	**	**
LA (%DM)	PM	ND	ND	ND	0.76 ^b	3.14 ^a	4.58 ^a	1.41 ^B	0.80	**	NS	NS
	MU	2.52 ^b	2.17 ^b	3.51 ^{ab}	3.69 ^{ab}	6.11 ^a	2.10 ^b	3.35 ^A	0.62	NS	NS	*
AA (%DM)	PM	3.02	2.84	2.10	4.00	2.01	2.17	2.69 ^A	0.31	NS	NS	NS
	MU	0.46 ^{ab}	ND	ND	0.24 ^b	0.83 ^a	ND	0.26 ^B	0.14	NS	*	**
PA (%DM)	PM	2.27 ^{ab}	1.76 ^{ab}	1.66 ^{ab}	3.52 ^a	0.53 ^b	1.00 ^{ab}	1.79 ^A	0.43	NS	NS	NS
	MU	0.5	ND	ND	0.48	ND	ND	0.16 ^B	0.10	NS	NS	NS
BA (%DM)	PM	ND	ND	ND	ND	ND	ND	ND	ND	NS	NS	NS
	MU	ND	ND	ND	ND	ND	ND	ND	ND	NS	NS	NS
NH ₃ -N (%TN)	PM	16.05 ^{ab}	17.88 ^a	17.02 ^a	12.42 ^{bc}	11.57 ^c	11.82 ^c	14.44 ^A	1.16	**	NS	NS
	MU	6.68 ^a	2.86 ^b	1.82 ^c	5.91 ^a	1.65 ^c	1.02 ^c	3.32 ^B	0.98	**	**	NS

PM, paper mulberry; MU, mulberry; LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid; NH₃-N, ammonia nitrogen; TN, total nitrogen; ND, not detected; SEM, standard error of the mean; NS, not significant; S0, ensiling without sucrose; S2, ensiling with 20 g/kg sucrose on a fresh matter basis; CK, ensiling without lactic acid bacteria additives; LP, ensiling with *Lactobacillus plantarum* at the level of 10⁶ cfu/g of fresh matter; LC, ensiling with *Lactobacillus casei* at the level of 10⁶ cfu/g of fresh matter; S, sucrose; Ad, lactic acid bacteria additives; *, significant at 0.05; **, significant at 0.01; means in the same row (a–e) or column (A–B) with different superscript letters differ significantly from each other ($p < 0.05$).

Table 3. The effect of sucrose and additives on chemical composition of woody forage silages.

Items	Species	S0			S2			Mean	SEM	p-Value		
		CK	LP	LC	CK	LP	LC			S	Ad	S × Ad
DM (%FW)	PM	27.0 ^{ab}	25.7 ^b	26.9 ^{ab}	27.4 ^{ab}	28.9 ^a	28.6 ^a	27.4 ^B	0.48	*	NS	NS
	MU	32.8 ^b	33.4 ^b	33.5 ^b	33.7 ^b	34.7 ^b	41.3 ^a	34.9 ^A	1.31	NS	NS	NS
CP (%DM)	PM	23.5	23.4	23.7	22.4	23.2	22.7	23.1 ^A	0.20	*	NS	NS
	MU	19.1 ^a	18.9 ^a	19.1 ^a	19.1 ^a	17.6 ^c	18.3 ^b	18.7 ^B	0.26	**	**	*
NDF (%DM)	PM	29.6 ^a	22.0 ^b	25.4 ^{ab}	20.6 ^b	27.0 ^{ab}	23.3 ^{ab}	24.7 ^A	1.36	NS	NS	*
	MU	21.1 ^a	18.8 ^{bc}	18.9 ^{bc}	20.1 ^{ab}	16.9 ^d	17.4 ^{cd}	18.9 ^B	0.64	**	**	NS
ADF (%DM)	PM	18.7 ^a	17.4 ^{ab}	18.4 ^{ab}	16.4 ^b	17.3 ^{ab}	17.5 ^{ab}	17.6 ^A	0.33	*	NS	NS
	MU	15.0 ^a	14.2 ^{ab}	13.5 ^{bc}	14.1 ^{ab}	12.8 ^{cd}	12.3 ^d	13.6 ^B	0.41	**	**	NS
ADL (%DM)	PM	4.0	2.9	4.0	3.2	3.2	4.1	3.6	0.21	NS	NS	NS
	MU	4.5 ^a	3.3 ^{ab}	4.0 ^{ab}	4.5 ^a	2.8 ^b	3.1 ^{ab}	3.7	0.30	NS	*	NS
HC (%DM)	PM	10.8 ^a	4.6 ^c	7.1 ^{abc}	4.2 ^c	9.7 ^{ab}	5.8 ^{bc}	7.0 ^A	1.12	NS	NS	**
	MU	6.2 ^a	4.6 ^{bc}	5.4 ^{abc}	6.0 ^{ab}	4.2 ^c	5.2 ^{abc}	5.3 ^B	0.32	NS	**	NS
CE (%DM)	PM	14.0 ^a	12.9 ^{ab}	13.3 ^a	11.8 ^b	12.8 ^{ab}	11.7 ^b	12.8 ^A	0.36	**	NS	NS
	MU	9.2 ^{ab}	9.5 ^a	8.5 ^{ab}	8.6 ^{ab}	8.3 ^b	8.2 ^b	8.7 ^B	0.21	*	NS	NS
WSC (%DM)	PM	0.6 ^{ab}	0.7 ^a	0.6 ^{bc}	0.6 ^{bc}	0.6 ^{bc}	0.5 ^c	0.6	0.02	NS	NS	NS
	MU	1.0 ^b	1.3 ^b	1.1 ^b	1.0 ^b	2.5 ^a	1.5 ^b	1.4	0.23	**	**	*

PM, paper mulberry; MU, mulberry; DM, dry matter; FW, fresh weight; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; HC, hemicellulose; CE, cellulose; WSC, water-soluble carbohydrates; SEM, standard error of the mean; NS, not significant; S0, ensiling without sucrose; S2, ensiling with 20 g/kg sucrose on a fresh matter basis; CK, ensiling without lactic acid bacteria additives; LP, ensiling with *Lactobacillus plantarum* at the level of 10⁶ cfu/g of fresh matter; LC, ensiling with *Lactobacillus casei* at the level of 10⁶ cfu/g of fresh matter; S, sucrose; Ad, lactic acid bacteria additives; *, significant at 0.05; **, significant at 0.01; means in the same row (a–d) or column (A–B) with different superscript letters differ significantly from each other ($p < 0.05$).

3.4. Protein Fractions of the Silages

The two woody forages showed similar profiles of protein fractions (Figure 1). The contribution of FB was at least 47.90% in PM and 55.19% in MU. Differences caused by treatments classified PM silages significantly ($p < 0.05$) only in terms of the FA, FB2 and FB3 fractions (Figure 1a), while they were found for MU silages in all fractions (Figure 1b). Comparing S0-CK samples of PM, S0-LP and S2-CK increased in the FA fraction and decreased in the FB3 fraction. S2-LP had a lower FB2 fraction and higher FA fraction in PM. Regardless of adding sucrose or not in MU silages, the FA fraction was decreased by LP and LC, and furthermore, the FC fraction was decreased by mixing with sucrose concurrently. Except for S0+LP treated samples in MU, the FB3 fraction of all treatments was higher than that in S0+CK. Moreover, LP caused MU silages to contain a lower FB1 fraction and higher FB2 fraction than LB.

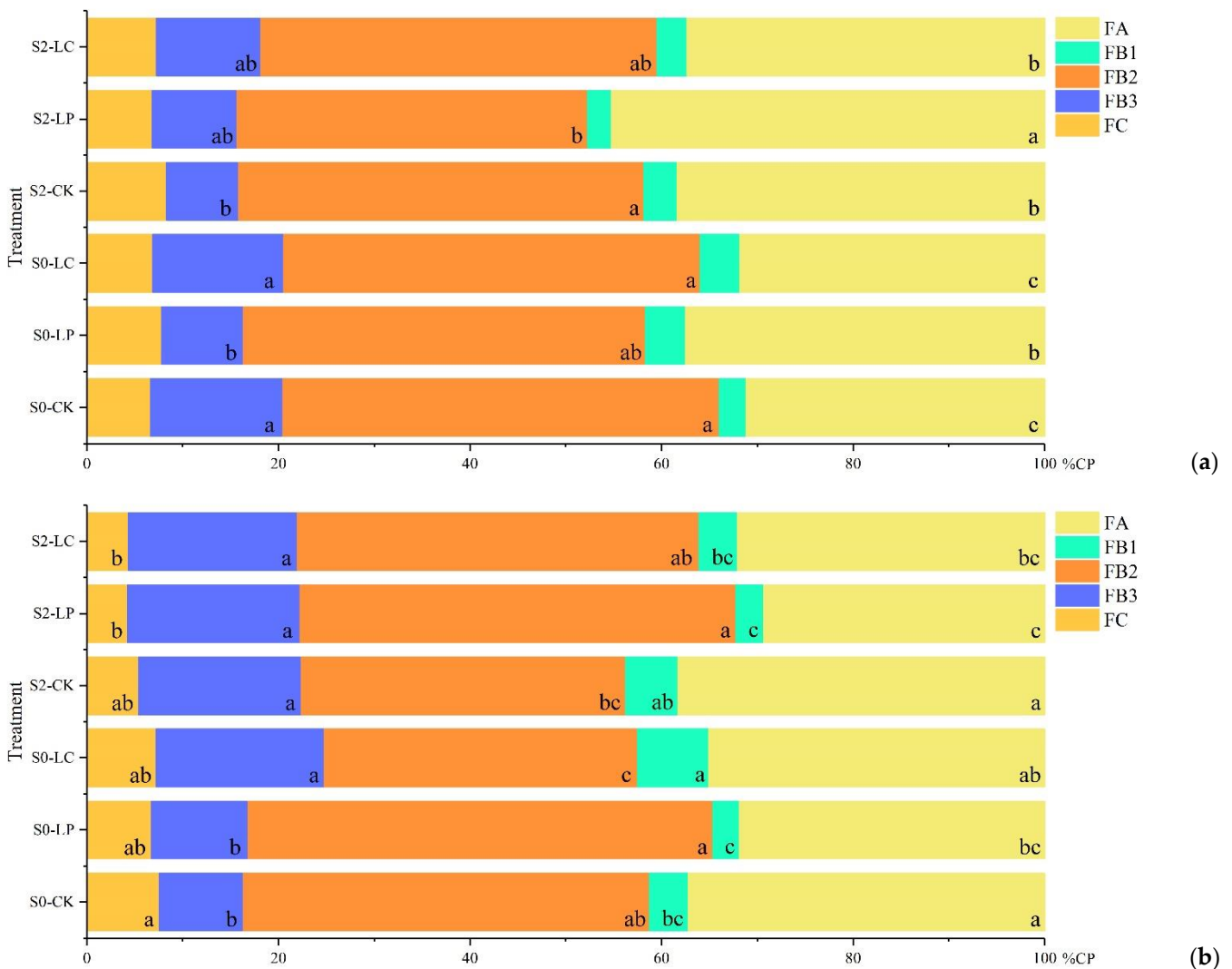


Figure 1. Protein fractions of leaf silage in paper mulberry (a) and mulberry (b). Different letters differ significantly from each other ($p < 0.05$) for the same color patch in each kind of silage. S0, ensiling without sucrose; S2, ensiling with 20 g /kg sucrose on a fresh matter basis; CK, ensiling without lactic acid bacteria additives; LP, ensiling with *Lactobacillus plantarum* at the level of 10^6 cfu/g of fresh matter; LC, ensiling with *Lactobacillus casei* at the level of 10^6 cfu/g of fresh matter; FA, fraction A; FB1, fraction B1; FB2, fraction B2; FB3, fraction B3; FC, fraction C.

4. Discussion

In our present study, two indigenous woody forages with the advantages of good resistance, convenient seedling sources, and uncomplicated management were planted. The results demonstrated that, for the leaves from PM and MU, we can reduce nutrient loss by ensiling, and the quality of silages may be improved by adding fermentable substrates and LAB additives. Both PM and MU are suitable for ensiling and have potential as high-protein forage silages for livestock.

All collected raw material samples showed a high CP content (25.97% DM and 19.38% DM), which was even higher than the content of common alfalfa varieties in southern China (from 16.5% DM to 20.4% DM) [21]. The carbohydrate components, including NDF, ADF, and ADL, were similar to the previously reported range [22,23]. In addition to the value of BC, the sharp gaps of WSC content might also be the reason for the difference of fermentation quality. PM and MU are deciduous broad-leaved species, and the wide variation in the WSC content of leaves was attributed to varieties and cycles of growth [24]. The WSC content of mulberry leaves from 45 germplasms and varieties ranged from 3.99% DM to 17.44% DM [8]. As a previous study showed, BC value and WSC content indicate the start of continuous fermentation, and endow silages with acceptable quality [3]. Thus, sucrose was reported as a supplement in the evaluation of the protein composition of silages, to balance the substrate limitations at early ensiling stages [25].

Previous studies have also attributed successful ensiling to the contribution made by LAB in anaerobic environments [26]. The low pH value and high LA content caused by enrichment of LAB are generally indicators of excellent silage quality. As the results showed, although PM silages only inoculated with LP or LC had a lower pH than the control, they were still over 6.00, while adding S (no matter whether in combination with LP or LC) would lead to an even lower pH of around 5.40–5.70. There is no significant difference of AA content between S0-CK and S2-CK, but trends of increasing AA by adding S may indicate that offering more WSC for PM may promote fermentation by the microorganisms attached to the plant surface. It should be noted that the contents of AA and PA in PM silage were higher than those in MU silage. Moreover, S2-treated PM silages showed lower PA contents than S0 with LAB, but the opposite occurred without LAB additives. This is likely because yeast seized more fermentation substrates without enough LAB load, resulting in the production of PA, which has been reported in high-moisture silage [27]. An analysis pointed out that AA and PA concentrations were significantly increased only when heterofermentative LAB was applied [28], suggesting another possibility: the majority of LAB in the surface microorganisms of PM might be heterofermentative bacteria, and may occupy a dominant position during ensiling. Our present study showed that exogenous sucrose improves LA content and decreases the pH value in silages of woody forage, in line with studies on alfalfa [29], kenaf [30] and king grass [10].

It has been reported that all epiphytic microorganisms on raw materials involved in the metabolic process are present at the beginning of fermentation, but LAB ensured that the fermentation would go in a positive direction [31]. Once the LAB load from the initial microbial population was greater than 1.0×10^5 colony forming unit/g of fresh weight, spoilage organisms could be inhibited [32]. The dominant LAB population provided by additives, both in PM and MU leaves, assisted the silages to show a lower pH value and higher LA content than CK. For MU silage, the pH value after ensiling was lower than PM silage. Adding different additives could drop the pH value to around 4.00. Furthermore, adding LC caused the silages show a lower pH value than LP, which might suggest that LC is a better additive for MU compared to LP. However, the high content of ammonia nitrogen may indicate that these LAB additives are not the best choice for PM ensiling, and we need to focus more on isolation of LAB specific to use with PM.

The high content of moisture in the silage was proven to be a possible condition for the activity of undesirable microorganisms, such as *Clostridium perfringens*, *Clostridium sporogenes*, *Clostridium ghonii*, and *Clostridium sartagoforme* [33], leading to spoilage more frequently in low DM silages. The DM contents both of raw materials and silages in

our study were from 25.72% FW to 41.32% FW, which are higher than adequate values (25% FW) considered by some researchers, and could inhibit the decomposition of the carbohydrate components [34]. However, the significant difference of DM content might still be an important factor leading to the different ensiling difficulty of the two woody forages. Lower NDF and ADF concentrations were observed in silages of MU than in PM. Carbohydrate components were reported to be disintegrated to a certain extent by LAB in the early ensiling stage, which might accelerate the domination of LAB. One kind of LAB has been proven to decrease the NDF content of alfalfa silage, because it produces ferulate esterase to assist degradation of the plant cell wall, and was aimed to release substrates and promote the reproduction of lactic acid bacteria [35]. The high ADL content of PM leaves might be due to the vigorous metabolic pathway in phenylpropanoid biosynthesis [7].

Limited proteolytic processes caused by the plants and microorganisms led to changes of protein fractions and the use of protein grading, presented as CNCPS, which could simulate and evaluate the digestion and utilization of protein in silages. According to previous studies, the protein degradability values significantly differed among forages. FA and FB2 were reported as the main fractions in alfalfa silage, comprising 46.2% CP and 36.5% CP, respectively [36]. However, for the silages of *Moringa oleifera* leaves, FB2 (55.2 % CP) was more than twice as abundant as FA (26.65% CP). In addition, the LAB additives have been reported to contribute to proteolysis inhibition by creating an acidic environment, resulting in a decrease of the FA concentration [37]. Generally, true protein is decomposed into peptides, free amino acids, ammonia, and other non-protein nitrogen by the action of plant proteases and microbial enzymes during silage production [38]. Most of the ammonia and amines are produced by microbial enzymes, not by plant proteases [39]. These results may be the main reason for the differences in enzyme activity between plant proteases and microbial enzymes. Alfalfa silages inoculated with LAB had a smaller proportion of FA than the control [40]. The pH value was negatively correlated with the fermentation time, and the enzyme activity decreased at a low pH value. In woody forage silages, when the LAB became the dominant bacteria, the decline rate of pH slowed. This process took 30–60 days in PM and MU silages, and 15 days in silages of *Moringa oleifera*. In the present study, the LC-treated silages showed a lower FA than LP in PM after ensiling for 60 days [4]. Furthermore, silages treated by S2 and LAB in MU showed a lower FC and higher FB3. A possible explanation for this shift is that the inoculants broke part of the chemical bonds during ensiling, untying the structure of some polyphenol-protein compounds [41]. Further research is needed, with more evaluations of the digestibility of the protein and carbohydrate components in PM and MU silages, including changes over time, which may provide more information for feeding livestock.

5. Conclusions

The present study showed that S2-treated PM silages had a lower pH value and higher LA content than S0, and LAB-treated MU silages had lower NH₃-N and FA concentrations than CK. In summary, adding S improved fermentation quality with no positive effect on protein fractions in PM silage, while LAB additives improved the potential utilization of protein in MU silage.

Author Contributions: Conceptualization, X.W. and F.Y.; methodology, X.W., Y.Z., and Y.X.; software, X.Y.; validation, X.W., Y.-L.Z. and Y.L.; formal analysis, X.W.; investigation, X.W., Y.X., and H.L.; data curation, X.W.; writing—original draft preparation, X.W.; writing—review and editing, N.W., K.N.; visualization, X.W. and Y.X.; supervision, F.Y.; project administration, F.Y. and Y.L.; funding acquisition, F.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Chinese Academy of Sciences (Strategic Priority Research Program Grant NO. XDA27000000), National Key R&D Program of China (Grant No. 2017YFD0502102), Chinese Universities Scientific Fund (Grant No. 2020TC116) and Research Innovation Fund for Graduate Students of China Agricultural University (Grant No. 2020XYZC30A).

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

References

1. Negrão, F.; Dantas, C.; Zanine, A.; Ferreira, D.; Ribeiro, M.; Souza, A.; Parente, M.; Parente, H.; Cunha, I.; Nascimento, T.; et al. Digestive Potential of Soybean Agro-Industry Byproducts. *Animals* **2020**, *10*, 911. [CrossRef] [PubMed]
2. Silva, M.D.A.; Carneiro, M.S.D.S.; Pinto, A.P.; Silva, D.S.; Coutinho, M.J.F.; Fontenele, R.M. Evaluation of the chemical composition of woody forage silages of the Brazilian semiarid. *Semin. Ciências Agrárias* **2015**, *36*, 571–578. [CrossRef]
3. Heinritz, S.N.; Martens, S.D.; Avila, P.; Hoedtke, S. The effect of inoculant and sucrose addition on the silage quality of tropical forage legumes with varying ensilability. *Anim. Feed Sci. Technol.* **2012**, *174*, 201–210. [CrossRef]
4. Zhang, Y.C.; Li, D.X.; Wang, X.K.; Lin, Y.L.; Zhang, Q.; Chen, X.Y.; Yang, F.Y. Fermentation dynamics and diversity of bacterial community in four typical woody forages. *Ann. Microbiol.* **2019**, *69*, 233–240. [CrossRef]
5. Su, Y.; Chen, G.; Cai, Y.; Gao, B.; Zhi, X.; Chang, F. Effects of Broussonetia Papyrifera Fermented Feed on the Growth Performance and Muscle Quality of Hu Sheep. *Can. J. Anim. Sci.* **2020**, *100*. [CrossRef]
6. Si, B.; Tao, H.; Zhang, X.; Guo, J.; Diao, Q.Y. Effect of Broussonetia papyrifera L. (paper mulberry) silage on dry matter intake, milk composition, antioxidant capacity and milk fatty acid profile in dairy cows. *Asian Australas. J. Anim. Sci.* **2018**, *31*, 1259. [CrossRef]
7. Sun, J.; Peng, X.; Fan, W.; Tang, M.; Liu, J.; Shen, S. Functional analysis of BpDREB2 gene involved in salt and drought response from a woody plant Broussonetia papyrifera. *Gene* **2014**, *535*, 140–149. [CrossRef]
8. Zheng, S.; Zeng, W.; Han, L.; Liu, C.; Yu, M.; Xiang, Z.; Zhao, A. Comprehensive evaluation of nutritional quality of leaves from 45 mulberry germplasms and varieties. *Food Sci.* **2017**, *38*, 159–163.
9. Cai, Y.M.; Benno, Y.; Ogawa, M.; Ohmomo, 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]
10. Li, M.; Zi, X.; Zhou, H.; Hou, G.; Cai, Y. Effects of sucrose, glucose, molasses and cellulase on fermentation quality and in vitro gas production of king grass silage. *Anim. Feed Sci. Technol.* **2014**, *197*, 206–212. [CrossRef]
11. Zhang, Q.; Yu, Z.; Yang, H.; Na, R.S. The effects of stage of growth and additives with or without cellulase on fermentation and invitro degradation characteristics of Leymus chinensis silage. *Grass Forage Sci.* **2016**, *71*, 595–606. [CrossRef]
12. Ni, K.; Zhao, J.; Zhu, B.; Su, R.; Pan, Y.; Ma, J.; Zhou, G.; Tao, Y.; Liu, X.; Zhong, J. Assessing the fermentation quality and microbial community of the mixed silage of forage soybean with crop corn or sorghum. *Bioresour. Technol.* **2018**, *265*, 563–567. [CrossRef]
13. Xu, C.; Cai, Y.; Murai, M. Fermentation quality and nutritive value of total mixed ration silage with barley tea grounds. *Jpn. J. Zootech. Sci.* **2004**, *75*, 185–191.
14. Porter, M.G.; Murray, R.S. The volatility of components of grass silage on oven drying and the inter-relationship between dry-matter content estimated by different analytical methods. *Grass Forage Sci.* **2010**, *56*, 405–411. [CrossRef]
15. 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]
16. Zhang, Q.; Zhao, M.; Wang, X.; Yu, Z.; Na, R. Ensiling alfalfa with whole crop corn improves the silage quality and invitro digestibility of the silage mixtures. *Grassl. Sci.* **2017**, *63*, 211–217. [CrossRef]
17. Cunniff, C.; Horwitz, W.; Latimer, G. Official Method of Analysis of AOAC International. *Trends Food Sci. Technol.* **2000**, *6*, 382.
18. Hattori, I.; Kumai, S.; Fukumi, R. Effect of water soluble carbohydrate (WSC) and lactic buffering capacity (LBC) on fermentative quality of silage. *Bull. Exp. Farm Coll. Agric. Ehime Univ.* **1996**, *39*–46. Available online: <https://agris.fao.org/agris-search/search.do?recordID=JP1998006752> (accessed on 2 February 2021).
19. Licitra, G.; Hernandez, T.M.; Van Soest, P.J. Standardization of procedures for nitrogen fractionation of ruminant feeds. *Anim. Feed Sci. Technol.* **1996**, *57*, 347–358. [CrossRef]
20. Sniffen, C.J.; O'Connor, J.D.; Van Soest, P.J.; Fox, D.G.; Russell, J.B. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. *J. Anim. Sci.* **1992**, *70*, 3562–3577. [CrossRef] [PubMed]
21. Li, Y.; Xu, Z.; Li, Z.; Li, Y. Comprehensive evaluation of yield and nutritional quality of 14 Alfalfa Varieties. *Grassl. Turf.* **2019**, *39*, 85–91.
22. Hao, Y.; Huang, S.; Liu, G.; Zhang, J.; Liu, G.; Cao, Z.; Wang, Y.; Wang, W.; Li, S. Effects of Different Parts on the Chemical Composition, Silage Fermentation Profile, In Vitro and In Situ Digestibility of Paper Mulberry. *Animals* **2021**, *11*, 413. [CrossRef] [PubMed]
23. Phetthavong, M.; Yachai, M.; Maneewan, C.; Panatuk, J. Effects of dried paper mulberry leaf silage supplementation in diets on growth performance of fattening pigs. *Khon Kaen Agric. J.* **2019**, *47*. Available online: <https://erp.mju.ac.th/openFile.aspx?id=MzQ5Mzk1> (accessed on 2 February 2021).
24. Wilson, K.B.; Baldocchi, D.D. Seasonal and interannual variability of energy fluxes over a broadleaved temperate deciduous forest in North America. *Agric. For. Meteorol.* **2000**, *100*, 1–18. [CrossRef]
25. Bai, C.; Zhang, R.; Jiang, C.; Yan, R.; Han, J.; Zhu, Y.; Zhang, Y. Characterization of carbohydrate fractions and fermentation quality in ensiled alfalfa treated with different additives. *Afr. J. Biotechnol.* **2011**, *10*, 9958–9968.
26. Muck, R.E. Silage microbiology and its control through additives. *Rev. Bras. Zootec.* **2010**, *39*, 183–191. [CrossRef]
27. Roberts, C.A.; Davis, D.K.; Looper, M.L.; Kallenbach, R.L.; Rottinghaus, G.E.; Hill, N.S. Ergot Alkaloid Concentrations in High- and Low-Moisture Tall Fescue Silage. *Crop Sci.* **2014**, *54*, 1887–1892. [CrossRef]
28. Blajman, J.E.; Paez, R.B.; Vinderola, C.G.; Lingua, M.S.; Signorini, M.L. A meta-analysis on the effectiveness of homofermentative and heterofermentative lactic acid bacteria for corn silage. *J. Appl. Microbiol.* **2018**, *125*, 1655–1669. [CrossRef]

29. Li, P.; Ji, S.; Hou, C.; Tang, H.; Wang, Q.; Shen, Y. Effects of chemical additives on the fermentation quality and N distribution of alfalfa silage in south of China. *Anim. Sci. J.* **2016**, *87*, 1472–1479. [[CrossRef](#)]
30. Ryu, C.; Park, M.; Jeon, E.; Kim, Y.S.; Lee, H.; Cho, S.; Choi, N.J. Effects of Different Forages and Kenaf Silage on in Vitro Rumen Fermentation and Growth Performance of Hanwoo Steer. *J. Anim. Sci.* **2017**, *95*, 304. [[CrossRef](#)]
31. Dunière, L.; Sindou, J.; Chaucheyras-Durand, F.; Chevallier, I.; Thévenot-Sergentet, D. Silage processing and strategies to prevent persistence of undesirable microorganisms. *Anim. Feed Sci. Tech.* **2013**, *182*, 1–15. [[CrossRef](#)]
32. Hartinger, T.; Kube, K.; Gresner, N.; Südekum, K.H. Varying ensiling conditions affect the fermentation quality and abundance of bacterial key players in lucerne silages. *J. Agric. Sci.* **2020**, *158*, 1–7. [[CrossRef](#)]
33. Zheng, M.; Niu, D.; Zuo, S.; Mao, P.; Meng, L.; Xu, C. The effect of cultivar, wilting and storage period on fermentation and the clostridial community of alfalfa silage. *Ital. J. Anim. Sci.* **2018**, *17*, 336–346. [[CrossRef](#)]
34. Amorim, D.S.; Loiola Edvan, R.; Do Nascimento, R.R.; Bezerra, L.R.; de Araújo, M.J.; Da Silva, A.L.; Mielezski, F.; Nascimento, K.D.S. Fermentation profile and nutritional value of sesame silage compared to usual silages. *Ital. J. Anim. Sci.* **2020**, *19*, 230–239. [[CrossRef](#)]
35. Li, D.; Wang, Y.; Zhang, Y.; Lin, Y.; Yang, F. Evaluation of lactic acid bacteria isolated from alfalfa for silage fermentation. *Grassl. Sci.* **2018**, *64*, 190–198. [[CrossRef](#)]
36. Li, X.; Tian, J.; Zhang, Q.; Jiang, Y.; Hou, J.; Wu, Z.; Yu, Z. Effects of applying *Lactobacillus plantarum* and Chinese gallnut tannin on the dynamics of protein degradation and proteases activity in alfalfa silage. *Grass Forage Sci.* **2018**, *73*, 648–659. [[CrossRef](#)]
37. Moselhy, M.A.; Borba, J.P.; Borba, A.E.S. Improving the nutritive value, in vitro digestibility and aerobic stability of *Hedychium gardnerianum* silage through application of additives at ensiling time. *Anim. Feed Sci. Technol.* **2015**, *206*, 8–18. [[CrossRef](#)]
38. Papadopoulos, Y.A.; Mckersie, B.D. A comparison of protein degradation during wilting and ensiling of six forage species. *Can. J. Plant Sci.* **1983**, *63*, 903–912. [[CrossRef](#)]
39. Heron, S.J.E.; Edwards, R.A.; McDonald, P. Changes in the nitrogenous components of gamma-irradiated and inoculated ensiled ryegrass. *J. Sci. Food Agric.* **1986**, *37*, 979–985. [[CrossRef](#)]
40. Wang, J.; Wang, J.Q.; Zhou, H.; Feng, T. Effects of addition of previously fermented juice prepared from alfalfa on fermentation quality and protein degradation of alfalfa silage. *Anim. Feed Sci. Technol.* **2009**, *151*, 280–290. [[CrossRef](#)]
41. Hervet-Hernández, D.; Pintado, C.; Rotger, R.; Goñi, I. Stimulatory role of grape pomace polyphenols on *Lactobacillus acidophilus* growth. *Int. J. Food Microbiol.* **2009**, *136*, 119–122. [[CrossRef](#)] [[PubMed](#)]