fermentation

MDPI

Article

Effects of Sucrose, Glucose and Molasses on Fermentation Quality and Bacterial Community of Stylo Silage

Xuejuan Zi [1,†], Yue Liu [1,†], Ting Chen [1], Mao Li [2,3,*], Hanlin Zhou [2,3] and Jun Tang [2,3]

[1] Key Laboratory of Ministry of Education for Genetics and Germplasm Innovation of Tropical Special Trees and Ornamental Plants, College of Forestry, Hainan University, Danzhou 571737, China; zixuejuan@hainanu.edu.cn (X.Z.); liuyue@hainanu.edu.cn (Y.L.); chenting@hainanu.edu.cn (T.C.)
[2] Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences, Danzhou 571737, China; zhouhanlin@catas.cn (H.Z.); tangjun@catas.cn (J.T.)
[3] Zhanjiang Experimental Station, Chinese Academy of Tropical Agricultural Sciences, Zhanjiang 524000, China
* Correspondence: limao@catas.cn
† These authors contributed equally to this work.

Abstract: To better understand the mechanism underlying water-soluble carbohydrates (WSC)-regulated silage fermentation, the bacterial community and fermentation quality of stylo (*Stylosanthes guianensis*) ensiled without (sterile distilled water) (CK) or with 10 g/kg sucrose (S), 10 g/kg glucose (G), and 10 g/kg molasses (M) were investigated. Each treatment was ensiled in three plastic bag silos for 30 days. The DM of stylo was 301.0 g/kg (fresh matter basis), while the contents of CP, WSC, NDF and ADF were 104.9, 12.3, 510.4 and 407.6 g/kg (dry matter basis), respectively. The bacterial community was characterized by using 16Sr DNA sequencing technology. The addition of WSC significantly altered both fermentation quality and the bacterial community of stylo silage. The additive treatment resulted in higher lactic acid (17.2 vs. 67.0 g/kgDM), lower pH (4.68 vs. 4.46), and lower acetic acid (37.0 vs. 28.9 g/kgDM) compared with the control. In addition, no propionic acid and butyric acid were detected in the additive treatment groups. These results indicated that WSC additives helped to produce greater quality stylo silage. Meanwhile, the Shannon index was higher, and the Simpson index was lower in the WSC additive-treated groups compared with the control, indicating that WSC improved microbial diversity. Furthermore, WSC treatments increased the abundance of acid-producing bacteria *Megamonas* and *Bacteroides*, decreased the abundance of *Weissella*, and inhibited the growth of the undesirable *Enterobacter*. Our results confirmed that sucrose, glucose and molasses have similar beneficial effects on both bacterial community and silage fermentation of stylo. Molasses was recommended to be used in stylo silage for economic benefit and resource utilization.

Keywords: stylo silage; sucrose; glucose; molasses; fermentation quality; bacterial community

Citation: Zi, X.; Liu, Y.; Chen, T.; Li, M.; Zhou, H.; Tang, J. Effects of Sucrose, Glucose and Molasses on Fermentation Quality and Bacterial Community of Stylo Silage. *Fermentation* **2022**, *8*, 191. https://doi.org/10.3390/fermentation8050191

Academic Editor: Michela Verni

Received: 22 February 2022
Accepted: 18 April 2022
Published: 24 April 2022

1. Introduction

Stylo (*Stylosanthes guianensis*) is an important legume forage and is widely distributed in tropical and subtropical regions. It has both a high biomass (15–22 t/ha per year, FM base) and abundant nutrients (15–16% crude protein, DM base), making it an ideal animal feed [1–3]. It has been reported that stylo supplemented in the rations of ruminants has positive effects on animal performance [4]. In addition, stylo is also used in pig feed and may improve the feed's digestibility [5]. Normally, Stylo grows fast in the summer or rainy seasons, but its growth is significantly hindered in the winter, resulting in the lack of ruminants' feed. High temperature and humidity in tropical regions of China limit the production of Stylo hay, thus blocking its intensive use in herbivorous livestock feeding. To overcome this obstacle, stylo is normally ensiled after harvest.

Ensiling is essential to ensure the supply of livestock feed throughout the year. However, stylo is a typical tropical forage with relatively low contents of water-soluble carbohy-

drates (WSC), which makes it hard to make high-quality silage [1]. Therefore, it is urgent to appropriately treat stylo for its ensiling and processing. Liu et al. (2012) isolated lactic acid bacteria (LAB) from stylo silage and found that these strains effectively improved fermentation quality [6]. Our previous study indicated that LAB treatment could improve the fermentation quality, chemical composition, and ruminal degradation of stylo silage [1]. Meanwhile, preparing stylo with other grass could improve silage quality and provide more balanced nutrition. Bureenok et al. (2016) reported that the quality of mixing silage of stylo with guinea was higher than that of the ensiled alone and that feeding the mixed silage improved the growth performance of goats [7]. In addition, *Moringa oleifera* (fresh leaf or hay) improved fermentation quality by altering the microbial community structure of stylo silage [8,9]. WSC is one of the major silage additives due to its promoting effect on the production of lactic acid by LAB, which as a result, drops the pH and then enhances the quality of silage.

WSC generally includes molasses, sucrose and glucose; molasses is a waste product of the sugar industry, glucose is a monosaccharide and sucrose is a disaccharide, and all have applications in silage modulation [10–13]. Sucrose can increase the abundance of *Lactobacillus* and *Pseudomonas*, and it also inhibits the growth of undesirable *Enterobacter* in mulberry leaf silage [12]. Meanwhile, Molasses enhances the amount of desirable Lactobacillus and inhibits the growth of undesirable microorganisms such as Clostridia and Enterobacter in soybean silage [13]. However, the effects of different WSC, which shape the silage microbial community and then improve fermentation quality, are varied, and stylo silage bacterial community structure is still unknown. We hypothesize that WSC in stylo could help to improve silage fermentation by shifting the silage microbial community during ensiling.

Therefore, in this study, the addition of sucrose, glucose and molasses was examined in stylo silage to explore their effects on bacterial community, fermentation, and then eventually, the silage quality was analyzed.

2. Materials and Methods

2.1. Preparation of Silage

Stylo was planted at our experimental base (109°58′ E, 19°52′ N, Chinese Academy of Tropical Agricultural Sciences, CATAS, Danzhou, China). The varieties were planted during the spring season (10 March 2019) at CATAS, and harvested on 10 July 2019 (vegetative stage); the first cut was at 30 cm (plant height was approximately 100 cm) from the ground surface. The harvested stylo was chopped into small pieces at about 2 cm. Four different treatments were conducted in our current study as follows: (1) no additive (CK), (2) 10 g/kg sucrose (S), (3) 10 g/kg glucose (G), and (4) 10 g/kg molasses (M), all on fresh matter basis. The added dosage was based on the result of some published studies [10–13]. Every treatment was carried out in triplicates. The application rate of sugar was on fresh matter basis. Briefly, 200 g of stylo was blended with additives before being placed and vacuumed in plastic bags (35 cm × 12 cm × 5 cm; Guozhong Packing Co., Ltd., Haikou, China). A total of 60 bags (four treatments × three replicates × 5 ensiling time points) were prepared and incubated at normal temperature (25 to 30 °C). The bags were opened for analyses of chemical composition and organic acid levels on days 1, 3, 7, 14 and 30. The composition of the bacterial community was analyzed on day 30.

2.2. Chemical Assay

Specimens were heated at 65 °C for 72 h, and dried materials were ground for chemical analysis. The contents of dry matter (DM), crude protein (CP), water-soluble carbohydrates (WSC), neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured as previously described [1]. The quality of silage fermentation was determined using distilled water extracts. Briefly, 50 g wet silage was blended with 200 mL distilled water, followed by incubation at 4 °C for 24 h and then filtration. Half of each extract sample was stored at −80 °C for analysis of the microbiota diversity. The pH was measured with a glass electrode

pH meter. The organic acid was measured by high-performance liquid chromatography using a Sodex RS Pak KC-811 column (Showa Denko K.K., Kawasaki, Japan) and eloquent of 3 mole/L $HClO_4$ at a flow rate of 1.0 L/min and 40 °C and detected at 210 NM using DAD detector SPD-20A (Shimadzu Co., Ltd., Kyoto, Japan) as reported previously by Liu et al. [6].

2.3. Analysis of Microbial Community

The above-mentioned extracts were used for the molecular analysis of the microbiota. Microbial DNA was isolated from silage specimens with the E.Z.N.A.® soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions. The concentration and purity of extracted DNA were assessed by NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, CA, USA), and DNA integrity was confirmed by electrophoresis on 1% agarose gel. Primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were adopted to amplify the V3-V4 hypervariable regions of the bacterial 16S rRNA gene using thermocycler PCR system (GeneAmp 9700, ABI, Foster City, CA, USA). After PCR products were purified and quantified, next-generation sequencing was carried out using Illumina MiSeq 2500 platform (Illumina, Inc., San Diego, CA, USA), and paired-end reads of 250 bp were generated.

The assembly of tags was carried out using filtered reads according to the principles as follows: overlap between paired-end reads should be more than 10-bp overlap and less than 2% mismatch. The unique tags were obtained by removing redundant tags using mothur software (version 1.40.45) [14]. The abundance was then determined using the resultant unique tags. The high-quality reads were grouped into operational taxonomic units (OTUs) defined at a similarity of 97%. Diversity metrics were determined using the core-diversity plug-in within QIIME2 (https://docs.qiime2.org/2019.1/, accessed on 15 March 2020) [15]. The microbial diversity within an individual sample was assessed using the alpha diversity indices, including observed OTUs, Chao1 richness estimator, Shannon diversity index, and Simpson and ACE index [16]. Beta diversity was analyzed to assess the structural variation of microbiota across specimens, and then principal coordinates analysis (PCoA) and Unweighted Pair-group Method with Arithmetic Mean (UPGMA) were conducted [17]. Appropriate methods were employed to identify the bacterial strains with different abundances among samples and groups [18]. Unless specified above, parameters used in the analysis were set as default. The sequencing data were deposited in the Sequence Read Archive (SRA) under the accession number PRJNA637681.

2.4. Statistics

The effects of WSC additives, ensiling days and their interaction were evaluated by two-way analysis of variance using the general linear model procedure of SAS 9.3 software (SAS Institute Inc., Cary, NC, USA) according to the model for a 4 × 5 factorial treatment design. Differences were compared using Duncan's multiple range test, and differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Chemical Composition of Stylo

The DM of stylo was 301.0 g/kg (fresh matter basis), while the contents of CP, WSC, NDF and ADF were 104.9, 12.3, 510.4 and 407.6 g/kg (dry matter basis), respectively.

3.2. Soluble Sugar Improves the Fermentation Quality of Stylo Silage

The stylo silage's fermentation characteristics during ensiling are listed in Table 1. The pH values in all silages in our study were significantly reduced ($p < 0.05$) after 7 days of ensiling, while no significant changes were observed in all silages on day 30 with the lowest pH. Meanwhile, the pH values of the G, S and M silages were significantly lower than that of the CK silage after ensiling ($p < 0.05$). The pH value of additive silages was close to 4.2, which ensured the good preservation of stylo silage. The level of lactic acid in additive-

treated silages was largely increased during ensiling ($p < 0.05$), which was significantly higher than that of the CK group on day 30 ($p < 0.05$). No significant differences were observed among different additive-treated groups. The levels of acetic acid in all silages were significantly increased during ensiling ($p < 0.05$), and those of additives silages were lower than that of the CK group on day 30 ($p < 0.05$). In this study, the contents of propionic acid and butyric acid were slowly increasing during the ensiling process in the CK group but not detectable in additive-treated silages, indicating that the stylo was well-preserved after being treated with soluble sugar. The total acid content showed a similar trend as those of lactic acid and acetic acid, while additive-treated silage had higher total acid content than the CK group after ensiling. These results indicated that supplementation of soluble sugars promoted the fermentation quality and that different additive-treated silages in this study were similarly effective.

Table 1. Fermentation quality of stylo silage.

Item	Treatment	Days of Ensiling					SEM	*p*-Value		
		1	3	7	14	30		D	T	D*T
pH	CK	5.31 a	5.09 b	4.88 bA	4.74 bcA	4.68 cA	0.06	<0.01	<0.01	<0.01
	S	5.34 a	5.14 b	4.84 bcA	4.56 cB	4.48 cB				
	G	5.21 a	5.01 b	4.73 cB	4.54 cdB	4.47 cB				
	M	5.19 a	4.95 b	4.74 cB	4.55 dB	4.42 dB				
Lactic acid (g/kg DM)	CK	8.8 dC	9.7 cdB	11.1 bcB	13.5 bB	17.2 aB	12.50	<0.01	<0.01	<0.01
	S	10.2 eB	19.0 dA	39.0 cA	53.8 bA	67.0 aA				
	G	10.1 eB	20.7 dA	38.4 cA	54.4 bA	65.1 aA				
	M	12.4 eA	24.7 dA	38.9 cA	56.9 bA	69.0 aA				
Acetic acid (g/kg DM)	CK	8.7 e	11.3 d	16.3 cA	24.4 bA	37.0 aA	2.20	<0.01	<0.01	<0.01
	S	8.7 e	11.5 d	14.6 cAB	20.5 bB	27.8 aB				
	G	8.9 e	12.9 d	16.9 cAB	21.1 bB	27.5 aB				
	M	9.0 e	13.6 d	19.1 cAB	25.8 bAB	31.3 aAB				
Propionic acid (g/kg DM)	CK	0.5 c	0.8 b	1.2 ab	1.3 ab	1.6 aA	0.39	<0.01	<0.01	<0.01
	S	ND	ND	ND	ND	0.0 B				
	G	ND	ND	ND	ND	0.0 B				
	M	ND	ND	ND	ND	0.0 B				
Butyric acid (g/kg DM)	CK	0.2 c	0.4 b	0.6 ab	0.8 a	0.8 aA	0.21	–	<0.01	–
	S	ND	ND	ND	ND	0.0 B				
	G	ND	ND	ND	ND	0.0 B				
	M	ND	ND	ND	ND	0.0 B				
Total acid (g/kg DM)	CK	18.2 eB	22.2 dC	29.2 cA	40.0 bB	56.6 aB	9.98	<0.01	<0.01	<0.01
	S	18.9 eB	30.5 dB	53.6 cA	74.3 bA	94.9 aA				
	G	19 eB	33.6 dB	55.3 cA	75.5 bA	92.6 aA				
	M	21.4 eA	38.3 dA	58.0 cA	82.7 bA	100.4 aA				

CK, control; S, sucrose; G, glucose; M, molasses. DM, dry matter; ND, not detected; "–", default. D, days of ensiling; T, treatment; D*T, interaction of ensiling time and treatment; SEM, standard error of means; Means with different letters in the same row (a–d) or column (A–D) differ ($p < 0.05$).

3.3. Effects of Soluble Sugars on the Microbial Community of Stylo Silages

On average, 75,611 clean tags and 62,803 effective tags were obtained in each silage sample from sequencing. Overall, 934,418 raw reads and 908,608 raw tags were generated. As shown in Figure 1, soluble sugar treatments affected the indices of OTUs, Ace, Chao 1, Shannon and Simpson of the microbial diversity and richness. 543 OTUs were observed in all stylo silages. Similar numbers of OUTs (540, 536, and 536) were observed in different soluble sugar treatment groups, but they were higher than that of the CK group (524) (Figure 1A). The treatment groups contained 514 common OTUs, while 2 specific OTUs were observed in the M group (Figure 1B). No significant differences were observed in the indices of richness (Chao 1 and ACE) between the CK group and the soluble sugar treatment groups (except ACE in CK group and G group) (Figure 1C,D). These indices

indicated that soluble sugar slightly affected the richness of the stylo silage microbial community. In contrast to the richness indices, however, the Shannon index was higher, while the Simpson index was lower, in the additive-treated groups than those of the CK group, indicating that soluble sugar treatments led to higher diversity (Figure 1E,F). The higher OTUs number, Shannon index, together with the lower Simpson index suggested higher microbial abundance and higher diversity. These results suggested that soluble sugar treatment may raise microbial diversity. The principal coordinate analysis (PCoA) and cluster tree were employed to examine the correlations among the community structures of the silage microbial community. Clear separation and differences in bacterial community were found in all four groups (Figure 2A), while the CK group and the S group had closer genetic distance compared with the G and M groups (Figure 2B), indicating that the microbial composition was changed by soluble sugar treatments during the ensiling process. Overall, our results demonstrated that supplementing soluble sugar affects the microbial diversity and community structure of stylo silage.

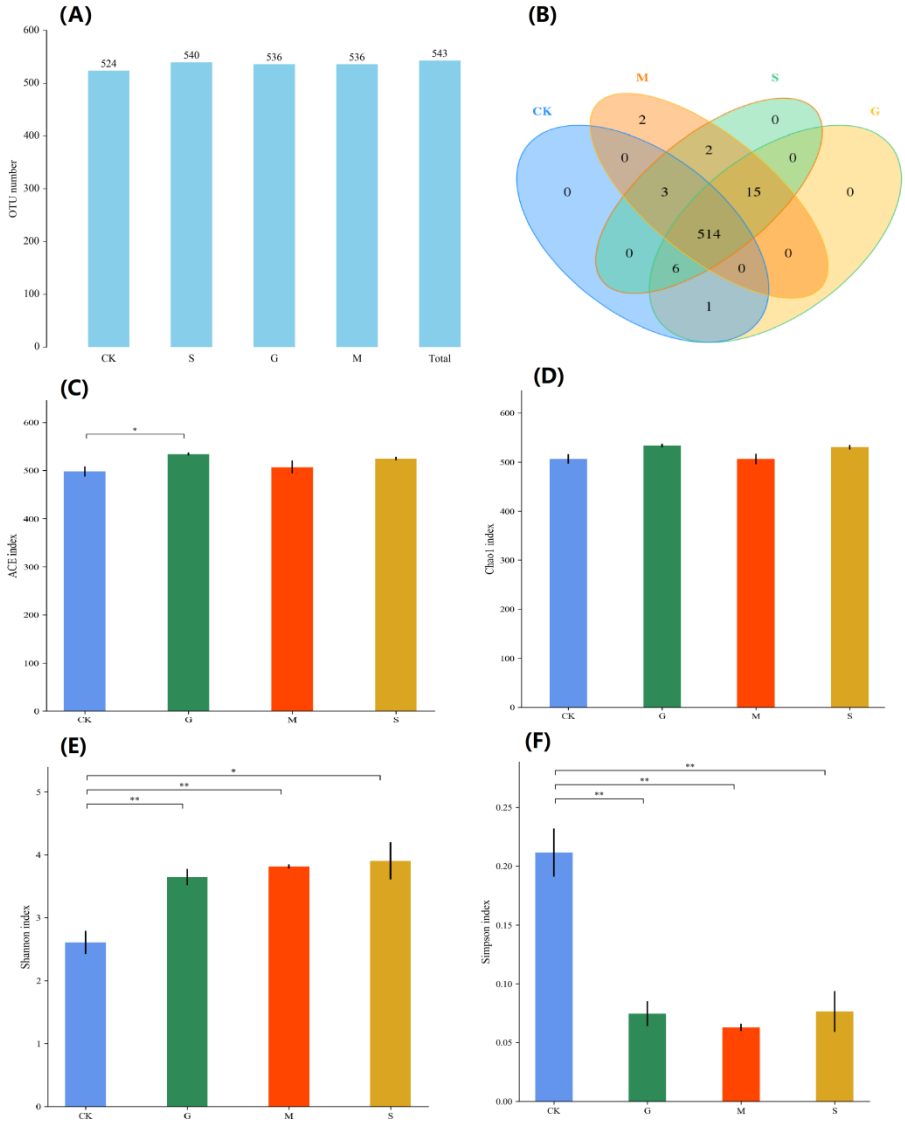

Figure 1. Venn analysis of OTUs and alpha-diversity of the bacterial community of stylo silage. '*' and '**' represent $p < 0.05$ and $p < 0.01$, respectively. CK, control; S, sucrose; G, glucose; M, molasses.(**A**) OTUs number of the bacterial community of stylo silage; (**B**) Venn analysis of OTUs in the bacterial community of stylo silage; (**C**–**F**) the ACE, Chao1, Shannon and Simpson index of the bacterial community of stylo silage, respectively.

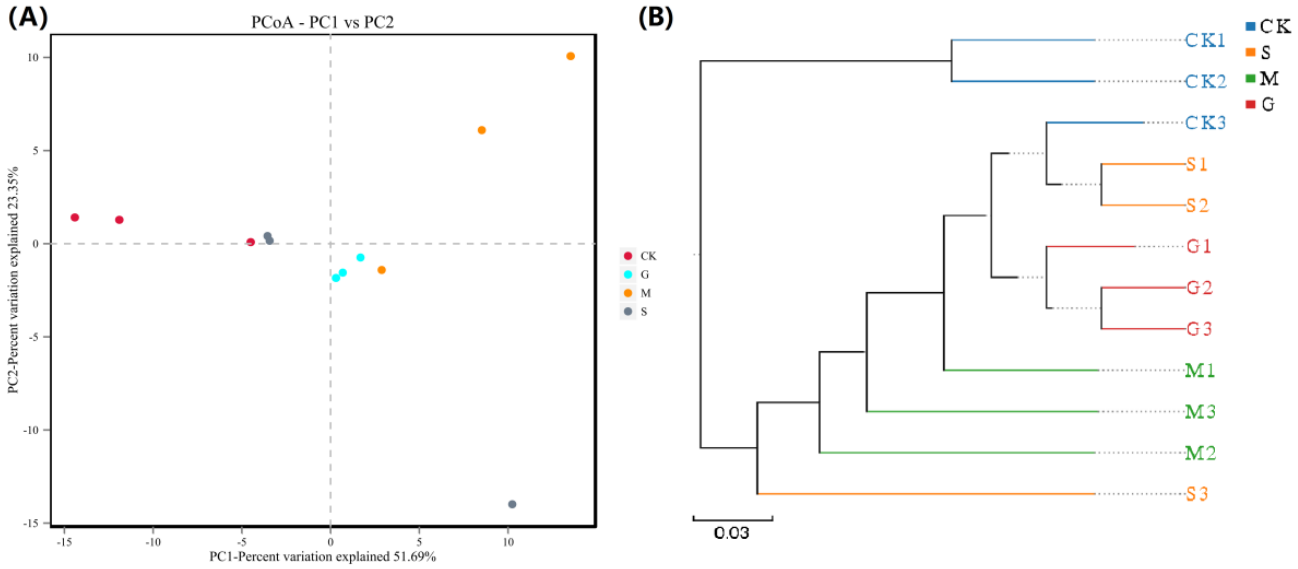

Figure 2. Beta-diversity of bacterial community of stylo silage. CK, control; S, sucrose; G, glucose; M, molasses.(**A**) PCoA and UPGMA (**B**) analysis of bacterial community of stylo silage.

Figure 3(A1,A2) describes the microbial community at the phylum level of all samples with each treatment. *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria* and *Cyanobacteria* were dominant in all treatments (>1% abundance). We also examined the bacterial structures of stylo silages at the genus level to further investigate the additives' effects on the microbial community (Figure 3(B1,B2)). *Enterobacter*, *Megamonas*, *Weissella*, *Pantoea*, *Bacteroides*, *Ruminococcus*, *Stenotrophomonas*, *Megasphaera*, *Faecalibacterium* and *Bifidobacterium* were the top 10 dominant genus in all treatments. Furthermore, *Weissella*, *Enterobacter*, *Pantoea* and *Stenotrophomonas* were predominant in the CK group, representing 47.71%, 21.39%, 8.05% and 3.95% of the total sequences, respectively. While in the S group, *Enterobacter*, *Megamonas*, *Weissella*, *Pantoea*, *Bacteroides*, *Ruminococcus*, *Stenotrophomonas*, *Megasphaera*, *Faecalibacterium* and *Bifidobacterium* were the top 10 dominant genera, representing 14.04%, 10.55%, 9.68%, 7.73%, 4.19%, 3.84%, 2.72%, 2.46%, 2.34% and 2.20%, respectively. In the G group, *Enterobacter*, *Megamonas*, *Weissella*, *Pantoea*, *Bacteroides*, *Ruminococcus*, *Megasphaera*, *Faecalibacterium* and *Bifidobacterium*, *Stenotrophomonas* were the top 10 dominant genera, representing 16.96%, 14.71%, 9.77%, 8.03%, 5.46%, 5.06%, 3.41%, 3.28%, 2.92% and 1.91%, respectively. In the M group, *Megamonas*, *Bacteroides*, *Enterobacter*, *Ruminococcus*, *Megasphaera*, *Weissella*, *Faecalibacterium*, *Bifidobacterium*, *Pantoea* and *Stenotrophomonas* were the top 10 dominant genera, representing 20.10%, 7.91%, 7.47%, 7.40%, 4.75%, 4.53%, 4.44%, 4.15%, 3.39% and 1.13%, respectively. In addition, the number of unclassified species increased from 18.13% (CK) to 40.25% (S), 28.22% (G) and 34.75% (M).

Figure 4 shows the effects of sucrose, glucose and molasses on the relative abundance of bacteria in the most dominant phylum (A) and genus (B) in stylo silage. The community was shifted along with soluble sugar treatments: the abundances of *Bacteroidetes* and *Actinobacteria* were both increased ($p < 0.05$), while the abundance of *Proteobacteria* was decreased ($p < 0.05$) in additive-treated groups compared with the CK group. The abundances of *Weissella*, *Enterobacter* and *Stenotrophomonas* were decreased by additive treatments (except *Pantoea* in the G group) ($p < 0.05$), while the abundances of *Megamonas*, *Bacteroides*, *Megasphaera*, *Faecalibacterium*, *Stenotrophomonas* and *Bifidobacterium* were increased by the treatments ($p < 0.05$).

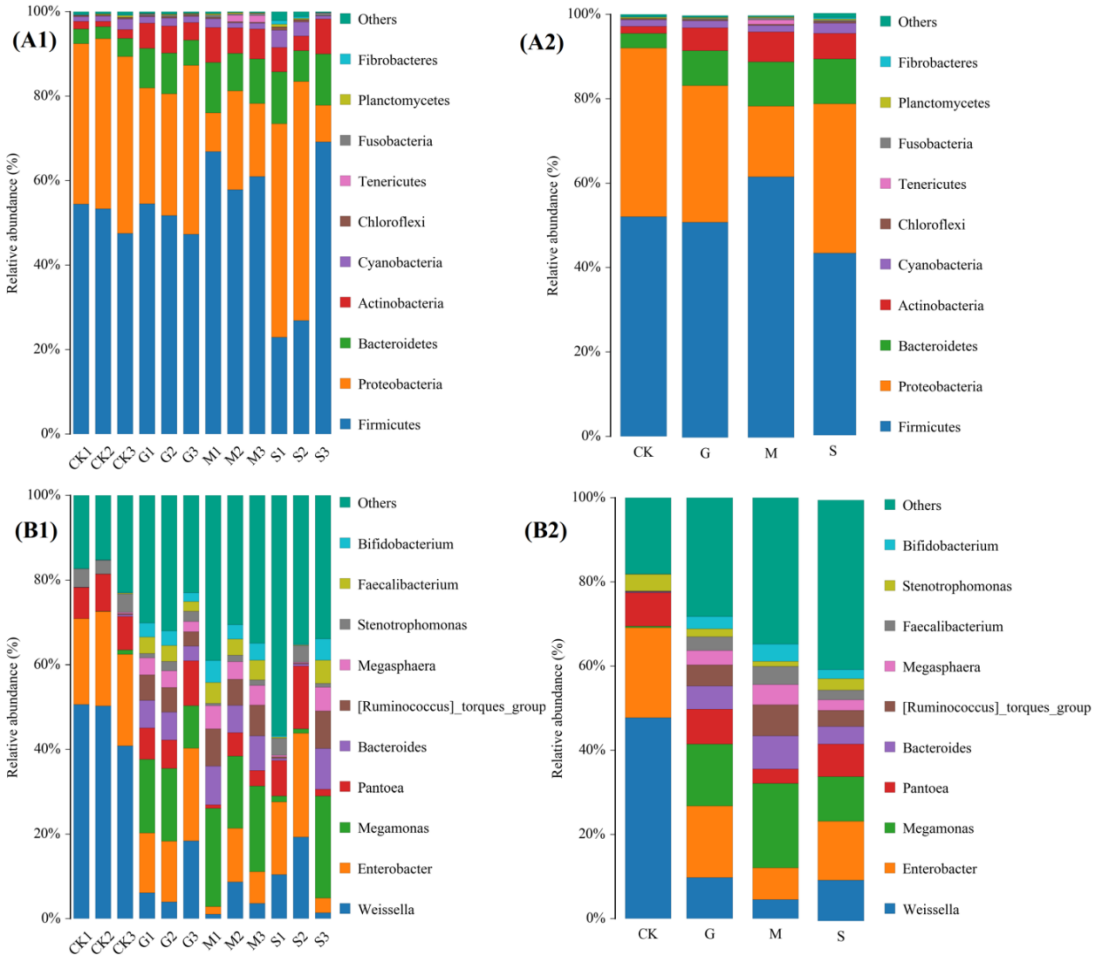

Figure 3. The bacterial abundance at the levels of phylum and genus in stylo silage. (**A1**) Relative abundance of bacteria at the phylum level of each sample; (**A2**) Relative abundance of bacteria at the phylum level of each group. (**B1**) Relative abundance of bacterial at the genus level of each group; (**B2**) Relative abundance of bacteria at the genus level. CK, control; S, sucrose; G, glucose; M, molasses.

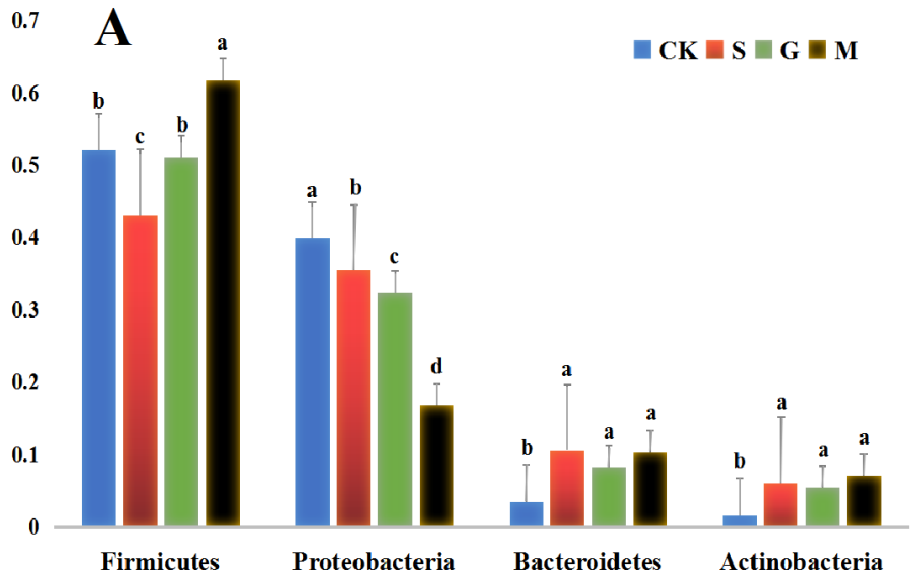

Figure 4. *Cont.*

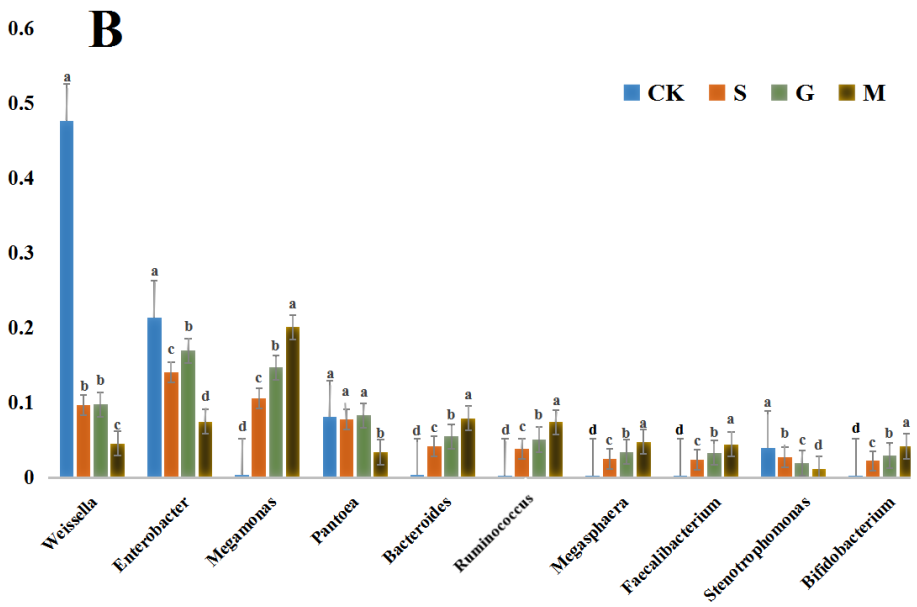

Figure 4. Effects of sucrose, glucose and molasses on the relative abundance of bacterial (**A**) in the most dominant phylum and (**B**) genus in stylo silage. Error bars represent the SD of three samples. Boxes with a different letter above the error bars are significantly different at $p < 0.05$. CK, control; S, sucrose; G, glucose; M, molasses.

4. Discussion

Generally, the chemical composition of raw materials is an important factor to determine the silage fermentation quality, especially DM and WSC content. Guyader et al. [19] suggested that the DM content of well-preserved raw material of silage was 30–35%. Meanwhile, the WSC is another key factor that affects the quality of fermentation. In this study, the DM of stylo was consistent with the previous results, but the WSC content was obviously lower than the minimum requirement for well-preserved silage [1,3,8,9]. However, some previous studies have confirmed that stylo ensiled alone can not achieve high fermentation quality [1,3,8,9]. Therefore, it might be helpful to use additives, such as soluble sugars, to provide more substrates for lactic acid bacteria and promote lactic fermentation in order to obtain well-preserved stylo silage.

The silage pH is an important indicator of fermentation quality, and silage at pH 4.2 or lower is considered well-fermented [20]. The pH value of additive silages was close to 4.2, which ensured good preservation of stylo silage. A previous study has shown higher pH values at 5.0 or above in stylo silage, which used gallic acid additive and the chemical characteristics were different compared with this study [21]. The lactic acid in silage is the dominant product from fermentation, thus an important index to evaluate silage quality. The addition of soluble sugar may promote the reproduction and growth of LAB and lead to increased lactic acid content. This is consistent with previously reported results [10–13], demonstrating that sucrose, glucose and molasses can remarkably increase the content of lactic acid. As the main metabolite of *acetobacter* fermentation in silage, acetic acid is also a crucial index for the evaluation of silage quality. Higher acetic acid level leads to higher pH, which benefits the undesirable microorganism *clostridium* and then reduces the quality of fermentation [22]. In this study, the contents of propionic acid and butyric acid were not detectable in additive-treated silages, indicating that the stylo was well-preserved after being treated with soluble sugar. This is consistent with the results from mulberry leaf silage treated with sucrose [13]. These results indicated that supplementation of soluble sugars promoted the fermentation quality, and that different additive-treated silages in this study were similarly effective.

In the present study, *Firmicutes*, *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were the dominant phylum in stylo silages. Xu et al. [23] and Dong et al. [24] have shown

similar results in corn stover and red clover silage. Another study found that *Firmicutes* and *Proteobacteria* were the most abundant phyla in silages for hydrolysis and acidification, with more than 90% of increase after fermentation [9]. This might be caused by the low pH and the anaerobic condition of silage.

Furthermore, the soluble sugar changed the microbial composition of stylo silage at the genus level. The abundance of *Megamonas* was significantly increased and became dominant in the soluble sugar treatment groups. *Megamonas* is commonly found in the intestines of humans and animals [25–27]. *Megamonas* can ferment many types of carbohydrates and produce lactic acid, acetic acid and propionic acid [27]. In this study, the soluble sugar treatments resulted in higher levels of lactic acid and total acids but lower abundance of lactic acid bacteria, probably caused by the higher abundance of *Megamonas.* In addition, *Bacteroides* is an anaerobic bacterium, usually lives in the intestines and the respiratory tract of humans and animals and produces organic acids such as lactic acid, acetic acid and propionic acid [28,29]. *Bacteroides*, a bacterium rarely reported in silage, was found to be dominant in this study in the soluble sugar treatment groups with higher levels of both lactic acid and total acids, compared with the control group. Both *Megamonas* and *Bacteroides* are usually found in humans and animals but only rarely reported to be in silages. Therefore, it is not clear how their abundances were significantly increased in well-conserved stylo silages, and further study is required to explore the relevant mechanisms. Furthermore, *Weissella*'s abundance was decreased by soluble sugar treatments but remained as one of the predominant microbial species in stylo silage, similar to what was observed with the silage of forage soybean mixed with crop corn or sorghum [30]. *Weissella* is a lactic acid-producing bacterium and the dominant group in fresh forage or at the beginning of silage fermentation before being replaced by other lactic acid-producing microorganisms after ensiling [31–33]. This may be explained by the fact that *Weissella* can ferment fewer kinds of sugars compared with *Megamonas*, leading to weakened competitiveness to and being replaced by *Megamonas. Enterobacter* is one of the major undesirable microorganisms in silage [12,13,21]. Besides, *Enterobacter* is the main competitor of *Megamonas* for soluble sugar, thus its decline is beneficial to the lactic acid-producing microorganisms [34]. This may explain the relatively higher lactic acid levels in the soluble sugar-treated silages. Moreover, in this study, the abundances of *Megasphaera*, *Faecalibacterium*, *Stenotrophomonas* and *Bifidobacterium* were significantly enhanced in soluble sugar treatment groups with higher fermentation quality than those in the control group with lower fermentation quality. We speculated that the increases of these microbial species balanced the micro-ecosystem and improved the quality of silage fermentation.

It should be noted that, the full chemical compositions of silage were very important for the evaluation of fermentation quality. Unfortunately, in this study, the silage chemical compositions were not analyzed, and only discussed the role of WSC in stylo silage from the perspective of fermentation quality and microorganisms. Therefore, the implications of these results for silage production are limited. Besides, the 16Sr DNA sequencing technology was conducted for the microbial diversity study, and the result was only at the genus level, which is not accurate enough. Hence, in-depth investigations are needed to clearly understand their roles in silage fermentation, for instance, using single molecule, real-time (SMRT) sequencing or metagenomic sequencing.

5. Conclusions

The sucrose, glucose, and molasses treatment resulted in significantly increased lactic acid content, reduced pH, acetic acid, propionic acid and butyric acid content, then improved the quality of fermentation of stylo silage. Meanwhile, the WSC additives altered the stylo silage bacterial community, raising the abundance of acid-producing bacteria *Megamonas*, *Bacteroides*, *Megasphaera*, *Faecalibacterium*, *Stenotrophomonas* and *Bifidobacterium*, while decreasing the abundance of *Weissella* and inhibiting the growth of undesirable *Enterobacter*, which led to the improved fermentation of stylo silage. The results obtained from this study confirmed that sucrose, glucose, and molasses have similar beneficial effects on

both the bacterial community and silage fermentation of stylo. Molasses was recommended to be used in stylo silage for economic benefits and resource utilization.

Author Contributions: M.L. and X.Z. did the experimental design work. X.Z., M.L., Y.L., T.C., H.Z. and J.T. conducted the experiments. X.Z., M.L., H.Z. and J.T. analyzed the data. X.Z., M.L., Y.L., T.C., H.Z. and J.T. wrote and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This study was funded by the NSFC (No. 31860680), CATAS(No. 1630032022011), the Ministry of Agriculture and Rural Affairs of the People's Republic of China (No. 12200012) and Integrated Demonstration of Key Techniques for the Industrial Development of Featured Crops in Rocky Desertification Areas of Yunnan-Guangxi-Guizhou Provinces.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest: The authors declare that they have no conflict of interest.

References

1. Li, M.; Zhou, H.; Zi, X.; Cai, Y. Silage fermentation and ruminal degradation of stylo prepared with lactic acid bacteria and cellulase. *Anim. Sci. J.* **2017**, *88*, 1531–1537. [CrossRef] [PubMed]
2. Palsaniya, D.R.; Tiwari, S.; Ram, S.N.; Shivkumar, B.G.; Ghosh, P.K. Stylosanthes Production, Processing and Utilization Technologies for Animal Production—A Review. *Anim. Nutr. Feed Technol.* **2017**, *17*, 501–514. [CrossRef]
3. Zhang, Y.; Li, M.; Zhou, H.; Hu, L.; Li, W.; Xu, T. Associative effects of stylo and king grass silage different ratios on in vitro Rumen Fermentation. *Legume Res.* **2018**, *41*, 584–588. [CrossRef]
4. Braga, G.J.; Ramos, A.K.B.; Carvalho, M.A.; Fonseca, C.E.L.; Fernandes, F.D.; Fernandes, C.D. Liveweight gain of beef cattle in Brachiaria brizantha pastures and mixtures with Stylosanthes guianensis in the Brazilian savannah. *Grass Forage Sci.* **2020**, *75*, 206–215. [CrossRef]
5. Mutiaka, B.K.; Boudry, C.; Picron, P.; Kiatoko, H.; Bindelle, J. Feeding value of hays of tropical forage legumes in pigs: Vigna unguiculata, Psophocarpus scandens, Pueraria phaseoloides and Stylosanthes guianensis. *Trop. Anim. Health Prod.* **2014**, *46*, 1497–1502.
6. Liu, Q.; Chen, M.; Zhang, J.; Shi, S.; Cai, Y. Characteristics of isolated lactic acid bacteria and their effectiveness to improve stylo (*Stylosanthes guianensis* Sw.) silage quality at various temperatures. *Anim. Sci. J.* **2012**, *83*, 128–135. [CrossRef]
7. Bureenok, S.; Sisaath, K.; Yuangklang, C.; Vasupen, K.; Schonewille, J.T.h. Ensiling characteristics of silages of Stylo legume (*Stylosanthes guianensis*), Guinea grass (*Panicum maximum*) and their mixture, treated with fermented juice of lactic bacteria, and feed intake and digestibility in goats of rations based on these silages. *Small Rumin. Res.* **2016**, *134*, 84–89. [CrossRef]
8. He, L.; Lv, H.; Xing, Y.; Wang, C.; You, X.; Chen, X.; Zhang, Q. The nutrients in *Moringa oleifera* leaf contribute to the improvement of stylo and alfalfa silage: Fermentation, nutrition and bacterial community. *Bioresour. Technol.* **2020**, *301*, 122733. [CrossRef]
9. Wang, C.; He, L.; Xing, Y.; Zhou, W.; Yang, F.; Chen, X.; Zhang, Q. Fermentation quality and microbial community of alfalfa and stylo silage mixed with *Moringa oleifera* leaves. *Bioresour. Technol.* **2019**, *284*, 240–247. [CrossRef]
10. Dong, Z.; Wang, S.; Zhao, J.; Li, J.; Shao, T. Effects of additives on the fermentation quality, in vitro digestibility and aerobic stability of mulberry (*Morus alba* L.) leaves silage. *Asian Australas. J. Anim. Sci.* **2020**, *33*, 1292–1300. [CrossRef]
11. 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]
12. Ni, K.; Wang, F.; Zhu, B.; Yang, J.; Zhou, G.; Pan, 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]
13. Wang, Y.; Chen, X.; Wang, C.; He, L.; Zhou, W.; Yang, F.; 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]
14. Schloss, P.D.; Westcott, S.L.; Ryabin, T.; Hall, J.R.; Hartmann, M.; Hollister, E.B.; Lesniewski, R.A.; Oakley, B.B.; Parks, D.H.; Robinson, C.J.; et al. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Appl. Environ. Microbiol.* **2009**, *75*, 7537–7541. [CrossRef]
15. Callahan, B.J.; Mcmurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S.P. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* **2016**, *13*, 581–583. [CrossRef]
16. Vázquez-Baeza Yoshiki, M.P.; Antonio, G.; Knight, B. EMPeror: A tool for visualizing high-throughput microbial community data. *GigaScience* **2013**, *2*, 16. [CrossRef]
17. Sakaki, T.; Takeshima, T.; Tominaga, M.; Hashimoto, H.; Kawaguchi, S. Recurrence of ICA-PCoA aneurysms after neck clipping. *J. Neurosurg.* **1994**, *80*, 58–63. [CrossRef]

18. Segata, N.; Abubucker, S.; Goll, J.; Schuber, A.M. Metagenomic Biomarker Discovery and Explanation. *Genom biolo* **2011**, *12*, R60. [CrossRef]
19. Guyader, J.; Baron, V.; Beauchemin, K. Corn forage yield and quality for silage in short growing season areas of the Canadian prairies. *Agronomy* **2018**, *8*, 164–175. [CrossRef]
20. McDonald, P.; Henderson, N.; Herson, S. *The Biochemistry of Silage*; Chalcombe Publications: Marlow, England, 1991.
21. He, L.; Chen, N.; Lv, H.; Wang, C.; Zhou, W.; Chen, X.; 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]
22. Zheng, M.L.; Niu, D.Z.; Jiang, D.; Zuo, S.S.; Xu, C.C. Dynamics of microbial community during ensiling direct-cut alfalfa with and without LAB inoculant and sugar. *J. Appl. Microbiol.* **2017**, *122*, 1456–1470. [CrossRef] [PubMed]
23. Xu, Z.; He, H.; Zhang, S.; Kong, J. Effects of inoculants Lactobacillus brevis and Lactobacillus parafarraginis on the fermentation characteristics and microbial communities of corn stover silage. *Sci. Rep.* **2017**, *7*, 13614. [CrossRef] [PubMed]
24. Dong, Z.; Li, J.; Chen, L.; Wang, S.; Shao, T. Effects of Freeze–Thaw Event on Microbial Community Dynamics During Red Clover Ensiling. *Front. Microbiol.* **2019**, *10*, 1559. [CrossRef] [PubMed]
25. Chen, Y.; Ni, J.; Li, H. Effect of green tea and mulberry leaf powders on the gut microbiota of chicken. *BMC Vet. Res.* **2019**, *15*, 77. [CrossRef]
26. Maaloum, M.; Diop, A.; Ndongo, S.; Nguyen, T.T.; Cadoret, F.; Raoult, D.; Fournier, P.E. Draft Genome Sequence of Megamonas funiformis Strain Marseille-P3344, Isolated from a Human Fecal Microbiota. *Genome Announc.* **2018**, *6*, e01459-17. [CrossRef]
27. Tian, D.; Xu, X.; Peng, Q.; Wen, Z.; Zhang, Y.; Wei, C.; Qiao, Y.; Shi, B. In vitro fermentation of arabinoxylan from oat (Avena sativa L.) by Pekin duck intestinal microbiota. *3 Biotech.* **2019**, *9*, 54. [CrossRef]
28. Li, M.; Zhou, H.; Pan, X.; Xu, T.; Zhang, Z.; Zi, X.; Jiang, Y. Cassava foliage affects the microbial diversity of Chinese indigenous geese caecum using 16S rRNA sequencing. *Sci. Rep.* **2017**, *7*, 45697. [CrossRef]
29. Xu, J.; Bjursell, M.K.; Himrod, J.; Deng, S.; Carmichael, L.K.; Chiang, H.C.; Hooper, L.V.; Gordon, J.I. A genomic view of the human-Bacteroides thetaiotaomicron symbiosis. *Science* **2003**, *299*, 2074–2076. [CrossRef]
30. 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]
31. Cai, Y.; Benno, Y.; Ogawa, M.; Ohmomo, S.; Kumai, S.; Nakase, T. Inflfluence 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]
32. Ding, Z.; Bai, J.; Xu, D.; Li, F.; Zhang, Y.; Guo, X. Microbial Community Dynamics and Natural Fermentation Profiles of Ensiled Alpine Grass Elymus nutans Prepared From Different Regions of the Qinghai-Tibetan Plateau. *Front. Microbiol.* **2020**, *11*, 855. [CrossRef] [PubMed]
33. Guan, H.; Yan, Y.; Li, X.; Li, X.; Shuai, Y.; Feng, G.; Ran, Q.; Cai, Y.; Li, Y.; Zhang, X. Microbial communities and natural fermentation of corn silages prepared with farm bunker-silo in Southwest China. *Bioresour. Technol.* **2018**, *265*, 282–290. [CrossRef] [PubMed]
34. Muck, R.E. Silage microbiology and its control through additives. *Rev. Bras. Zootecn.* **2010**, *39*, 183–191. [CrossRef]