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Effect of Mixing Alfalfa with Whole-Plant Corn in Different Proportions on Fermentation Characteristics and Bacterial Community of Silage

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Abstract: The influence of mixing alfalfa with whole-plant corn in different proportions on the fermentation characteristics and bacterial community of silage was investigated. Alfalfa and whole-plant corn, harvested at dry matter content of 276.47 and 328.43 g/kg fresh weight, accordingly, were chopped to approximately 2 cm and mixed at ratios of 100:0 (C0, control), 80:20 (C20), 60:40 (C40), 40:60 (C60), 20:80 (C80) and 0:100 (C100) on a fresh weight basis, respectively. Silos of each treatment were produced in triplicate and anaerobically fermented in darkness for 100 days at room temperature (20–21 °C). At silo opening, silage fermentation characteristics and bacterial composition and diversity were analyzed. The C0 silage was weakly preserved, evidenced by a low lactic acid concentration and a high value of pH, acetic acid, propionic acid, butyric acid and ammonia nitrogen. With corn proportion in the mixture increasing from 0% to 40%, silage pH, acetic acid, butyric acid and ammonia nitrogen level decreased, whereas the value of lactic acid and lactic acid to acetic acid ratio increased. The C40, C60, C80 and C100 silages' Flieg score, used to evaluate the overall fermentation quality, was above 80 and higher than C0 (25) and C20 (61) silages. The C0 silage contained a complex bacterial community at the genus level, consisting mainly of *Enterococcus* (38.86%), *Enterobacteria* (20.61%), *Rhizobium* (8.45%), *Lactobacillus* (8.15%), *Methylobacterium* (5.54%) and *Weissella* (5.24%). As corn percentage increased from 0% to 40%, the relative abundance of desirable *Lactobacillus* increased and undesirable *Rhizobium* and *Methylobacterium* population reduced. With corn proportion in the mixture increasing from 0% to 40%, inclusion of corn to alfalfa at ensiling significantly improved silage fermentation quality and shifted the bacterial community for better silage preservation. Overall, high quality silage was produced when alfalfa was combined with at least 40% whole-plant corn on a fresh weight basis.

Keywords: conservation characteristics; forages mixing; microflora; *Medicago sativa*; *Zea mays*



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1. Introduction

Alfalfa (*Medicago sativa* L.) has been planted widely in northern China and many other regions of the world due to its high content in many essential vitamins, minerals and protein [1,2]. Ensiling is a common approach of conserving a forage and can reduce the shortage of green feed for ruminants in the countries with restricted growth seasons [3]. However, it is challenging to directly ensile alfalfa principally owing to a high buffering capacity (BC) [4,5] and a low water-soluble carbohydrates (WSC) and dry matter (DM) concentration [5,6]. The conventional methods to improve alfalfa's ensilability consist of

wilting [7], application of silage additives [8] and combining it with a forage crop rich in carbohydrates [9]. Several studies have indicated that high fermentation quality silage can be made by co-ensiling alfalfa and corn (*Zea mays* L.) [2,6,10].

Microbes, including lactic acid bacteria, play a key role in silage fermentation, and are divided into two kinds: desirable and undesirable ones [4]. Profiling microbial community in silage is of great importance to learn about which microbes are involved in top-quality fermentation. Commonly used culture-based techniques largely underestimated the microbial diversity present during ensiling [11]. Improved characterizing silage microbiota has been achieved through molecular technologies, including denaturing gradient gel electrophoresis [12], random amplified polymorphic DNA [13] and terminal restriction fragment length polymorphism [14]. Nevertheless, these approaches only identify some of the operational taxonomic units (OTUs) present due to poor detection limits [15]. Recently, next-generation sequencing techniques have been used to increase our understanding of silage microbiota [8] and have been applied to characterize the bacterial community of alfalfa and corn silages [7,16]. Besides, we previously evaluated the bacterial composition and diversity of silage prepared with alfalfa, corn stalk and their mixture by Illumina MiSeq sequencing, after 65 days (d) of ensiling, and found that the relative abundance of desirable *Lactobacillus* increased, whereas undesirable *Enterobacter* abundance decreased as corn stalk percentage ranged from 0% to 60% [5]. Furthermore, the *Lactobacillus* population in silage was positively correlated with lactic acid concentration and was negatively correlated with pH and ammonia nitrogen (NH₃-N) level. However, to the best of our knowledge, most of the studies on co-ensiling alfalfa and corn focus on silage fermentation parameters and chemical characteristics, and very few works have evaluated the bacterial community in alfalfa-corn mixture silage.

Therefore, this study aimed to evaluate the impact of mixing alfalfa with corn in different proportions on the bacterial community and fermentation characteristics of silage. It was hypothesized that combining alfalfa with corn would increase silage lactic acid concentration and decrease pH and NH₃-N levels, and that the relative abundance of the major lactic acid bacteria involved in silage fermentation, such as *Lactobacillus* members, may be increased with a higher corn proportion in the mixture.

2. Materials and Methods

2.1. Forages Harvesting and Ensiling

Alfalfa and corn fields were located at the Zhuozhou Experimental Station (N 39°35'25"–39°36'05", E 115°42'12"–116°14'35") of China Agricultural University, Hebei, China. A second regrowth of alfalfa (cultivar "WL343HQ") at the early bloom stage was distributed in five plots (about 9 m²), of which three plots were selected randomly for harvesting alfalfa used in this work. The alfalfa was cut by hand, wilted outdoors (cloudy weather) for 4 h on a clean plastic sheet to DM content of 276.47 g/kg fresh weight (FW) and chopped to about 2 cm by a paper cutter on 17 September 2017. Wilted and chopped alfalfa was mixed thoroughly and divided into 18 piles. Meanwhile, whole-plant corn (cultivar "Beinong368") in three plots (about 15 m²) was at about the 1/3 milk line stage and manually harvested at DM content of 328.43 g/kg FW, leaving a stubble height of 15 cm. The harvested corn was chopped to 2 cm by a forage chopper, fully mixed and grouped into 18 piles. Chopped alfalfa and corn were sampled individually, and mixed at ratios of 100:0, 80:20, 60:40, 40:60, 20:80 and 0:100 on an FW basis, thereby resulting in corresponding treatments of C0 (control), C20, C40, C60, C80 and C100, respectively. For each of three replicates with C0 treatment, 200 g of alfalfa was thoroughly blended with 0 g of corn in a plastic basin (35.5 cm in diameter, 14 cm in height). The remaining treatments were C20 (160 g + 40 g), C40 (120 g + 80 g), C60 (80 g + 120 g), C80 (40 g + 160 g) and C100 (0 g + 200 g), accordingly, and the mixing process of these five treatments was done according to C0 treatment. The 200 g of mixed materials was packed into a plastic film bag and vacuumed by a sealer. Silos of each treatment were produced in triplicate and stored in darkness for 100 d at room

temperature (20–21 °C). The chopped alfalfa and corn materials were taken before forages mixture was ensiled.

2.2. Silage Fermentation Profile and Chemical Determination

After 100 d of fermentation, mini silos of each treatment were opened. The 10 g of silage was placed into a blender jar, diluted with distilled water to 100 g and homogenized for 35 s in a high-speed blender. The homogenate was filtered through two layers of medical gauze and pH was immediately determined. About 2 mL of filtrate was centrifuged at $8000 \times g$ for 15 min at 4 °C and was used for organic acids and $\text{NH}_3\text{-N}$ analysis. Silage organic acids were determined by high performance liquid chromatograph [5]. The $\text{NH}_3\text{-N}$ was analyzed according to Broderick and Kang [17]. Another 100 g of silage or ensiling material was dried for 72 h at 65 °C to analyze DM content, ground through a 1 mm sieve and stored in a desiccator at room temperature prior to chemical analysis. Neutral detergent fiber (NDF, method 2002.04), acid detergent fiber (ADF, method 973.18) and total nitrogen (TN, method 990.03) were determined according to Horwitz and Latimer [18]. Hemicellulose was estimated by the difference between NDF and ADF. Crude protein (CP) was calculated via multiplying TN by 6.25. Concentration of WSC was determined according to Murphy [19]. The BC was determined by the lactic acid titration method [5].

2.3. Silage Bacterial Diversity and Composition Analysis

The bacterial diversity and composition of silage was determined as recorded in detail by Wang et al. [5]. For extraction of DNA, 20 g of silage stored at -20 °C was blended with 80 mL of sterile saline solution and macerated in a shaker for 2 h at 150 rpm. The homogenate was filtered through two layers of cheesecloth and centrifuged at $8000 g$ for 15 min at 4 °C. The universal primer pair of 338-F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806-R (5'-GGACTACHVGGGTWTCTAAT-3') was employed to amplify the V3 and V4 region of the bacterial 16S rRNA gene [7].

2.4. Estimations

The fermentation coefficient (FC) of an ensiling material was calculated by Knicky and Spörndly [20], where $\text{FC} = \text{DM} + 8 \text{WSC}/\text{BC}$, and its value showed the ensilability of an ensiling material (≥ 45 , strong; ≤ 35 , weak). Flieg score was employed to assess the overall silage fermentation quality (>80 , excellent; 61–80, good; 41–60, medium; 21–40, weak; 0–20, poor) according to Woolford [21].

2.5. Statistical Analysis

The experiment was a completely randomized design with a 6×3 (6 treatments and 3 duplicates) factorial arrangement. One mini-silo was used as an experimental unit. Experimental data were analyzed using a general linear model procedure (SAS Inc. 2002–2012, Release 9.4; SAS Institute Inc., Cary, NC, USA) of SAS, with corn proportion in the mixture as a fixed effect and duplicates as a random effect. Least squares means and standard error of the means were reported per treatment. The sum of squares was further divided into orthogonal linear, quadratic and cubic contrasts in order to evaluate the effect of corn proportion on silage parameters. Significant difference was declared at $p < 0.05$ and the Duncan multiple comparisons test was employed to compare means. Unweighted UniFrac principal coordinate analysis (PCoA) was done and charted by R 3.5.2 installation package.

3. Results

3.1. Chemical Composition of Alfalfa and Corn Prior to Ensiling

Compared with corn, alfalfa prior to ensiling numerically contained a lower DM and WSC concentration but a higher BC level according to Table 1. The FC value of corn was in number higher than alfalfa.

Table 1. Chemical composition of alfalfa and corn prior to ensiling.

Item	DM (g/kg FW)	g/kg DM						FC
		WSC	NDF	ADF	Hemicellulose	CP	BC (mEq g/kg DM)	
Alfalfa	276.47	31.68	473.87	313.69	160.18	210.30	39.97	33.99
Corn	328.43	125.50	449.46	228.84	220.62	99.15	20.22	82.49

Note: DM, dry matter; FW, fresh weight; WSC, water-soluble carbohydrates; NDF, neutral detergent fiber; ADF, acid detergent fiber; CP, crude protein; BC, buffering capacity; FC, fermentation coefficient.

3.2. Fermentation Characteristics and Chemical Composition of Silage

The fermentation profile and chemical composition of silage are presented in Table 2. The C0 silage was weakly fermented, indicated by a low lactic acid concentration and a high value of pH, acetic acid, propionic acid, butyric acid and NH₃-N. With corn proportion in the mixture increasing from 0% to 40%, silage pH, acetic acid, butyric acid and NH₃-N level decreased ($p < 0.05$), whereas the value of lactic acid and lactic acid to acetic acid ratio increased ($p < 0.05$). The C40, C60, C80 and C100 silages' Flieg score was above 80 and higher than that for C0 and C20 silages ($p < 0.05$). The WSC and CP content in C0 silage was 8.4 times lower and 2.1 times higher than C100 silage.

Table 2. Fermentation characteristics and chemical composition of silage prepared with mixtures of alfalfa and corn.

Item ¹	Treatment ^{2,3}						SEM ⁴	p-Value ⁵		
	C0	C20	C40	C60	C80	C100		Linear	Quadratic	Cubic
pH	5.56 ^a	4.78 ^b	4.16 ^c	3.93 ^d	3.81 ^e	3.65 ^f	0.01	<0.01	<0.01	<0.01
Lactic acid (g/kg DM)	30.77 ^d	74.54 ^c	90.00 ^{ab}	87.13 ^b	92.04 ^a	94.14 ^a	0.95	<0.01	<0.01	<0.01
Acetic acid (g/kg DM)	37.29 ^a	34.66 ^b	27.27 ^c	19.75 ^d	15.76 ^e	14.01 ^f	0.52	<0.01	<0.01	<0.01
Lactic acid to acetic acid ratio	0.83 ^f	2.15 ^e	3.30 ^d	4.42 ^c	5.84 ^b	6.72 ^a	0.08	<0.01	0.12	0.43
Propionic acid (g/kg DM)	6.01 ^a	3.27 ^c	3.36 ^c	3.52 ^c	3.62 ^c	4.72 ^b	0.22	0.03	<0.01	0.01
Butyric acid (g/kg DM)	3.11 ^a	2.11 ^b	0.00 ^c	0.00 ^c	0.00 ^c	0.00 ^c	0.07	<0.01	<0.01	0.42
NH ₃ -N (g/kg TN)	196.89 ^a	156.44 ^b	95.05 ^c	59.07 ^d	42.09 ^e	27.72 ^f	1.88	<0.01	<0.01	<0.01
Flieg score	25 ^e	61 ^d	95 ^c	98 ^b	100 ^a	100 ^a	0.48	<0.01	<0.01	<0.01
DM (g/kg FW)	268.86 ^f	283.97 ^e	306.49 ^d	322.46 ^c	338.31 ^b	347.58 ^a	1.61	<0.01	<0.01	0.04
WSC (g/kg DM)	7.68 ^f	12.53 ^e	23.35 ^d	37.85 ^c	46.62 ^b	64.83 ^a	0.67	<0.01	<0.01	0.25
CP (g/kg DM)	183.90 ^a	173.71 ^b	159.36 ^c	135.24 ^d	109.40 ^e	85.09 ^f	1.49	<0.01	<0.01	0.36

Note: ¹ DM, dry matter; NH₃-N, ammonia nitrogen; TN, total nitrogen; FW, fresh weight; WSC, water-soluble carbohydrates; CP, crude protein. ² Alfalfa and corn were mixed at proportions of 100:0 (C0), 80:20 (C20), 60:40 (C40), 40:60 (C60), 20:80 (C80) and 0:100 (C100) on a fresh weight basis, respectively. ³ Means with different superscript letters (a–f) in a row were significantly different ($p < 0.05$) based on Duncan test. ⁴ SEM, standard error of the means. ⁵ Linear, quadratic and cubic effects of corn proportion on silage parameters.

3.3. Bacterial Diversity and Composition of Silage

The total valid sequences of 18 samples were 882,349 with an average of 49,019 reads per sample after quality control. Rarefaction curves (Figure 1) showed that the number of sequences determined was adequate for identifying OTU because these curves approached plateau as reads number increased. The Good's coverage in 18 samples was around 99.9% (Table 3), from which it was inferred that the depth of this sequencing was sufficient for bacterial diversity and composition analysis. As shown in Table 3, C80 and C100 silages had a higher Chao, Shannon and OTU number value relative to C0 and C20 silages ($p < 0.05$), whereas the Shannon index level in C100 silage was lower than C80 silage ($p < 0.05$). The PCoA plot (Figure 2) displayed differences in the bacterial community of 18 samples, subsequently classified into six different clusters, namely C0, C20, C40, C60, C80 and C100.

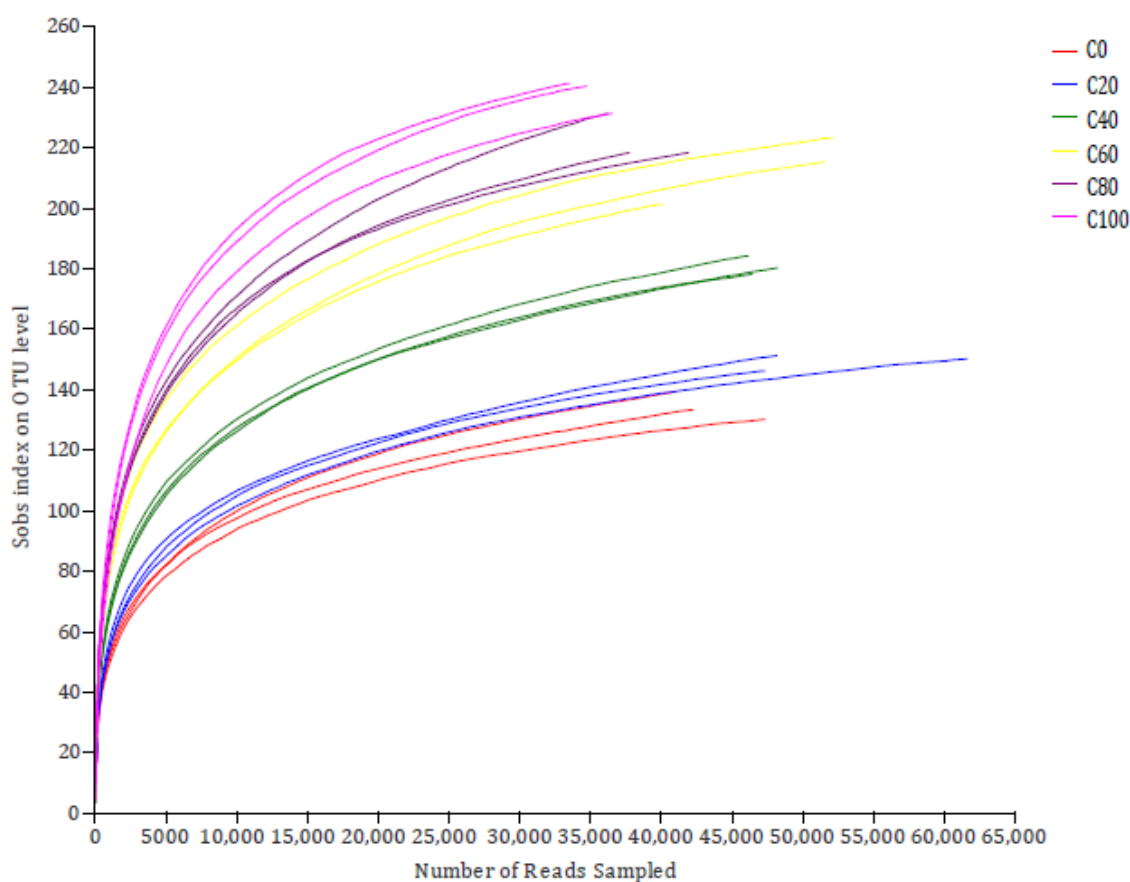


Figure 1. Rarefaction curves for samples in silage prepared with mixtures of alfalfa and corn. Alfalfa and corn were mixed at proportions of 100:0 (C0), 80:20 (C20), 60:40 (C40), 40:60 (C60), 20:80 (C80) and 0:100 (C100) on a fresh weight basis, respectively.

Table 3. Statistics of high-throughput sequencing data and bacterial community diversity.

Item ¹	Treatment ^{2,3}						SEM ⁴	p-Value ⁵		
	C0	C20	C40	C60	C80	C100		Linear	Quadratic	Cubic
Reads	50,226 ^{ab}	60,139 ^a	53,456 ^a	53,651 ^a	40,260 ^{bc}	36,385 ^c	3393.70	<0.01	0.01	0.16
Length	445 ^b	445 ^c	443 ^c	444 ^{bc}	444 ^{bc}	446 ^a	0.37	0.01	<0.01	0.63
Chao	163 ^c	171 ^c	226 ^b	238 ^{ab}	259 ^a	256 ^a	8.15	<0.01	0.03	0.10
Shannon	2.45 ^d	2.72 ^c	2.94 ^b	3.19 ^a	3.31 ^a	2.93 ^b	0.04	<0.01	<0.01	<0.01
Simpson	0.18 ^a	0.11 ^b	0.10 ^{bc}	0.08 ^{cd}	0.07 ^d	0.11 ^b	0.01	<0.01	<0.01	0.59
OTU number	134 ^e	149 ^d	181 ^c	213 ^b	222 ^b	237 ^a	3.66	<0.01	0.02	0.03
Coverage (%)	99.93	99.95	99.92	99.93	99.89	99.91	0.01	<0.01	0.90	0.07

Note: ¹ OTU, operational taxonomic unit. ² Alfalfa and corn were mixed at proportions of 100:0 (C0), 80:20 (C20), 60:40 (C40), 40:60 (C60), 20:80 (C80) and 0:100 (C100) on a fresh weight basis, respectively. ³ Means with different superscript letters (a–e) in a row were significantly different ($p < 0.05$) based on Duncan test. ⁴ SEM, standard error of the means. ⁵ Linear, quadratic and cubic effects of corn proportion on silage parameters.

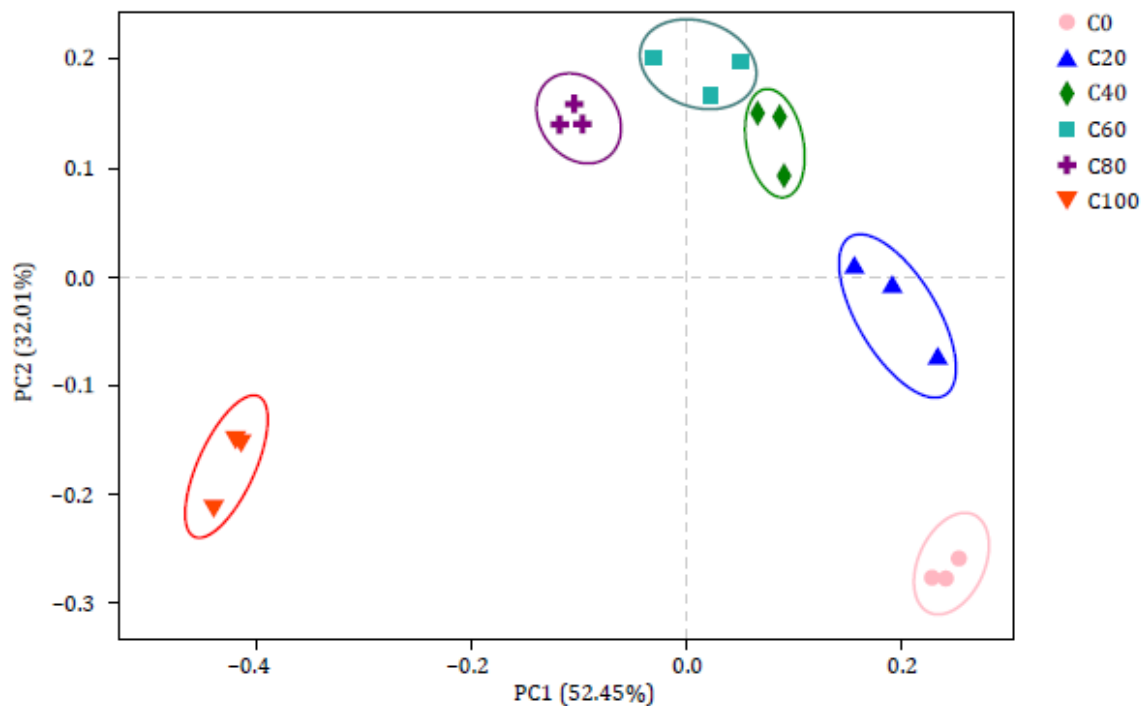


Figure 2. Unweighted UniFrac principal coordinate analysis (PCoA) plot of individual samples of silage prepared with mixtures of alfalfa and corn. Alfalfa and corn were mixed at proportions of 100:0 (C0), 80:20 (C20), 60:40 (C40), 40:60 (C60), 20:80 (C80) and 0:100 (C100) on a fresh weight basis, respectively.

The relative abundances of silage bacterial community at the phylum, family and genus level are shown in Tables 4–6, respectively. According to Table 4, silage bacterial community was represented mainly by *Firmicutes* (37.44–72.83%), *Proteobacteria* (21.76–54.09%), *Actinobacteria* (2.28–7.92%) and *Bacteroidetes* (0.09–1.89%). *Firmicutes* relative abundance dropped ($p < 0.05$) and *Proteobacteria* population increased ($p < 0.05$) when corn percentage increased from 0% to 80%. However, *Firmicutes* (72.83%) dominated the microbiota in C100 silage, followed by *Proteobacteria* (21.76%). As illustrated in Table 5, *Lactobacillaceae* and *Leuconostocaceae* abundance increased ($p < 0.05$) and *Enterococcaceae*, *Methylobacteriaceae* and *Rhizobiaceae* population declined ($p < 0.05$) with a higher corn inclusion. The C0 silage contained a complex bacterial community at the genus level (Table 6), composed mainly of *Enterococcus* (38.86%), *Enterobacteria* (20.61%), *Rhizobium* (8.45%), *Lactobacillus* (8.15%), *Methylobacterium* (5.54%) and *Weissella* (5.24%). As corn percentage increased from 0% to 40%, the relative abundance of *Lactobacillus* increased ($p < 0.05$) and *Rhizobium* and *Methylobacterium* population reduced ($p < 0.05$).

Table 4. Relative abundance (%) of the four most relatively abundant bacterial phyla in silage prepared with mixtures of alfalfa and corn.

Item	Treatment ^{1,2}						SEM ³	p-Value ⁴		
	C0	C20	C40	C60	C80	C100		Linear	Quadratic	Cubic
<i>Actinobacteria</i>	2.35 ^c	2.28 ^c	7.92 ^a	7.15 ^a	7.04 ^a	3.40 ^b	0.34	<0.01	<0.01	<0.01
<i>Bacteroidetes</i>	0.09 ^c	0.21 ^c	0.44 ^c	1.13 ^b	1.89 ^a	1.43 ^{ab}	0.21	<0.01	0.68	0.02
<i>Firmicutes</i>	55.65 ^b	43.53 ^c	42.49 ^c	37.44 ^c	39.92 ^c	72.83 ^a	1.63	<0.01	<0.01	<0.01
<i>Proteobacteria</i>	41.89 ^b	53.91 ^a	49.04 ^a	54.09 ^a	50.95 ^a	21.76 ^c	1.69	<0.01	<0.01	<0.01

Note: ¹ Alfalfa and corn were mixed at proportions of 100:0 (C0), 80:20 (C20), 60:40 (C40), 40:60 (C60), 20:80 (C80) and 0:100 (C100) on a fresh weight basis, respectively. ² Means with different superscript letters (a–c) in a row were significantly different ($p < 0.05$) based on the Duncan test. ³ SEM, standard error of the means. ⁴ Linear, quadratic and cubic effects of corn proportion on silage parameters.

Table 5. Relative abundance (%) of the 16 most relatively abundant bacterial families in silage prepared with mixtures of alfalfa and corn.

Item	Treatment ^{1,2}						SEM ³	p-Value ⁴		
	C0	C20	C40	C60	C80	C100		Linear	Quadratic	Cubic
<i>Microbacteriaceae</i>	1.50 ^c	1.98 ^c	7.13 ^a	6.19 ^b	5.90 ^b	1.97 ^c	0.28	<0.01	<0.01	<0.01
<i>Bacillaceae</i>	0.24 ^c	0.23 ^c	0.78 ^{bc}	1.29 ^{ab}	1.81 ^a	0.29 ^c	0.23	0.02	<0.01	<0.01
<i>Enterococcaceae</i>	38.86 ^a	15.15 ^b	5.40 ^c	2.61 ^d	1.12 ^d	0.50 ^d	0.87	<0.01	<0.01	<0.01
<i>Lactobacillaceae</i>	8.24 ^c	20.99 ^b	27.92 ^{ab}	26.31 ^{ab}	25.68 ^{ab}	33.46 ^a	2.23	<0.01	0.02	<0.01
<i>Lachnospiraceae</i>	0.00 ^b	0.00 ^b	0.03 ^b	0.10 ^b	0.54 ^b	4.64 ^a	0.24	<0.01	<0.01	<0.01
<i>Leuconostocaceae</i>	5.32 ^c	5.04 ^c	7.03 ^{bc}	5.94 ^c	9.62 ^b	25.06 ^a	0.93	<0.01	<0.01	<0.01
<i>Paenibacillaceae</i>	0.01 ^b	0.06 ^b	0.07 ^b	0.54 ^b	0.81 ^b	7.94 ^a	0.61	<0.01	<0.01	<0.01
<i>Aurantimonadaceae</i>	2.55 ^d	7.23 ^{ab}	8.34 ^a	6.11 ^{bc}	4.91 ^c	0.82 ^d	0.57	<0.01	<0.01	0.06
<i>Enterobacteriaceae</i>	23.16 ^c	30.71 ^a	27.29 ^{ab}	26.24 ^{bc}	22.95 ^c	12.68 ^d	1.16	<0.01	<0.01	0.71
<i>Methylobacteriaceae</i>	5.58 ^a	3.85 ^b	2.32 ^c	2.37 ^c	2.33 ^c	1.04 ^d	0.22	<0.01	<0.01	<0.01
<i>Moraxellaceae</i>	0.02 ^c	0.02 ^c	0.46 ^{bc}	1.53 ^a	2.06 ^a	0.82 ^b	0.18	<0.01	<0.01	<0.01
<i>Pseudomonadaceae</i>	0.02 ^b	0.01 ^b	0.25 ^b	3.20 ^a	4.17 ^a	0.25 ^b	0.35	<0.01	<0.01	<0.01
<i>Rhizobiaceae</i>	8.46 ^a	8.37 ^a	4.35 ^b	3.82 ^b	3.31 ^b	0.81 ^c	0.45	<0.01	0.64	0.91
<i>Rhodobacteraceae</i>	0.64 ^c	1.43 ^b	2.56 ^a	2.43 ^a	1.82 ^b	0.09 ^d	0.14	0.18	<0.01	0.03
<i>Sphingomonadaceae</i>	0.37 ^d	0.84 ^c	1.12 ^c	1.65 ^b	2.23 ^a	0.98 ^c	0.08	<0.01	<0.01	<0.01
<i>Xanthomonadaceae</i>	0.30 ^c	0.35 ^c	1.58 ^b	5.44 ^a	5.44 ^a	0.66 ^c	0.18	<0.01	<0.01	<0.01

Note: ¹ Alfalfa and corn were mixed at proportions of 100:0 (C0), 80:20 (C20), 60:40 (C40), 40:60 (C60), 20:80 (C80) and 0:100 (C100) on a fresh weight basis, respectively. ² Means with different superscript letters (a–d) in a row were significantly different ($p < 0.05$) based on the Duncan test. ³ SEM, standard error of the means. ⁴ Linear, quadratic and cubic effects of corn proportion on silage parameters.

Table 6. Relative abundance (%) of the 16 most relatively abundant bacterial genera in silage prepared with mixtures of alfalfa and corn.

Item	Treatment ^{1,2}						SEM ³	p-Value ⁴		
	C0	C20	C40	C60	C80	C100		Linear	Quadratic	Cubic
<i>Curtobacterium</i>	0.36 ^c	0.59 ^c	1.98 ^a	1.89 ^{ab}	1.69 ^b	0.47 ^c	0.08	<0.01	<0.01	<0.01
<i>Bacillus</i>	0.23 ^c	0.22 ^c	0.74 ^{bc}	1.23 ^{ab}	1.77 ^a	0.28 ^c	0.23	0.02	<0.01	<0.01
<i>Enterococcus</i>	38.86 ^a	15.15 ^b	5.40 ^c	2.61 ^d	1.12 ^d	0.50 ^d	0.87	<0.01	<0.01	<0.01
<i>Lactobacillus</i>	8.15 ^c	20.94 ^b	27.87 ^{ab}	26.23 ^{ab}	25.65 ^{ab}	33.45 ^a	2.29	<0.01	0.03	<0.01
<i>Leuconostoc</i>	0.08 ^d	0.15 ^d	0.78 ^d	1.97 ^c	4.86 ^b	13.73 ^a	0.38	<0.01	<0.01	<0.01
<i>Paenibacillus</i>	0.00 ^b	0.06 ^b	0.07 ^b	0.54 ^b	0.80 ^b	7.84 ^a	0.62	<0.01	<0.01	<0.01
<i>Weissella</i>	5.24 ^{bc}	4.89 ^{bc}	6.26 ^b	3.98 ^c	4.77 ^{bc}	11.34 ^a	0.71	<0.01	<0.01	<0.01
<i>Acinetobacter</i>	0.02 ^c	0.02 ^c	0.46 ^{bc}	1.53 ^a	2.06 ^a	0.82 ^b	0.18	<0.01	<0.01	<0.01
<i>Aureimonas</i>	2.55 ^d	7.23 ^{ab}	8.34 ^a	6.11 ^{bc}	4.91 ^c	0.82 ^d	0.57	<0.01	<0.01	0.06
<i>Enterobacter</i>	20.61 ^b	24.88 ^a	19.52 ^b	19.18 ^b	17.41 ^b	11.51 ^c	1.04	<0.01	<0.01	0.57
<i>Methylobacterium</i>	5.54 ^a	3.81 ^b	2.31 ^c	2.36 ^c	2.31 ^c	1.02 ^d	0.22	<0.01	<0.01	<0.01
<i>Pantoea</i>	1.48 ^d	4.39 ^c	6.58 ^a	5.53 ^b	3.97 ^c	0.47 ^e	0.28	0.01	<0.01	0.58
<i>Pseudomonas</i>	0.02 ^b	0.01 ^b	0.25 ^b	3.20 ^a	4.17 ^a	0.25 ^b	0.35	<0.01	<0.01	<0.01
<i>Rhizobium</i>	8.45 ^a	8.36 ^a	4.34 ^b	3.81 ^b	3.26 ^b	0.69 ^c	0.45	<0.01	0.72	0.87
<i>Sphingomonas</i>	0.31 ^d	0.72 ^c	0.99 ^c	1.42 ^b	1.90 ^a	0.86 ^c	0.08	<0.01	<0.01	<0.01
<i>Stenotrophomonas</i>	0.30 ^c	0.34 ^c	1.56 ^b	5.32 ^a	5.24 ^a	0.54 ^c	0.17	<0.01	<0.01	<0.01

Note: ¹ Alfalfa and corn were mixed at proportions of 100:0 (C0), 80:20 (C20), 60:40 (C40), 40:60 (C60), 20:80 (C80) and 0:100 (C100) on a fresh weight basis, respectively. ² Means with different superscript letters (a–e) in a row were significantly different ($p < 0.05$) based on Duncan test. ³ SEM, standard error of the means. ⁴ Linear, quadratic and cubic effects of corn proportion on silage parameters.

4. Discussion

4.1. Chemical Composition of Alfalfa and Corn Prior to Ensiling

The ensilability of an ensiling material was chemically affected by its BC, WSC and DM [4]. According to Knicky and Spörndly [20], corn in this work had a strong fermentability, whereas alfalfa showed a weak fermentability, which was similar to the finding reported by Wang et al. [5].

4.2. Fermentation Characteristics and Chemical Composition of Silage

High quality alfalfa silage is difficult to produce due to a poor fermentability, caused by a high BC value and a low WSC and DM concentration [5]. Wilting prior to ensiling improved alfalfa silage quality due to a faster pH drop during fermentation [3]. However, it will be challenging to obtain an ideal DM content (about 350–500 g/kg FW) when cloudy weather occurs during wilting [2,3,6]. In this regard, combining high-moisture alfalfa with a forage crop rich in WSC at ensiling may be an alternative to enhance its fermentability.

It is widely accepted that a low pH of 3.6–4.5 is one of the main attributes of well-fermented silage [22]. In the present study, C0 silage was weakly preserved, demonstrated by a low lactic acid concentration (30.77 g/kg DM) and a great value of pH (5.56), acetic acid (37.29 g/kg DM), propionic acid (6.01 g/kg DM) and butyric acid (3.11 g/kg DM). In addition, a large amount of NH₃-N (196.89 g/kg TN) was detected. Lactic acid is the desirable organic acid in silage, and acetic acid, propionic acid and butyric acid are undesirable [4]. Non-protein nitrogen in silage, including NH₃-N, mainly results from proteolysis during ensiling originating from activities of plant proteases and undesirable microbes [7]. The presence of NH₃-N formation decreases silage quality [23]. In the present work, the pH and NH₃-N level in silage dropped to 4.16 and 95.05 g/kg TN, accordingly, and lactic acid concentration increased to 90.00 g/kg DM, as corn percentage increased from 0% to 40%. In addition, silage Flieg score was 95 and no butyric acid was detected when corn percentage reached 40%, which revealed that top-quality fermentation appeared. This finding was in accordance with others [2,6,10]. Similarly, our previous study indicated that ensiling alfalfa with fresh corn stalk significantly improved silage fermentation [5]. Unlike alfalfa, corn (stalk) is rich in WSC and low in BC [6,24], making these forage species complementary in terms of ensilability. When corn (stalk) is included into alfalfa, the forages mixture contains a higher WSC content and a lower BC value [2,5,6,10], which makes a contribution to lactic acid fermentation. As a result, there may be a faster drop in pH during the early period of ensiling and a better fermentation appears during the subsequent ensiling process.

4.3. Bacterial Diversity and Composition of Silage

It is widely acknowledged that lactic acid bacteria regularly involved in silage fermentation belong to the phylum *Firmicutes* and to the genera *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus* and *Weissella* [8,25,26]. Our present work revealed that 55.65% of total sequences in C0 silage were *Firmicutes*, consisting mainly of the families *Enterococcaceae*, *Lactobacillaceae* and *Leuconostocaceae*, and genera *Enterococcus*, *Lactobacillus* and *Weissella*. The 41.89% of C0 silage bacterial community belonged to the phylum *Proteobacteria*, composed principally of the families *Enterobacteriaceae*, *Rhizobiaceae* and *Methylobacteriaceae*, and genera *Enterobacter*, *Rhizobium* and *Methylobacterium*. Similarly, our research team evaluated the diversity and composition of alfalfa silage and found that the predominant genera were *Lactobacillus*, *Weissella* and *Enterobacter* [5]. Likewise, Ogunade et al. [7] stated that *Lactobacillus*, *Weissella*, *Pediococcus* and *Pantoea* dominated the microflora of alfalfa silage after 100 d of fermentation. Nevertheless, Zhang et al. [8] reported that *Garciella* was the only predominant genus in alfalfa silage. The difference in the microbial community of alfalfa silage between our work and others may be attributed to several factors, including geographical location, forage variety, growth stage, DM concentration, storage temperature and ensiling time.

Ensiling is based on lactic acid fermentation that occurs spontaneously under anaerobic conditions due to activities of a complex community of forage epiphytic microbes [27]. Bacterial community reflects silage fermentation properties, and more *Lactobacillus* members in silage generally results in a higher fermentation quality [5]. In the present study, C0 silage was poorly fermented and contained a complex community, composed mainly of *Enterococcus* (38.86%), *Enterobacter* (20.61%), *Rhizobium* (8.45%), *Lactobacillus* (8.15%), *Methylobacterium* (5.54%) and *Weissella* (5.24%). In addition, *Lactobacillus* and *Weissella* abundance was low, whereas *Enterobacter*, *Rhizobium* and *Methylobacterium* population was

comparatively high. The appearance of the *Enterobacter* species in silage is undesirable since they compete with lactic acid bacteria for substrates during ensiling due to their facultative anaerobic nature. Moreover, *Enterobacter* members can release $\text{NH}_3\text{-N}$ formed from protein degradation and the reduction of NO_3 , thereby increasing the BC of the ensiling material and showing a slow drop in pH [28]. In the present study, a silage bacterial community was reconstructed when corn was included into alfalfa. With corn proportion increasing from 0% to 40%, *Lactobacillus* abundance increased and *Rhizobium* and *Methylobacterium* number declined, although few changes of the principal genera occurred. Because silage pH got lower and more undesirable microbes were inhibited, with a higher corn proportion in the mixture. *Lactobacillus* members play a critical role in enhancing lactic acid concentration and reducing pH, thereby inhibiting the activities of undesirable microbes, such as *Enterobacter* and *Rhizobium* [26]. Lactic acid-producing cocci, such as *Weissella* or *Enterococcus* species are regarded as early colonizers [29] as they are outcompeted by acid-tolerant *Lactobacillus* owing to the pH drop as ensiling advances [30]. Our research team reported that adding corn stalk to alfalfa significantly shifted the bacterial community of silage, by means of enriching *Lactobacillus* relative abundance and decreasing *Enterobacter* population [5]. Similarly, Ni et al. [26] found that *Lactobacillus* abundance in silage was increased when forage soybean (*Glycine max* Merr.) was ensiled with corn or sorghum (*Sorghum bicolor* L.). Ensiling legumes in a mixture with a herbage rich in WSC improved silage fermentation quality, probably by increasing the relative abundance of the predominant lactic acid bacteria, such as *Lactobacillus* members, and thereby accelerating acidification in the early stage of ensiling.

In the current work, we only evaluated the bacterial diversity and composition of silage after 100 d of ensiling, and did not examine its dynamic changes over the entire fermentation process. Future work will track the dynamic changes of the predominant lactic acid bacteria involved in silage fermentation during ensiling mixtures of alfalfa and corn.

5. Conclusions

With corn proportion in the mixture increasing from 0% to 40%, inclusion of corn to alfalfa at ensiling significantly improved silage fermentation quality, evidenced by a lower level of pH, acetic acid, butyric acid and $\text{NH}_3\text{-N}$, and a higher value of lactic acid and lactic acid to acetic acid ratio, and shifted the bacterial community for better silage preservation, by means of increasing the relative abundance of desirable *Lactobacillus* and reducing undesirable *Rhizobium* and *Methylobacterium* population. Overall, high quality silage was produced when alfalfa was combined with at least 40% whole-plant corn on a fresh weight basis.

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