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

Musen Wang 1,2,*, Run Gao 2,*, Marcia Franco 3, David B. Hannaway 4, Wencan Ke 1, Zitong Ding 1, Zhu Yu 2,*, and Xusheng Guo 1,4,*

1 School of Life Sciences, Lanzhou University, Lanzhou 730000, China; wangms@lzu.edu.cn (M.W.); kewc12@lzu.edu.cn (W.K.); dingwr@lzu.edu.cn (Z.D.)
2 College of Grassland Science and Technology, China Agricultural University, Beijing 100193, China; gaorun@cau.edu.cn
3 Natural Resources Institute Finland (Luke), Tietotie 2 C, FI-31600 Jokioinen, Finland; marcia.franco@luke.fi
4 Department of Crop and Soil Science, Oregon State University, Corvallis, OR 97331, USA; david.hannaway@oregonstate.edu
* Correspondence: 02059@cau.edu.cn (Z.Y.); guoxsh07@lzu.edu.cn (X.G.)

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, respectively, 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

1. Introduction

Alfalfa (Medicago sativa L.) has been planted widely in 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 × g for 15 min at 4 °C and was used for organic acids and NH₃-N analysis. Silage organic acids were determined by high performance liquid chromatograph [5]. The NH₃-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 FC = DM + 8 WSC/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) | WSC  | NDF  | ADF  | Hemicellulose | CP   | BC (mEq g/kg DM) | FC   |
|----------|--------------|------|------|------|---------------|------|-----------------|------|
| 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 NH$_3$-N. With corn proportion in the mixture increasing from 0% to 40%, silage pH, acetic acid, butyric acid and NH$_3$-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.

| Treatment | C0 | C20 | C40 | C60 | C80 | C100 | SEM  | Linear | Quadratic | Cubic |
|-----------|----|-----|-----|-----|-----|------|------|--------|-----------|-------|
| Item      | 2,3 |     |     |     |     |      | p-Value |        |           |        |
| pH        | 5.56$^a$ | 5.47$^b$ | 5.46$^c$ | 5.39$^d$ | 5.31$^e$ | 5.28$^f$ | 0.01 | <0.01  | <0.01     | <0.01 |
| Lactic acid (g/kg DM) | 30.77$^d$ | 29.33$^e$ | 28.45$^f$ | 27.29$^g$ | 26.85$^h$ | 26.55$^i$ | 0.95 | <0.01  | <0.01     | <0.01 |
| Acetic acid (g/kg DM) | 37.29$^a$ | 36.73$^b$ | 36.15$^c$ | 35.62$^d$ | 35.18$^e$ | 34.72$^f$ | 1.52 | <0.01  | <0.01     | <0.01 |
| Lactic acid to acetic acid ratio | 0.83$^f$ | 0.83$^g$ | 0.83$^h$ | 0.83$^i$ | 0.83$^j$ | 0.83$^k$ | 1.08 | <0.01  | 0.12      | 0.43  |
| Propionic acid (g/kg DM) | 6.01$^a$ | 5.13$^b$ | 4.72$^c$ | 4.37$^d$ | 4.05$^e$ | 3.74$^f$ | 0.07 | <0.01  | <0.01     | <0.01 |
| Butyric acid (g/kg DM) | 3.11$^a$ | 3.11$^b$ | 3.11$^c$ | 3.11$^d$ | 3.11$^e$ | 3.11$^f$ | 0.03 | <0.01  | <0.01     | 0.01  |
| NH$_3$-N (g/kg TN) | 196.89$^a$ | 196.89$^a$ | 196.89$^a$ | 196.89$^a$ | 196.89$^a$ | 196.89$^a$ | 0.01 | <0.01  | <0.01     | <0.01 |
| Flieg score | 25$^a$ | 25$^a$ | 25$^a$ | 25$^a$ | 25$^a$ | 25$^a$ | 1.88 | <0.01  | <0.01     | <0.01 |
| DM (g/kg FW) | 268.86$^f$ | 268.86$^f$ | 268.86$^f$ | 268.86$^f$ | 268.86$^f$ | 268.86$^f$ | 0.48 | <0.01  | <0.01     | <0.01 |
| WSC (g/kg DM) | 7.68$^f$ | 7.68$^f$ | 7.68$^f$ | 7.68$^f$ | 7.68$^f$ | 7.68$^f$ | 0.67 | <0.01  | <0.01     | 0.25  |
| CP (g/kg DM) | 183.90$^a$ | 183.90$^a$ | 183.90$^a$ | 183.90$^a$ | 183.90$^a$ | 183.90$^a$ | 1.49 | <0.01  | <0.01     | 0.36  |

Note: 1 DM, dry matter; NH$_3$-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.
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.

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

| Item | Treatment 2,3 | p-Value 5 |
|------|---------------|-----------|
|      | C0   | C20  | C40  | C60  | C80  | C100 | SEM 4 | 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 | <0.01 |
| 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 | 0.05 d  | 0.11 b  | 0.01 | <0.01 | <0.01 | 0.59 |
| OTU number | 134 a  | 149 b  | 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 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.
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            | C0       | C20      | C40       | C60       | C80       | C100      | SEM  | Linear | Quadratic | Cubic |
|-----------------|----------|----------|-----------|-----------|-----------|-----------|------|--------|-----------|-------|
| Actinobacteria  | 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 |
| Bacteroidetes   | 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  |
| Firmicutes      | 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 |
| Proteobacteria  | 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                  | C0   | C20  | C40  | C60  | C80  | C100 | SEM  | Linear | Quadratic | Cubic |
|-----------------------|------|------|------|------|------|------|------|--------|-----------|-------|
| Microbacteriaceae     | 1.50 | 1.98 | 7.13 | 6.19 | 5.90 | 1.97 | 0.28 | <0.01 | <0.01     | <0.01 |
| Bacillaceae           | 0.24 | 0.23 | 0.78 | 1.29 | 1.81 | 0.29 | 0.23 | 0.02   | <0.01     | <0.01 |
| Enterobacteriaceae    | 38.86| 15.15| 5.40 | 2.61 | 1.12 | 0.50 | 0.87 | <0.01 | <0.01     | <0.01 |
| Lactobacillaceae      | 8.24 | 20.99| 27.92| 26.31| 25.68| 33.46| 2.23 | 0.02   | <0.01     | <0.01 |
| Lachnospiraceae       | 0.00 | 0.00 | 0.03 | 0.10 | 0.54 | 4.64 | 0.24 | <0.01 | <0.01     | <0.01 |
| Leuconostocaceae      | 5.32 | 5.04 | 7.03 | 5.94 | 9.62 | 25.06| 0.93 | <0.01 | <0.01     | <0.01 |
| Paenibacillaceae      | 0.01 | 0.06 | 0.07 | 0.54 | 0.81 | 7.94 | 0.61 | <0.01 | <0.01     | <0.01 |
| Aurantimonadaceae     | 2.55 | 7.23 | 8.34 | 6.11 | 4.91 | 0.82 | 0.57 | <0.01 | <0.01     | 0.06  |
| Enterobacteriaceae    | 23.16| 30.71| 27.29| 26.24| 22.95| 12.68| 1.16 | <0.01 | <0.01     | 0.71  |
| Methylbacteriaceae    | 5.58 | 3.85 | 3.22 | 2.37 | 2.33 | 1.04 | 0.22 | <0.01 | <0.01     | <0.01 |
| Moraxellaceae         | 0.02 | 0.02 | 0.46 | 1.53 | 2.06 | 0.82 | 0.18 | <0.01 | <0.01     | <0.01 |
| Pseudomonadaceae      | 0.02 | 0.01 | 0.25 | 3.20 | 4.17 | 0.25 | 0.35 | <0.01 | <0.01     | <0.01 |
| Rhizobacteaceae       | 8.46 | 8.37 | 4.35 | 3.82 | 3.31 | 0.81 | 0.45 | <0.01 | 0.64       | 0.91  |
| Rhodobacteriaceae     | 0.64 | 1.43 | 2.56 | 2.43 | 1.82 | 0.09 | 0.14 | 0.38   | <0.01     | 0.03  |
| Sphingomonadaceae     | 0.37 | 0.84 | 1.12 | 1.65 | 2.23 | 0.98 | 0.08 | <0.01 | <0.01     | <0.01 |
| Xanthomonadaceae      | 0.30 | 0.35 | 1.58 | 5.44 | 5.44 | 0.66 | 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 Mixed model analysis with 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                  | C0   | C20  | C40  | C60  | C80  | C100 | SEM  | Linear | Quadratic | Cubic |
|-----------------------|------|------|------|------|------|------|------|--------|-----------|-------|
| Curtobacterium        | 0.36 | 0.59 | 1.98 | 1.89 | 1.69 | 0.47 | 0.08 | <0.01 | <0.01     | <0.01 |
| Bacillus              | 0.23 | 0.22 | 0.74 | 1.23 | 1.77 | 0.28 | 0.23 | 0.02   | <0.01     | <0.01 |
| Enterococcus          | 38.86| 15.15| 5.40 | 2.61 | 1.12 | 0.50 | 0.87 | <0.01 | <0.01     | <0.01 |
| Lactobacillus         | 8.15 | 20.94| 27.92| 26.31| 25.65| 33.45| 2.29 | <0.01 | 0.03       | <0.01 |
| Leuconostoc           | 0.08 | 0.15 | 0.78 | 1.97 | 4.86 | 13.73| 0.38 | <0.01 | <0.01     | <0.01 |
| Paenibacillus         | 0.00 | 0.00 | 0.07 | 0.54 | 0.80 | 7.84 | 0.62 | <0.01 | <0.01     | <0.01 |
| Weissella             | 5.24 | 4.89 | 6.26 | 3.98 | 4.77 | 11.34| 0.71 | <0.01 | <0.01     | <0.01 |
| Acinetobacter         | 0.02 | 0.02 | 0.46 | 1.53 | 2.06 | 0.82 | 0.18 | <0.01 | <0.01     | <0.01 |
| Aureimonas            | 2.55 | 7.23 | 8.34 | 6.11 | 4.91 | 0.82 | 0.57 | <0.01 | <0.01     | 0.06  |
| Enterobacter          | 20.61| 24.88| 19.52| 19.18| 17.41| 11.51| 1.04 | <0.01 | <0.01     | 0.57  |
| Methylbacterium       | 5.54 | 3.81 | 2.31 | 2.31 | 1.02 | 0.22 | <0.01 | <0.01 | <0.01     | <0.01 |
| Pantoena              | 1.48 | 4.39 | 6.58 | 5.53 | 3.97 | 0.47 | 0.28 | 0.01   | 0.01       | 0.58  |
| Pseudomonas           | 0.02 | 0.01 | 0.25 | 3.20 | 4.17 | 0.25 | 0.35 | <0.01 | <0.01     | <0.01 |
| Rhizobium             | 8.45 | 8.36 | 4.34 | 3.81 | 3.26 | 0.69 | 0.45 | <0.01 | 0.72       | 0.87  |
| Sphingomonas          | 0.31 | 0.72 | 0.99 | 1.42 | 1.90 | 0.86 | 0.08 | <0.01 | <0.01     | <0.01 |
| Stenotrophomonas      | 0.30 | 0.34 | 1.56 | 5.32 | 5.24 | 0.54 | 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 the Duncan test. 3 SEM, standard error of the means. 4 Mixed model analysis with 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 NH\textsubscript{3}-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 NH\textsubscript{3}-N, mainly results from proteolysis during ensiling originating from activities of plant proteases and undesirable microbes [7]. The presence of NH\textsubscript{3}-N formation decreases silage quality [23]. In the present work, the pH and NH\textsubscript{3}-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 fermentability [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 Garciaella 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 NH$_3$-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 NH$_3$-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.

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

Funding: This work was funded by the China Forage and Grass Research System (CARS–34) and National Natural Science Foundation of China (31901390; 31672487).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Acknowledgments: The authors thank Jinbao Pan from College of Plant Science and Technology, Beijing University of Agriculture, China for supplying corn seeds.

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

1. Wen, A.; Yuan, X.; Wang, J.; Desta, S.T.; Shao, T. Effects of four short-chain fatty acids or salts on dynamics of fermentation and microbial characteristics of alfalfa silage. *Anim. Feed Sci. Technol.* 2017, 233, 141–148. [CrossRef]

2. Zhang, Q.; Zhao, M.; Wang, X.; Yu, Z.; Na, R. Ensiling alfalfa with whole crop corn improves the silage quality and *in vitro* digestibility of the silage mixtures. *Grassl. Sci.* 2017, 63, 211–217. [CrossRef]

3. Santos, M.C.; Kung, J. The effects of dry matter and length of storage on the composition and nutritive value of alfalfa silage. *J. Dairy Sci.* 2016, 99, 5466–5469. [CrossRef]

4. Ozturk, D.; Kizilsimsek, M.; Kamalak, A.; Canbolat, O.; Ozkan, C.O. Effects of ensiling alfalfa with whole-crop maize on the fermentation dynamics and bacterial diversity of mixed lucerne and sweet corn stalk silage ensiled at six ratios. *Grass Forage Sci.* 2019, 74, 264–273. [CrossRef]

5. Wang, M.; Yu, Z.; Wu, Z.; Hannaway, D.B. Effect of *Lactobacillus plantarum* ’KR107070’ and a propionic acid-based preservative on the fermentation characteristics, nutritive value and aerobic stability of alfalfa-corn mixed silage ensiled with four ratios. *Grassl. Sci.* 2018, 64, 51–60. [CrossRef]

6. Ogunade, I.M.; Jiang, Y.; Pech Cervantes, A.A.; Kim, D.H.; Oliveira, A.S.; Vyas, D.; Weinberg, Z.G.; Jeong, K.C.; Adesogan, A.T. Effects of ensiling alfalfa with whole-crop maize on the microbial characteristics of alfalfa silage as analyzed by Illumina MiSeq sequencing: Effects of *Escherichia coli* O157:H7 and silage additives. *J. Dairy Sci.* 2018, 101, 2048–2059. [CrossRef] [PubMed]

7. Zhang, Q.; Yu, Z.; Wang, X.; Tian, J. Effects of inoculants and environmental temperature on fermentation quality and bacterial diversity of alfalfa silage. *Anim. Sci. J.* 2018, 89, 1085–1092. [CrossRef]

8. Chen, L.; Dong, Z.; Ji, L.; Shao, T. Ensiling characteristics, *in vitro* rumen fermentation, microbial communities and aerobic stability of low-dry matter silages produced with sweet sorghum and alfalfa mixtures. *J. Sci. Food Agric.* 2019, 99, 2140–2151. [CrossRef]

9. Ozturk, D.; Kizilisimsek, M.; Kamalak, A.; Canbolat, O.; Ozkan, C.O. Effects of ensiling alfalfa with whole-crop maize on the chemical composition and nutritive value of silage mixtures. *Asian-Australas. J. Anim. Sci.* 2006, 19, 526–532. [CrossRef]

10. McCabe, M.S.; Cormican, P.; Keogh, K.; O’Connor, A.; O’Hara, E.; Palladino, R.A.; Kenny, D.A.; Waters, S.M. Illumina MiSeq phylogenetic amplicon sequencing shows a large reduction of an uncharacterised *Succinivibrionaceae* and an increase of the *Methanobrevibacter gottschalkii* clade in feed restricted cattle. *PLoS ONE* 2015, 10, 1–25. [CrossRef] [PubMed]

11. Wu, B.; Zhang, Q.; Liu, Z.; Yu, Z.; Nishino, N. Bacterial communities in alfalfa and corn silages produced in large-scale stack and bunker silos in China. *Grassl. Sci.* 2014, 60, 247–251. [CrossRef]

12. Rossi, F.; Dellaglio, F. Quality of silages from Italian farms as attested by number and identity of microbial indicators. *J. Appl. Microbiol.* 2007, 103, 1707–1715. [CrossRef]

13. McEniry, J.; O’Kiely, P.; Clipson, N.J.W.; Forristal, P.D.; Doyle, E.M. Bacterial community dynamics during the ensilage of wilted grass. *J. Appl. Microbiol.* 2008, 105, 359–371. [CrossRef]

14. McGarvey, J.A.; Franco, R.B.; Palumbo, J.D.; Hnasko, R.; Stanker, L.; Mitloehner, F.M. Bacterial population dynamics during the ensiling of *Medicago sativa* (alfalfa) and subsequent exposure to air. *J. Appl. Microbiol.* 2013, 114, 1661–1670. [CrossRef]

15. Gharechahi, J.; Kharazian, Z.A.; Sarikhian, S.; Jouzani, G.S.; Aghdasi, M.; Salekdeh, G.H. The dynamics of the bacterial communities developed in maize silage. *Microb. Biotechnol.* 2017, 10, 1663–1676. [CrossRef]

16. Broderick, G.A.; Kang, J.H. Automated simultaneous determination of ammonia and total amino acids in ruminal fluid and *in vitro* media. *J. Dairy Sci.* 1980, 63, 64–75. [CrossRef]

17. Horwitz, W.; Latimer, G.W.; AOAC International. *Official Methods of Analysis AOAC International*, 18th ed.; AOAC International: Gaithersburg, MD, USA, 2005.

18. Murphy, R.P. A method for the extraction of plant samples and the determination of total soluble carbohydrates. *J. Sci. Food Agric.* 1958, 9, 714–717. [CrossRef]

19. Knicky, M.; Spörndly, R. The ensiling capability of a mixture of sodium benzoate, potassium sorbate, and sodium nitrate. *J. Dairy Sci.* 2011, 94, 824–831. [CrossRef]

20. Woollford, M.K. The Silage Fermentation; Marcel Dekker, Inc.: New York, NY, USA, 1984.

21. Kung, L.; Robinson, J.R.; Ranjit, N.K.; Chen, J.H.; Golt, C.M.; Pesek, J.D. Microbial populations, fermentation end-products, and aerobic stability of corn silage treated with ammonia or a propionic acid-based preservative. *J. Dairy Sci.* 2000, 83, 1479–1486. [CrossRef]

22. Flythe, M.D.; Russell, J.B. The effect of pH and a bacteriocin (bovicin HCS) on *Clostridium sporogenes* MD1, a bacterium that has the ability to degrade amino acids in ensiled plant materials. *FEMS Microbiol. Ecol.* 2004, 47, 215–222. [CrossRef]

23. Liu, Q.; Shao, T.; Zhang, J. Determination of aerobic deterioration of corn stalk silage caused by aerobic bacteria. *Anim. Feed Sci. Technol.* 2013, 183, 124–131. [CrossRef]

24. Cai, Y.; Benno, Y.; Ogawa, M.; Ohmomo, S.; Kumai, S.; Nakase, T. Influence of *Lactobacillus* spp. from an inoculant and of *Weissella* and *Leuconostoc* spp. from forage crops on silage fermentation. *Appl. Environ. Microb.* 1998, 64, 2982–2987. [CrossRef]

25. Li, 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] [PubMed]

26. Muck, R.E. Recent advances in silage microbiology. *Agric. Food Sci.* 2013, 22, 3–15. [CrossRef]

27. Spoelstra, S.F. Degradation of nitrate by enterobacteria during silage fermentation of grass. *Neth. J. Agric. Sci.* 1987, 35, 43–54. [CrossRef]
29. Dellaglio, F.; Torriani, S. DNA-DNA homology, physiological characteristics and distribution of lactic acid bacteria isolated from maize silage. *J. Appl. Bacteriol.* **1986**, *60*, 83–92. [CrossRef]

30. Graf, K.; Ulrich, A.; Idler, C.; Klocke, M. Bacterial community dynamics during ensiling of perennial ryegrass at two compaction levels monitored by terminal restriction fragment length polymorphism. *J. Appl. Microbiol.* **2016**, *120*, 1479–1491. [CrossRef] [PubMed]