

Effect of maize straw additives on the nutritional quality and bacterial communities of ensiled forage rape for animal feed

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ABSTRACT

The impact of forage rape (*Brassica napus* L.) with maize (*Zea mays* L.) straw addition on the changes of nutritional quality and bacterial community was investigated by using high-throughput sequencing analysis of the 16S rRNA. The six ensilage treatments were set based on different mass ratios of forage rape (S) and maize stalk (Y): S1 (forage rape:maize stalk 10:0), S7Y3 (7:3), S6Y4 (6:4), S5Y5 (5:5), S4Y6 (4:6), and S3Y7 (3:7). The results showed that compared with S1, the DM content of S7Y3, S6Y4, S5Y5, S4Y6 and S3Y7 increased by 10.71%, 16.02%, 22.56%, 24.37% and 30.19% after the maize straw addition, respectively. Contents of crude protein (CP), ether extracts (EE) and water-soluble carbohydrate (WSC) contents in S7Y3 were significantly higher than that of other maize straw additive treatments ($P < 0.05$), which were 11.11% DM, 4.54% DM and 12.50% DM, respectively. On the 60th day of fermentation, the lowest pH value was found in S7Y3, which was 3.56. The V-score of each treatment were above 80. Bacterial Shannon and Chao indices were highest in S7Y3. The dominant bacterial phyla across all silage samples were *Firmicutes*, *Proteobacteria*, *Cyanobacteria* and *Actinobacteria*. The dominant genera are *Lactobacillus* and *Weissella*. Redundancy analysis (RDA) suggested that DM, CP, EE and WSC are the main factors in shaping bacterial communities. This study demonstrated that forage rape with maize straw addition can affect the nutritional quality and bacterial community of silage, and the best mixing ratio of maize straw and forage rape ensilage is 7:3.

Key words: Bacterial community, ensilage quality, forage rape, maize straw additives.

INTRODUCTION

Forage rape (*Brassica napus* L.) is one of the most important oilseed crops globally, which has been widely used for human food supply and animal forage (Lu et al., 2019). Forage rape is a new type of high-protein forage (Kaur et al., 2010), content of crude protein is 270-330 g kg⁻¹ DM (Islam and Garcia, 2012). Given the strong cold tolerance and rapid growth, forage rape is widely grown in north European and China (Iqbal, 2014). In recent years, shortage of forage has become a limiting factor constraining the economic development of animal husbandry in China.

Ensilage is a well-developed technology which can preserve the nutrient component to a large extent. However, water content of forage rape is higher during the flowering period, furthermore the direct silage can easily lead to the fermentation of butyric acid and loss of nutrients, resulting in failure of fermentation and economic loss (Guo et al., 2013). Yan et al. (2019) observed that the mixing of water content of the silage and dry grain by-products is an effective way to solve those problems. Maize (*Zea mays* L.) straw is a rich agricultural by-product widely used as a feed resource worldwide. Maize, as one of major crops, is widely planted in north China and its straw is used as an important animal husbandry forage (Hu et al., 2018). Lamb and Evvard (1916) tried to mix forage rape with other forage grasses, including

whole corn plant and corn grain, and pointed a mixture of rape and a legume produces the best quality of silage. Kaur et al. (2009) reported that the feeding time and sequence of forage rape and maize silage does not affect digestibility and rumen parameters in sheep. Additives are also applied to the fermentation of forage rape silage. These studies provide us with valuable experience in the research on mixed silage of forage rape and maize straw. Previous studies mainly focused on forage rape and fresh corn, and did not discuss the appropriate silage ratio of forage rape mix with dry maize straw. In addition, high-throughput sequencing technology provides an opportunity for us to understand the bacterial community under the mixed silage of forage rape and maize straw.

Bacteria, the most diverse and abundant organisms on Earth. The bacteria play a crucial role in the process of silage fermentation (Zhang et al., 2018). Affected by plant epiphytic bacteria, the bacterial community composition of different silages is different (Santoso et al., 2011). In addition, the rapid growth and reproduction of epiphytic lactic acid bacteria is beneficial to lower the pH of the silage and protect the silage from deterioration (Gharechahi et al., 2017). Therefore, high-quality silage raw materials are more conducive to the success of silage fermentation. Maize is widely used in the preparation of silage, which includes whole plant maize, maize straw and maize kernels. It was reported that bacterial community shift from *Enterobacter* to *Lactococcus* and *Lactobacillus* after fermentation in maize silage (Parvin et al., 2010). *Lactobacillus* stable presence contributes to the rising of lactic and acetic acid during whole-plant maize ensiling (Hu et al., 2018). The mixed silage of maize straw and other forage grass not only increases the nutrient content of silage, but also improves the fermentation flora and promotes stability of silage fermentation (Wang et al., 2019). Although predecessors conducted research on the mixed silage of whole maize and forage rape (Lamb and Evvard, 1916), knowledge on the impact of forage rape with maize straw addition on bacterial community remains poorly understood. Ni et al. (2018) indicated that changes in microbial communities may be responsible for differences in silage quality. Accordingly, studying the bacterial community structure of forage rape mixed with maize straw silage can help us understand the changes of beneficial or harmful bacteria, and provide data support for determining a reasonable silage ratio.

Therefore, the present study aims to investigate the effect of different maize straw and forage rape ensilage mixed ratios on silage nutritional quality and bacterial community. The objectives were to assess silage nutritional quality changes, reveal the shifts of bacterial community, and explore possible reasons leading to the changes in bacterial community and provide the rational mixing ratio of forage rape and maize straw.

MATERIAL AND METHODS

Site descriptions and plant material

This study was conducted at Zhongjiazhuang township, which is on the alluvial plain of the Manasi River Basin in Xinjiang (44°45' N, 85°40' E). This region is one of the main farming-pastoral zones in northwest China. In order to make full use of the high-protein, high-fat and low-fiber of rapeseed nutritional characteristics, and solve the problem of storage, processing and utilization of rapeseed, we designed an ensilage experiment based on forage rape and supplemented by maize straw.

The forage rape (*Brassica napus* L.) 'Huayouza 62' seeds were provided by the Huazhong Agricultural University and silage maize straw (*Zea mays* L.) 'Xinsiyu 11' seeds were provided by the Shihezi University, they were harvested during the flowering period as the silage raw materials. The six ensilage treatments were set based on different mass ratios of forage rape (S) and maize stalk (Y): S1 (forage rape:maize stalk 10:0), S7Y3 (7:3), S6Y4 (6:4), S5Y5 (5:5), S4Y6 (4:6), and S3Y7 (3:7).

Experimental preparation of raw materials

The post-harvest forage rape and maize straw were manually cut into 2 cm sections by a forage cutter (model 9ZP-3.6, Kaiyue Machinery Company, Xuzhou, China). These two raw materials were mixed according to the ratios of S1, S7Y3, S6Y4, S5Y5, S4Y6 and S3Y7 for ensilage preparation with total weight of 1.5 kg. After being well mixed, samples were filled into an ensilage bag for vacuum preservation and then stored at room temperature (23-30 °C), in the dark for 60 d. Treatments were conducted in triplicate, 5 bags per serving, a total of 15 bags. To determinate the nutrition and fermentation index, three bags were taken randomly from each treatment on 3, 7, 15, 30, 60 d of the fermentation, respectively. The nutrient content of raw material before ensiling is shown in Table 1.

Table 1. Chemical properties of raw material of forage rape and maize stalk used for ensiling.

| Treatments ¹ | DM | CP | EE | NDF | ADF | WSC |
|-------------------------|--------------|--------------|-------------|--------------|--------------|--------------|
| | % | | | % DM | | |
| S1 | 15.04 ± 0.09 | 15.16 ± 0.12 | 6.41 ± 0.35 | 42.59 ± 0.88 | 33.21 ± 0.60 | 13.91 ± 0.42 |
| S7Y3 | 27.75 ± 0.20 | 11.11 ± 0.03 | 4.54 ± 0.25 | 57.23 ± 0.29 | 41.12 ± 0.28 | 12.50 ± 0.29 |
| S6Y4 | 31.24 ± 0.12 | 10.21 ± 0.04 | 4.26 ± 0.03 | 57.95 ± 0.33 | 42.78 ± 1.59 | 12.03 ± 0.25 |
| S5Y5 | 37.60 ± 0.32 | 9.32 ± 0.05 | 3.30 ± 0.05 | 61.06 ± 0.87 | 42.80 ± 0.31 | 11.56 ± 0.21 |
| S4Y6 | 39.41 ± 0.37 | 8.43 ± 0.06 | 3.23 ± 0.16 | 63.84 ± 0.58 | 42.84 ± 0.57 | 11.10 ± 0.08 |
| S3Y7 | 45.23 ± 0.47 | 7.54 ± 0.07 | 3.03 ± 0.02 | 66.33 ± 0.42 | 43.12 ± 0.47 | 10.61 ± 0.13 |

¹Forage rape (S) and maize stalk (Y) ratio (S:Y). S1: 10:0; S7Y3: 7:3; S6Y4: 6:4; S5Y5: 5:5; S4Y6: 4:6; S3Y7: 3:7.

DM: Dry matter; CP: crude protein; EE: ether extracts; NDF: neutral detergent fiber; ADF: acid detergent fiber; WSC: water-soluble carbohydrate.

Analytical methods

Determination of fermentation nutrition index. Approximately 100 g samples were analyzed for DM content by oven drying at 65 °C for 48 h and then ground to pass a 1 mm screen by a laboratory knife mill (FW100, Taisite Instrument, Tianjin, China). Crude protein (CP) was measured using the Kjeldahl N analyzer (KDY-9830, Ketuo Instrument, Beijing, China) according to the methods of Association of Official Analytical Chemists (AOAC, 2012). The ether extracts (EE) content was determined by Soxhlet extraction. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were analyzed without amylase, using the sulphite method. The water-soluble carbohydrate (WSC) content was determined according to the thracenone-sulphuric acid method (AOAC, 2012).

Organic acid and chemical composition. A sample of fresh silage (20 g) was added to 180 mL distilled water and shaken for 5 min, allowed to stand for 50 min, after 3 cycles stand for 10 min, pH value of filtrate from silage was measured by electrode pH meter (PHSJ-5; LEICI, Shanghai, China). Ammonia N (NH₃-N) was measured by phenol-hypochlorous acid colorimetric method. Volatile fatty acid (VFA) was measured by high pressure liquid chromatography (HPLC) system (LCMS-2020; Shimadzu, Tokyo, Japan). The samples were investigated using an isocratic elution 0.2% H₃PO₄ at 1 mL min⁻¹ and column temperature of 50 °C. The wavelength was 210 nm (Yu et al., 2016). The V-score is an important index for comprehensively evaluate ensilage fermentation quality, which was calculated to evaluate the fermentative quality based on the values of organic acids and NH₃-N content.

Bacterial diversity analysis

DNA extraction and PCR amplification. After 60 d of ensiling, the triplicate samples from each treatment were opened, well mixed for analysis of bacterial community. Total DNA was extracted from silage samples using a Power Soil DNA Isolation kit (MP Biomedicals, Santa Ana, California, USA) following kit instructions. The V3-V4 hypervariable regions of bacterial 16S rRNA gene were amplified using the primer pair 338F/806R. The V3-V4 regions of each silage sample were amplified using PCR (27 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 45 s, a final extension at 72 °C for 10 min, and holding at 10 °C). PCR reactions were performed in triplicate, 20 µL mixture containing 4 µL 5 × FastPfu buffer, 2 µL 2.5 mM dNTPs, 0.8 µL each primer (5 µM), 0.4 µL FastPfu Polymerase and 10 ng template DNA. The resulted PCR products were extracted from a 2% agarose gel and further purified using the AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, California, USA) and quantified using a fluorometer (QuantiFluor-ST, Promega, Madison, Wisconsin, USA) according to the manufacturer's protocol.

Illumina MiSeq sequencing. Purified amplicons were pooled in equimolar and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, California, USA) according to the standard protocols by Majorbio Bio-Pharm Technology (Shanghai, China).

Processing of sequencing data. Raw FASTQ files (Illumina) were demultiplexed, quality filtered by Trimmomatic and merged by FLASH software (Center for Computational Biology, Johns Hopkins University, Washington D.C, USA. <https://ccb.jhu.edu/software/FLASH/index.shtml>) with the following criteria: (i) reads were truncated at any site receiving an average quality score < 20 over a 50 bp sliding window, (ii) primers were exactly matched allowing 2 nucleotide

mismatching and reads containing ambiguous bases were removed, (iii) sequences whose overlap longer than 10 bp were merged according to their overlap sequence. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed by RDP classifier algorithm against the Silva (SSU123) 16S rRNA database (SILVA ribosomal RNA database project; Max Planck Institute for Marine Microbiology and Jacobs University, Bremen, Germany) using confidence threshold of 70%.

Statistical analysis

The data from ensiling was statistically analyzed using SPSS 19.0 software (SPSS Inc., Chicago, Illinois, USA). One-way ANOVA was used for comparisons. Using the Bary Curts metric matrix, a hierarchical clustering tree was constructed to compare the composition of bacterial communities in all samples. CANOCO 5 was used to perform the redundancy analysis (RDA) analysis. The figures of the nutrition-fermentation quality changes and relative abundance of bacterial community were performed using Origin version 9.0 (Microcal Software, Northampton, Massachusetts, USA).

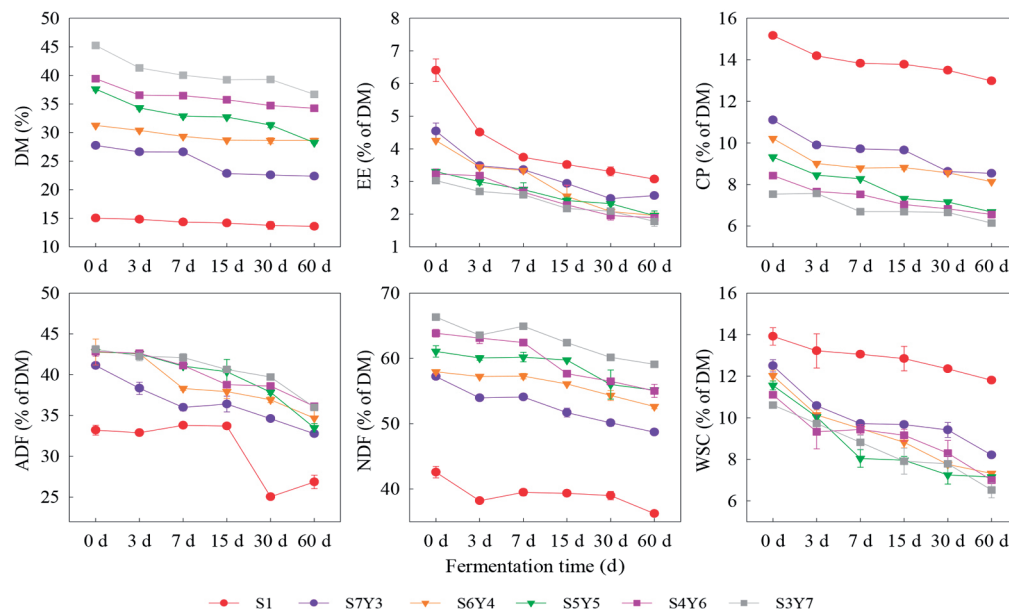
RESULTS

Changes in nutrition quality during silage fermentation

In this study, DM content increased significantly after maize straw addition, the highest value was found in S3Y7, while it was lowest in S1. But CP, EE and WSC contents decreased with the increasing maize straws proportion (Table 1), their contents in S7Y3 was higher than maize straw additive treatments ($P < 0.05$).

The changes of nutrient quality under different treatments during fermentation are shown in Figure 1. The content of DM, EE and CP under different treatments was gradually decreased as the fermentation process progresses. On the 60th day, DM content was the highest in S3Y7, while it was lowest in S1. The highest EE and CP contents were 3.07% and 12.98% in S1, respectively. Contents of EE and CP in S7Y3 were significantly higher than that of other maize straw additive treatments ($P < 0.05$). On the 60th day, the significant highest WSC content was found in S7Y3 treatment ($P < 0.05$). In this study, NDF and ADF content under different treatments decreased significantly with the increasing fermentation days ($P < 0.05$). On the 60th day, the content of ADF and NDF in S7Y3 was significantly lower than those in other treatments ($P < 0.05$).

Figure 1. The changes of nutrition-fermentation quality during silage fermentation.



Forage rape (S) and maize stalk (Y) ratio. S1: 10:0; S7Y3: 7:3; S6Y4: 6:4; S5Y5: 5:5; S4Y6: 4:6; S3Y7: 3:7.

DM: Dry matter; EE: ether extracts; CP: crude protein; ADF: acid detergent fiber; NDF: neutral detergent fiber; WSC: water-soluble carbohydrate.

Fermentative quality of silages

As shown in Table 2, the maize straw addition had significant effect on fermentation characteristics of forage rape silage ($P < 0.05$). On the 60th day of fermentation, pH values of all treatments were below 4.0, with the lowest in S7Y3 and highest in S5Y5. The changes of lactic acid (LA), acetic acid (AA) and propionic acid (PA) content had same trend under different treatments, namely, content of LA, AA and PA increased gradually with the increasing maize straw addition. The higher LA and AA contents were observed in all treatments, and a small amount of butyric acid (BA) (less than 0.05% DM). We found no differences in $\text{NH}_3\text{-N}$ content between S1, S7Y3 and S3Y7 ($P > 0.05$). The higher $\text{NH}_3\text{-N}$ content was observed in S6Y4 and S4Y6 ($P < 0.05$). The V-score scoring system calculated the score respectively and total score to obtain final evaluation score based on the content of VFA and $\text{NH}_3\text{-N}$ in silage. The scores of each treatment were above 80 (Table 2). The V-score decreased after the maize straw addition, S1 treatment exhibited significantly higher V-score compare to other treatments. With the increase of maize straw addition ratio, LA content increased gradually.

Silage fermentation bacterial community diversity and composition

As shown in Table 3, there were a total of 902 OTUs from six treatments. The number of high-quality sequences per sample varied from 58841 to 66202 with an average of 62521 sequences. Microbial diversity index can reflect the diversity and richness of biological populations. Shannon and Simpson indexes can reflect the community diversity, and Chao index can reflect the community richness. The species richness based on Chao1 index showed that S7Y3 and S1 exhibited the highest richness (Table 3). S7Y3 and S1 had the highest Shannon index in this study. In contrast, these two treatments displayed the lowest Simpson indices (0.17 and 0.17, respectively). The coverage of all samples is above 0.99, suggesting that the sequencing reads were sufficient for this analysis.

The dominant phyla across all samples were *Firmicutes*, *Proteobacteria*, *Cyanobacteria* and *Actinobacteria* (Figure 2a). The highest relative abundance of *Firmicutes* (88.47%) was observed in S3Y7. Abundance of *Firmicutes* increased as the proportion of maize straws increases. However, the relative abundance of *Proteobacteria* decreased as the proportion of maize straws increases. Abundance of *Firmicutes* and *Proteobacteria* performed the opposite trend. There was nonsignificant change in the relative abundance of *Cyanobacteria* and *Actinobacteria*.

Table 2. Effect of different maize stalk additions on fermentation characteristics of forage rape silage.

| Items | Treatments ¹ | | | | | | SEM |
|-------------------------------|-------------------------|--------|--------|--------|--------|--------|------|
| | S1 | S7Y3 | S6Y4 | S5Y5 | S4Y6 | S3Y7 | |
| pH | 3.72ab | 3.56c | 3.65b | 3.76a | 3.70ab | 3.69ab | 0.02 |
| $\text{NH}_3\text{-N}$, % TN | 8.69b | 8.63b | 9.77a | 7.72c | 9.84a | 8.55b | 0.16 |
| LA, % DM | 0.57e | 0.94d | 1.19c | 1.19c | 1.42b | 1.53a | 0.02 |
| AA, % DM | 0.44e | 0.73d | 0.90c | 0.88c | 1.09b | 1.16a | 0.02 |
| PA, % DM | 0.08d | 0.11c | 0.14b | 0.15b | 0.17a | 0.18a | 0.01 |
| BA, % DM | 0.00e | 0.01d | 0.01c | 0.01b | 0.01b | 0.01a | 0.00 |
| V-score | 89.89a | 87.15b | 83.16c | 87.42b | 81.34e | 83.28c | 0.39 |

Means with different letters within each row are significantly different at $P < 0.05$.

¹Forage rape (S) and maize stalk (Y) ratio (S:Y). S1: 10:0; S7Y3: 7:3; S6Y4: 6:4; S5Y5: 5:5; S4Y6: 4:6; S3Y7: 3:7.

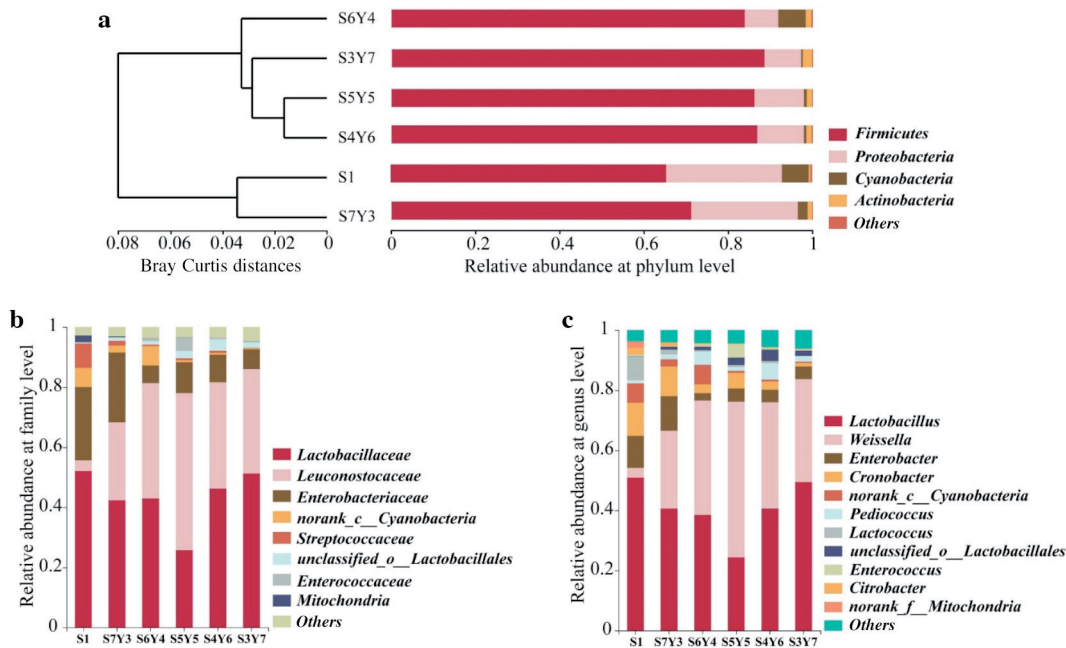
SEM: Standard error of the mean; LA: lactic acid; AA: acetic acid; PA: propionic acid; BA: butyric acid; TN: Total nitrogen; DM: dry matter.

Table 3. General information of sequence and bacterial diversity.

| Sample ID ¹ | Number of read | Number of OTUs | Shannon index | Simpson index | Chao1 index | Coverage index |
|------------------------|----------------|----------------|---------------|---------------|-------------|----------------|
| S1 | 65124 | 156 | 2.28 | 0.17 | 181.59 | 0.99 |
| S7Y3 | 66202 | 153 | 2.21 | 0.17 | 188.06 | 0.99 |
| S6Y4 | 63851 | 155 | 1.99 | 0.24 | 168.80 | 0.99 |
| S5Y5 | 58841 | 130 | 1.91 | 0.30 | 170.63 | 0.99 |
| S4Y6 | 62819 | 156 | 2.15 | 0.21 | 172.87 | 0.99 |
| S3Y7 | 65230 | 152 | 1.82 | 0.28 | 163.55 | 0.99 |

¹Forage rape (S) and maize stalk (Y) ratio (S:Y). S1: 10:0; S7Y3: 7:3; S6Y4: 6:4; S5Y5: 5:5; S4Y6: 4:6; S3Y7: 3:7.

Figure 2. Relative abundance of soil bacterial community at the phylum (a), family (b), and genus (c) levels across the different treatments.



Forage rape (S) and maize stalk (Y) ratio (S:Y). S1: 10:0; S7Y3: 7:3; S6Y4: 6:4; S5Y5: 5:5; S4Y6: 4:6; S3Y7: 3:7.

At the family levels (Figure 2b), *Lactobacillaceae* and *Leuconostocaceae* were dominant families. The total relative abundances of *Lactobacillaceae* and *Leuconostocaceae* were more than 55.67% under different treatments. The relative abundance of *Lactobacillaceae* decreased after adding maize straws, the highest relative abundance was observed in S1 (52.12%). However, the relative abundance of *Leuconostocaceae* increased after adding maize straws, the highest relative abundance was observed in S5Y5 (52.34%). In summary, the total relative abundances of *Lactobacillaceae* and *Leuconostocaceae* increased as the proportion of maize straws increases.

Further comparison of the bacterial communities was conducted at the genus levels (Figure 2c). The comparisons of the relative abundances of the top 11 classified bacterial genus (> 0.02%) showed differences among the treatments (Figure 2c). The dominant genus *Lactobacillus* and *Weissella* were enriched in all samples. Members of the genus *Enterobacter*, *Cronobacter*, *Pediococcus*, *Lactococcus*, *Enterococcus* and *Citrobacter* were also present in all samples. We observed that the total relative abundance of *Enterobacter* and *Cronobacter* decreased gradually after maize straw addition. The relative abundance of *Enterobacter* was 10.63% in S1. *Enterobacter* abundance was 2.47% in S6Y4.

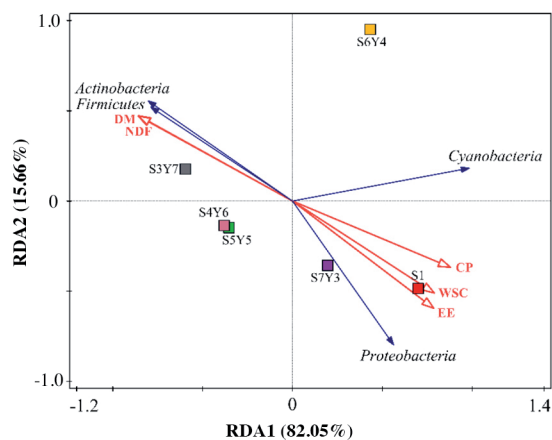
The genus *Lactobacillus* and *Weissella*, which belongs to phylum *Firmicutes*, were enriched in all treatments (Figure 2). The abundance of *Lactobacillus* was 50.92% in S1 and 24.35% in S5Y5. While the abundance of *Weissella* was only 3.25% in S1. After maize straws addition, the relative abundance of *Weissella* increased significantly, and the highest abundance was 51.82% in S5Y5.

Correlations between silage nutritional quality and bacterial community

The nutritional quality of silage has a significant impact on the bacterial community. Results of RDA analysis indicated a significant correlation between silage nutrition quality and abundant phyla ($P < 0.05$). The first two axes of RDA axis explained 97.71% of total variation (Figure 3, Table 4). The first component (RDA1) separated samples from different treatments and explained 82.05% of variation. A much better discrimination exhibited at phyla level, and S3Y7, S4Y6, S5Y5 treatment cluster together, where S6Y4 and S7Y3 distinctly separated from other treatments. This result indicated that maize straw addition has a significant impact on bacterial community at phyla level. The RDA plots of bacterial community structure clearly also showed that CP, WSC, EE, DM, NDF and ADF are the longer arrows, with their direction closely correlated to the RDA1 axis, respectively (Figure 4). We have also observed that CP, WSC and EE positively

correlated with the relative abundance of *Proteobacteria*, and negatively correlated with *Firmicutes* and *Actinobacteria* (Figure 5a). But DM, NDF and ADF positively correlated with *Firmicutes* and *Actinobacteria*, and negatively correlated with *Proteobacteria*.

Figure 3. Redundancy analysis (RDA) to show correlation between silage nutritional quality and dominant bacterial phyla.



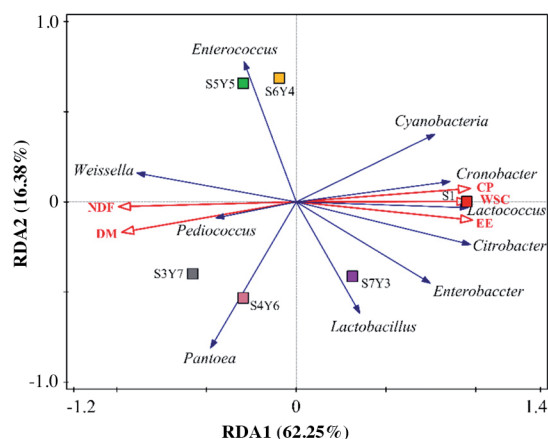
Forage rape (S) and maize stalk (Y) ratio (S:Y). S1: 10:0; S7Y3: 7:3; S6Y4: 6:4; S5Y5: 5:5; S4Y6: 4:6; S3Y7: 3:7.
DM: Dry matter; CP: crude protein; WSC: water-soluble carbohydrate; EE: ether extracts; NDF: neutral detergent fiber.

Table 4. Correlation between nutritional quality parameters and redundancy analysis (RDA) axes at phyla level.

| Nutritional quality parameters | RDA1 | RDA2 | R ² | P values |
|--------------------------------|--------|--------|----------------|----------|
| DM | 0.956 | -0.295 | 0.995 | 0.001 |
| CP | -0.936 | 0.363 | 0.865 | 0.010 |
| EE | -0.998 | 0.071 | 0.967 | 0.003 |
| NDF | 0.957 | -0.289 | 0.920 | 0.011 |
| WSC | -0.964 | 0.267 | 0.853 | 0.003 |

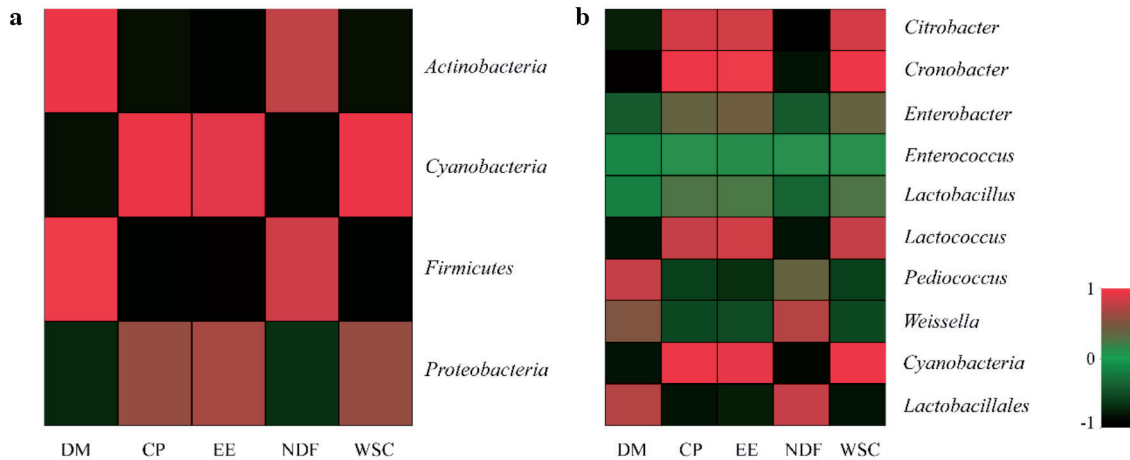
DM: Dry matter; CP: crude protein; EE: ether extracts; NDF: neutral detergent fiber; WSC: water-soluble carbohydrate.

Figure 4. Redundancy analysis (RDA) results of abundant genus and silage nutritional quality for samples from different treatments.



Forage rape (S) and maize stalk (Y) ratio (S:Y). S1: 10:0; S7Y3: 7:3; S6Y4: 6:4; S5Y5: 5:5; S4Y6: 4:6; S3Y7: 3:7.
DM: Dry matter; CP: crude protein; WSC: water-soluble carbohydrate; EE: ether extracts; NDF: neutral detergent fiber.

Figure 5. Correlation between silage nutritional quality indices and bacterial community at phyla (a) and genus (b) levels.



DM: Dry matter; CP: crude protein; EE: ether extracts; NDF: neutral detergent fiber; WSC: water-soluble carbohydrate.

RDA analysis of the silage nutritional quality and abundant genus indicated that RDA1 and RDA2 explain 62.25% and 16.38% bacterial community variation, respectively (Figure 4, Table 5). S1 separated S6Y4, S3Y7, S4Y6 and S5Y5 on the RDA1 axis, indicating that maize straw addition not only has significant effect on CP, WSC, EE, DM, NDF and ADF, but also impact bacterial community. Pearson correlation coefficient showed that CP, WSC and EE contents positively correlated with *Cronobacter*, *Lactococcus* and *Citrobacter*, contents positively correlated with *Enterobacter* (Figure 5b, $P < 0.05$). While the content of DM, NDF and ADF negatively *Cronobacter*, *Lactococcus* and *Citrobacter*, NDF and ADF content positively correlated with *Weissella*. While the content of DM negatively correlated with *Enterobacter*. The CP positively correlated with *Cyanobacteria* and EE positively correlated with *Weissella*, $\text{NH}_3\text{-N}$ contents positively correlated with *Pediococcus*.

DISCUSSION

Effect of nutrition quality

Mixed silage is an effective way to solve the problem of higher water content and insufficient nutrient content in single forage silage. To a large extent, the ensilage fermentation process is influenced by the DM content of biomass, and lactic acid bacteria (LAB) responsible for fermentation require moisture for their growth and reproduction. Zhao et al. (2019) reported increased DM content and reduced moisture by mixing lucerne and sweet corn stalk silage. In this study, DM content increased after maize straw addition to ensilage, this shows that adding maize straw helps to increase DM content of forage rape and promotes fermentation. However, the CP, EE and WSC content of silage decreased with the increasing maize straws proportion before silage because maize straws contained lower CP, EE and WSC contents (Zhao et al., 2019). In addition, due to bacterial growth and reproduction require nutrients under anaerobic conditions (Kung et al., 2018), content of CP, EE and WSC under different treatments was gradually decreased as the fermentation process

Table 5. Correlation between nutritional quality parameters and redundancy analysis (RDA) axes at genus level.

| Nutritional quality parameters | RDA1 | RDA2 | R ² | P values |
|--------------------------------|--------|--------|----------------|----------|
| DM | -0.688 | -0.726 | 0.952 | 0.010 |
| CP | 0.886 | 0.463 | 0.893 | 0.033 |
| EE | 0.823 | 0.568 | 0.996 | 0.008 |
| NDF | -0.838 | -0.545 | 0.943 | 0.018 |
| WSC | 0.872 | 0.490 | 0.921 | 0.014 |

DM: Dry matter; CP: crude protein; EE: ether extracts; NDF: neutral detergent fiber; WSC: water-soluble carbohydrate.

progresses. Generally, the higher the content of CP, EE and WSC in silage, the greater nutritional value. Therefore, the high ratio of maize straws is not conducive to maintaining the nutrient content of silage. NDF has been characterized as the most important factor affecting the total diet organic matter digestibility (Britos et al., 2018). The lower ADF indicated the higher digestibility (Nazli et al., 2019). In our study, NDF and ADF content under different treatments decreased significantly with the increasing fermentation days ($P < 0.05$), which could be explained by the produced organic acids hydrolyzing the more digestible cell wall fraction during ensiling (Larsen et al., 2017). On the 60th day, the significantly highest CP, EE and WSC content were found in S7Y3 treatment ($P < 0.05$), the content of ADF and NDF in S7Y3 was significantly lower than those in other maize straw additive treatments ($P < 0.05$). This due to that maize straw contained higher NDF and ADF contents, and the lowest maize straw content in S7Y3 treatment. These results indicated that S7Y3 may have a higher nutritional value and digestibility. This provides a basis for determining the reasonable mixed silage ratio of forage rape and maize straw.

Fermentative quality of the silages

Lower pH is conducive to successful fermentation. The drop of pH in this study could be explained by increasing lactic acid (LA) and acetic acid (AA) content during the fermentation. Contents of LA, AA and PA increased with the increasing maize straw addition. The higher LA and AA contents have been observed in all treatments and the lowest pH values in S7Y3. This could be explained by more organic acids produced when silage was mixed with high WSC and moisture content forages (Wang et al., 2018). Xue et al. (2017) also indicated that desert wormwood ensiled with high-moisture maize (corn) straw had lower pH and AA concentration and higher LA concentration as the proportion of desert wormwood decreased. However, Yan et al. (2019) reported that AA content of the Italian ryegrass silages was low or less than 0.01% DM. This inconsistent result in our study is probably attributed to the bacterial communities of the silages being from different conditions, materials, or microbe on the raw materials (Guan et al., 2018). Conversion of LA to BA is one of the most wasteful anaerobic fermentations in silage. Muck (2010) indicated that BA is undesirable during ensilage fermentation because it reduces the intake of livestock if over 0.05% DM. A small amount of BA was detected in this study indicating that maize straw addition cannot introduce undesirable fermentation.

The amount of $\text{NH}_3\text{-N}$ present in silage is a reliable indicator of the extent of proteolytic clostridial activity, this may also be the performance of CP reduction (Zheng et al., 2017). This has also been confirmed in our study, as the CP content decreases, $\text{NH}_3\text{-N}$ content gradually increases. Simultaneously, increasing $\text{NH}_3\text{-N}$ content lowers V-score. The V-score method was adopted to evaluate the silage quality by calculating $\text{NH}_3\text{-N}$ and VFA contents (Li et al., 2017). This method is widely used in the evaluation of fermentation quality of other silage. Lower scores indicate the higher the rate of hydrolysis of the crude protein, the higher VFA content of heterofermentative (Zhao et al., 2019). The higher V-score, the better silage quality (Cao et al., 2010). And we found that V-score decreased after the maize straw addition, this indicates that an excessive increase in the proportion of maize straw will not improve the silage quality.

Diversity and composition of silage fermentation bacterial community

To a large extent, the silage quality is determined by microbes during silage fermentation. Shannon and Chao1 indices decreased gradually after maize straw addition, this is due to addition of corn stalks reduced the nutrients required for bacterial reproduction and LAB dominates and inhibits reproduction of other bacteria.

Abundance of *Firmicutes* increased as the proportion of maize straws increases, but the relative abundance of *Proteobacteria* decreased. Hu et al. (2018) noted that *Firmicutes* was the dominant species for whole-plant maize fermentation. The addition of maize straw may be the main cause of the *Firmicute* and *Proteobacteria* differences. The genus *Lactobacillus* and *Weissella*, which belong to phyla *Firmicutes*, were enriched in all treatments (Figure 2). These two genera were also documented in similar study (Guan et al., 2018), suggesting that they were beneficial bacteria to ensilage fermentation. *Weissella* is strictly heterofermentative, producing a mixture of LA and AA by metabolizing WSC and certain LAB have the ability to transform LA to AA (Graf et al., 2016). In this study, each treatment contained a higher content of AA, and the relative abundance of *Weissella*, and both of them first increased and then decreased along with the increasing of maize straw proportion. This further confirmed that the increase in AA was due to the increase in the relative abundance of LAB and *Weissella* in our study. Members of the genus *Enterobacter*, *Cronobacter*, *Cyanobacteria*, *Lactococcus*, *Enterococcus* and *Citrobacter* were also present in all samples, but at very low abundance. In fact, the

above bacteria were found in various maize and grass ensilages (Yang et al., 2017), indicating that these genera were not unique in our study. Among them, *Enterobacter* and *Cyanobacteria* are often considered as harmful indicators affecting the ensilage fermentation (Duniere et al., 2017). Fortunately, we observed that the relative abundance of *Enterobacter* and *Cyanobacteria* decreased gradually after maize straw addition.

Correlations between silage nutritional quality and bacterial community

The addition of maize straw has an important impact on the bacterial community composition at the end of fermentation. The reason for this difference may be related to the epiphytic bacteria of maize straw. Relevant studies have pointed out that the epiphytic lactic acid bacteria of corn stover grows rapidly after the start of anaerobic fermentation, and occupy a dominant position (Gharechahi et al., 2017). When silage is exposed to the air, it will promote the growth and reproduction of aerobic bacteria and cause the silage decay (Da Silva et al., 2015). Different compaction densities of silage also have an important impact on the composition of the bacterial community. Tian et al. (2020) pointed out that increasing the compaction density will inhibit growth and reproduction of harmful bacteria. Compared with single silage, mixed silage is more susceptible to the influence of epiphytic bacteria and nutritional quality of silage raw materials. The RDA analysis of the silage nutritional quality and abundant genus indicated that CP, WSC, EE, DM, NDF and ADF were significantly associated with bacterial community at phyla and genus levels (Figure 5). This relationship may be mutual. For example, WSC can be used easily by bacteria and plays a major role in shaping bacterial community structure (Duniere et al., 2017). During the fermentation process, some microorganisms consume WSC and CP to decompose the NDF and ADF by producing cellulase (Yan et al., 2019). It is generally supposed that *Lactobacillus* quickly come to dominate by consuming nutrients in silage (McGarvey et al., 2013). We investigated the bacterial component at the fermentation end point, *Lactobacillus* also dominate. This is consistent with many reports on silage bacterial community (Yang et al., 2016). Maize straw addition has significant effect on the content of CP, WSC, EE and DM, this may lead to differences in the fermentation substrate of the bacteria during the fermentation process and bacterial communities at the fermentation end point. Regrettably, we did not pay attention to changes in bacterial communities throughout the silage process. This makes it impossible for us to understand in detail the succession changes of epiphytic bacteria during the silage process. Epiphytic bacteria also have a great influence on the fermentation of silage. Gharechahi et al. (2017) reported that the *Weissella* is the dominant genus of maize straw epiphytic bacteria. We observed that CP, WSC and EE negatively correlated with *Weissella*. But NDF and ADF positively correlated with *Weissella*. The relative abundance of *Weissella* decreased with the increasing maize straws proportion. These results showed that maize straws affect the bacterial community of silage through epiphytic bacteria and nutrient content. Previous studies have observed that pH is the main predictor of bacterial community during silage fermentation (Guan et al., 2018). But there was nonsignificant relationship between pH and bacterial communities in our study, since the former did not change markedly (ranged from 3.56 to 3.76) in response to maize straw addition.

CONCLUSIONS

Silage nutritional quality and bacterial community were affected by maize straw addition. Appropriate ratio of maize straw addition not only ensured the proper moisture, but also ensured contents of crude protein, ether extracts and water-soluble carbohydrate content, this will be helpful to obtain high quality silage. The dominant genera are *Lactobacillus* and *Weissella* in silage. The best mixing ratio of forage rape and maize straw ensilage is 7:3.

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