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Postprint: Detection of Microbial Diversity in Whole-Plant Corn Silage During Ensiling and After Aerobic Exposure Using Metagenomics Technology

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Abstract

This experiment aimed to analyze the fermentation quality and microbial diversity of whole-plant corn silage during fermentation and after exposure to air, and to monitor the dynamic changes in its microbial community composition. Samples were collected at three fermentation stages: day 5 of ensiling (F5 group), day 40 of ensiling (F40 group), and day 3 after opening the bag and exposing to air following 40 days of ensiling (A3 group), with three bags serving as three replicates each time. Metagenomic technology was employed to determine the 16S rDNA V3-V4 region sequences of microorganisms in whole-plant corn silage during fermentation and after air exposure, compare the composition and abundance information of microbial communities in samples from the three fermentation stages, and investigate and compare the microbial diversity of whole-plant corn silage during fermentation and after air exposure through Alpha diversity and principal component analysis. The results showed that 40 days of ensiling fermentation of whole-plant corn resulted in significant decreases in pH and contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) ($P<0.05$), a significant increase in lactic acid content ($P<0.05$), demonstrating good fermentation quality and nutritional value, and short-term opening of the bag and exposure to air after 40 days of fermentation had no significant effect on its fermentation quality and nutritional value ($P>0.05$). The three groups of samples obtained a total of 122,371 high-quality valid sequences through the Illumina Miseq sequencing platform, which were clustered into 239 operational taxonomic units and taxonomically assigned to 16 phyla and 163 genera. At the phylum level, Firmicutes remained dominant, with its abundance in the F5, F40, and A3 groups being 57.57%, 74.65%, and 78.82%, respectively, showing an increasing trend. The dominant bacterial genus in the early fermentation stage (F5 group), late fermentation stage (F40 group), and aerobic exposure

period (A3 group) was Lactobacillus, with abundances of 49.78%, 64.46%, and 45.34%, respectively; however, the proportion of Sporolactobacillus increased significantly during the aerobic exposure period, reaching 28.46%. In summary, natural fermentation of whole-plant corn silage increased the abundance of lactic acid-producing Lactobacillus, which is beneficial for improving the fermentation quality of whole-plant corn silage, but opening the bag and exposing to air for 3 days affected the microbial diversity of whole-plant corn silage, and through Miseq high-throughput sequencing technology, the changes in microbial community composition and abundance during whole-plant corn silage and after air exposure can be comprehensively understood.

Full Text

Microbial Diversity of Whole-Plant Maize during Ensilage and after Air Exposure Analyzed by Metagenomics Technology

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Abstract

This study aimed to analyze the fermentation quality and microbial diversity of whole-plant maize during ensilage and after air exposure, and to monitor the dynamic changes in microbial community composition. Samples were collected at three fermentation stages: day 5 of ensilage (F5 group), day 40 of ensilage (F40 group), and day 3 after opening the bag following 40 days of ensilage (A3 group), with three bags sampled as replicates at each time point. The 16S rDNA V3–V4 region sequences of microorganisms were determined using metagenomics technology to compare the composition and abundance of microbial communities across the three fermentation stages. Alpha diversity and principal component analysis were conducted to investigate microbial diversity. The results showed that after 40 days of ensilage, pH and the contents of neutral detergent fiber (NDF) and acid detergent fiber (ADF) decreased significantly ($P<0.05$), while lactic acid content increased significantly ($P<0.05$), indicating good fermentation quality and nutritional value. Short-term air exposure after 40 days of ensilage had no significant effect on fermentation quality or nutritional value ($P>0.05$). A total of 122,371 high-quality effective sequences were obtained via Illumina MiSeq sequencing, clustering into 239 operational taxonomic units (OTUs) that were taxonomically assigned to 16 phyla and 163 genera. At the phylum level, Firmicutes was consistently dominant, with abundances of 57.57%, 74.65%, and 78.82% in the F5, F40, and A3 groups, respectively, showing an increasing trend. The dominant genus at all three stages was *Lactobacillus*, with abundances of 49.78%, 64.46%, and 45.34% in the F5, F40, and A3 groups,

respectively. However, the proportion of *Sporolactobacillus* increased markedly during the aerobic period, reaching 28.46%. In conclusion, natural fermentation of whole-plant maize silage increased the abundance of lactic acid-producing *Lactobacillus*, which improved fermentation quality. However, three days of air exposure after opening affected the microbial diversity. MiSeq high-throughput sequencing technology provides a comprehensive understanding of changes in microbial community composition and abundance during ensilage and after air exposure.

Keywords: silage; whole-plant maize; metagenomics; microbial diversity; fermentation quality

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Silage is a crucial source of nutrition and feed for ruminants, particularly in regions with long winters. Whole-plant maize silage is among the most important silage feeds for ruminants and has been widely used in Europe and North America for many years [1-2]. With the strategic adjustment of agricultural structure and rapid development of animal husbandry in China, promoting the cultivation and utilization of whole-plant silage corn through microbial fermentation technology is essential for improving silage quality, enhancing livestock production performance, and improving animal product quality [3-4]. Consequently, the composition and dynamics of microorganisms during ensilage and their impact on fermentation quality have become focal points for researchers.

Traditional culture methods have long been used to study microbial diversity and community changes in silage. However, due to limitations in culture conditions, these methods are time-consuming, labor-intensive, and incomplete, failing to fully resolve microbial composition, abundance, and dynamics while underestimating microbial diversity [5]. Over the past decade, advances in molecular biology have enabled numerous culture-independent methods based on polymerase chain reaction (PCR) technology, such as 16S rRNA clone library construction, restriction fragment length polymorphism (RFLP), and denaturing gradient gel electrophoresis PCR (DGGE-PCR). These methods have been widely applied to study microbial diversity in various environments, fermented foods, silage, and other microecosystems [6-8]. High-throughput pyrosequencing technology based on microbial metagenomics offers advantages of high throughput, speed, and reduced labor, and has been increasingly applied to detect and study microbial diversity in soil, gut, water, and fermented foods [9-11]. However, few studies have reported the application of high-throughput pyrosequencing technology to silage fermentation feed. Li et al. [12] used high-throughput sequencing to detect changes in community structure of over 30 bacterial genera during ensilage. Liu [13] applied MiSeq high-throughput sequencing technology to detect microbial diversity in switchgrass silage after 60 days. Tao et al. [14] combined laboratory detection methods with MiSeq high-throughput sequencing to analyze silage quality and changes in microbial community composition and abundance, providing insights into microbial composition during fermentation. Bao et al. [15] used third-generation single-molecule real-time (SMRT)

sequencing technology to detect changes in microbial composition before and after alfalfa ensilage and their effects on alfalfa quality, demonstrating that the SMRT platform can assess microbial composition changes and silage quality. However, no studies have reported the use of high-throughput sequencing to detect microbial diversity during whole-plant maize ensilage. Therefore, this study employed metagenomics technology to investigate and analyze the microbial community structure and succession patterns during whole-plant maize ensilage and after air exposure, providing a theoretical foundation and new methodology for comprehensively understanding microbial composition and dynamics during natural ensilage, identifying beneficial epiphytic microorganisms, and improving the nutritional value and quality of silage.

1.1 Experimental Materials and Sample Collection

Whole-plant maize was obtained from the Chajintai First Farm in Tongliao City, Inner Mongolia, from mechanically harvested samples (the harvested whole-plant maize was automatically cut into 1–2 cm pieces by machine). The ensilage experiment was conducted in September 2016 at the laboratory of the College of Animal Science and Technology, Inner Mongolia University for the Nationalities. Samples were packed into double-layer polyethylene bags (45 cm × 30 cm), with air expelled, compacted, and sealed. Each bag contained approximately 3.0 kg with a compaction density of about 550 kg/m^3. The silage bags were placed in plastic storage boxes for fermentation at room temperature. Samples were collected at three time points: day 5 of ensilage (F5 group), day 40 of ensilage (F40 group), and day 3 after opening the bag following 40 days of ensilage (A3 group). At each sampling, three bags were taken as replicates (nine bags total). During sampling, bags were opened, the top layer was discarded, and samples were taken from the middle portion. One portion was placed in sterile 50 mL cryogenic centrifuge tubes and stored at -80 °C for microbial diversity analysis. Another portion (approximately 60 g) was placed in sealed bags and stored at -20 °C for determination of fermentation quality and nutritional components.

1.2 Determination of Fermentation Quality and Nutritional Components

For each of the three silage stages, 20 g of whole-plant maize silage sample was mixed with 180 mL distilled water, stirred evenly, and homogenized for 1 min using a tissue blender. The mixture was filtered sequentially through four layers of gauze and qualitative filter paper to obtain the extract, which was divided into four portions. pH and ammonia nitrogen (AN) were measured using a pH meter and the phenol-hypochlorite colorimetric method [14], respectively. Acetic acid and lactic acid contents were determined by the p-hydroxybiphenyl colorimetric method and neutralization titration method [16], respectively. Dry matter (DM), total nitrogen (TN), crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) contents were determined using oven drying, Kjeldahl nitrogen determination, and Van Soest detergent fiber methods

as described in reference [17]. Water-soluble carbohydrate (WSC) content was measured by the anthrone spectrophotometric method [18].

1.3.1 Extraction of Bacterial Genomic DNA

At each sampling time, the three replicate samples were mixed together. Bacterial genomic DNA was extracted from the silage using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer' s instructions. The extracted genomic DNA was assessed for concentration and purity using a UV spectrophotometer, and DNA integrity was verified by 0.8% agarose gel electrophoresis.

1.3.2 Amplification of 16S rDNA

Bacterial 16S rRNA gene V3–V4 region primers 338F (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGGGTWTCTAAT) [19] were used to amplify sequences from the DNA template. The PCR reaction system (20 µL) contained 4.0 µL of 5×FastPfu Buffer, 2.0 µL of dNTPs (dATP, dTTP, dGTP, and dCTP at 2.5 mmol/L each), 0.4 µL of FastPfu DNA Polymerase, 20.0 µL of template DNA, 0.8 µL of forward primer 338F (5 µmol/L), 0.8 µL of reverse primer 806R (5 µmol/L), and 0.2 µL of bovine serum albumin (BSA) (0.1%), brought to 20 µL with sterile ddH_2O. PCR conditions were: initial denaturation at 95 °C for 3 min; 27 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s; and final extension at 72 °C for 10 min.

1.3.3 Sequencing

PCR products were examined by 2% agarose gel electrophoresis. Products with appropriate concentration and correct target band size were sequenced using the Illumina MiSeq sequencing platform (Illumina Corporation, San Diego, USA) by Shanghai Majorbio Bio-Pharm Technology Co., Ltd.

1.3.4 Bioinformatics and Data Analysis

1.3.4.1 Assembly and Quality Control of Raw Paired-End Reads This process involved removing impure reads and assembling paired-end sequences using FLASH and Trimmomatic software. First, bases in 50 bp windows at read tails with average quality values below 20 were trimmed. Reads shorter than 50 bp were filtered out, and reads containing N bases were removed. Assembly was performed based on the overlap relationship between paired-end reads, requiring a minimum overlap of 10 bp and a base mismatch rate below 0.2%. Finally, effective sequences were obtained by distinguishing samples based on barcodes and primers at both ends of the sequences, with no barcode mismatches allowed.

1.3.4.2 Sequence Filtering and Operational Taxonomic Unit (OTU) Clustering Sequence filtering and OTU clustering were performed using the

Usearch platform (v7.0, http://drive5.com/uparse/). Redundant analysis of non-repetitive sequences from the effective sequences obtained in section 1.3.3 was conducted to remove singletons and obtain optimized sequences. OTU clustering of optimized sequences was performed at 97% similarity, with chimeras removed during the process to generate OTU representative sequences. These were then compared with optimized sequences to produce OTU tables.

1.3.4.3 Bioinformatics Analysis Taxonomic classification was performed using the RDP classifier Bayesian algorithm (version 2.2, http://sourceforge.net/projects/rdp-classifier/) and the Qiime platform (http://qiime.org/scripts/assign_{taxonomy}.html) with a confidence threshold of 0.7. OTU representative sequences were BLAST-aligned against the Silva database (Release128, http://www.arb-silva.de) to obtain taxonomic information, and community composition was analyzed at various taxonomic levels. Alpha diversity and rarefaction curve analysis were performed using mothur (version v.1.30.1, http://www.mothur.org/wiki/Schloss_{SOP}#Alpha_{diversity}). Venn diagrams, Heatmaps, and principal component analysis (PCA) were generated using relevant R software packages.

2.1 Changes in Fermentation Quality and Nutritional Components during Ensilage and after Air Exposure

As shown in Table 1 , compared with day 5 of ensilage, ammonia nitrogen/total nitrogen, lactic acid, and acetic acid contents increased significantly (P<0.05) at day 40 of ensilage and day 3 after opening the bag, while pH showed the opposite trend with significant decreases (P<0.05). No significant differences were observed between day 40 of ensilage and day 3 after opening (P>0.05). Changes in nutritional components before ensilage, at day 5, day 40, and day 3 after opening (Table 1) indicated that DM, CP, NDF, ADF, and WSC contents tended to decrease with prolonged ensilage time. Although slight increases were observed after opening, these were not significantly different from values at day 40 (P>0.05). WSC content showed the most pronounced decrease, declining by 38.60%, 68.17%, and 65.86% at day 5, day 40, and day 3 after opening, respectively, compared with pre-ensilage levels (P<0.05).

2.2 Sequence Filtering and Quality Control

After sequence filtering, extraction, and paired-end assembly, a total of 122,371 effective sequences were obtained from the three sample groups, comprising 54,758,721 total bases with an average length of 447 bp. Following optimization of effective sequences, 110,173 optimized sequences were obtained for OTU clustering and taxonomic analysis, representing an effective optimization rate of 90.03% (Table 2).

OTU clustering at >97% sequence similarity yielded 239 OTUs. However, the number of OTUs with >1% sequence abundance in individual samples was small:

12 (F5 group), 8 (F40 group), and 10 (A3 group). Coverage for all three samples exceeded 0.99. Both rarefaction and Shannon curves (Figure 1 [Figure 1: see original paper]) plateaued, indicating that sequencing depth was saturated and sufficient to capture the vast majority of bacterial species information. The numbers of effective and optimized sequences increased with ensilage time, and OTU numbers also increased, suggesting continuous microbial proliferation and increasing diversity during ensilage and after air exposure. However, the number of OTUs with >1% abundance not only decreased but was also lower in the later ensilage stage than in the early stage, indicating that ensilage reduced diversity among dominant microbial groups.

2.3 Principal Component Analysis of Microbial Communities during Ensilage and after Air Exposure

Principal component analysis (Figure 2 [Figure 2: see original paper]) revealed that the A3, F40, and F5 groups were distinctly separated from each other, forming a triangular pattern, indicating substantial differences in microbial composition among the three groups representing different fermentation stages: early fermentation (F5), late fermentation (F40), and aerobic period (A3). The F40 group was relatively close to both the A3 and F5 groups, suggesting that late fermentation shared greater similarity with both early fermentation and the aerobic period, reflecting the succession relationship among the three stages.

2.4 Microbial Community Structure Analysis at the Phylum Level

OTU clustering at >97% similarity yielded 239 OTUs assigned to 163 genera, 93 families, 54 orders, 26 classes, and 16 phyla. Microbial communities in all samples comprised 16 phyla. As shown in Figure 3 [Figure 3: see original paper], five phyla had abundances >1%: Firmicutes, Proteobacteria, Bacteroidetes, Cyanobacteria, and Actinobacteria, which accounted for the vast majority of sequences. In the early fermentation stage (F5 group), the dominant phyla were Firmicutes (57.57%) and Proteobacteria (27.54%), followed by Cyanobacteria (7.05%), Bacteroidetes (6.49%), and Actinobacteria (1.08%), with a combined abundance of 99.73%. In the late fermentation stage (F40 group), three phyla exceeded 1% abundance: Firmicutes (74.65%), Proteobacteria (20.36%), and Bacteroidetes (3.10%), with a combined abundance of 98.10%. In the aerobic period (A3 group), the dominant phyla were Firmicutes (78.82%) and Proteobacteria (17.02%), followed by Bacteroidetes (2.17%) and Actinobacteria (1.20%), with a combined abundance of 99.21%. Comparison across fermentation stages revealed that Firmicutes abundance increased continuously during ensilage and subsequent air exposure, while Proteobacteria and Bacteroidetes proportions decreased. Cyanobacteria were present only in the F5 group, and Actinobacteria exceeded 1% abundance only during early fermentation and the aerobic period.

2.5 Microbial Community Structure Analysis at the Genus Level

Figure 4 [Figure 4: see original paper] shows changes in microbial community structure at the genus level during ensilage and after air exposure (abundance >1%). A total of 163 genera were identified across the three samples. In the early fermentation stage (F5 group), 133 genera were identified, with 11 genera (including Cyanobacteria) exceeding 1% abundance and accounting for 86.33% of total abundance. *Lactobacillus* was dominant at 49.78%, followed by *Klebsiella* (9.08%), Cyanobacteria (7.05%), *Rahnella* (3.45%), *Chryseobacterium* (3.14%), *Pediococcus* (3.18%), *Lactococcus* (2.67%), *Enterobacter* (2.52%), *Sphingomonas* (2.34%), *Pantoea* (1.78%), and *Sphingobacterium* (1.33%). Numerous other genera were present at low abundances.

In the late fermentation stage (F40 group), 146 genera were identified, with seven genera exceeding 1% abundance and accounting for 85.60% of total abundance. *Lactobacillus* remained dominant at 64.46%, followed by *Sporolactobacillus* (7.79%), *Klebsiella* (5.48%), *Pantoea* (4.34%), *Stenotrophomonas* (1.28%), *Sphingomonas* (1.24%), and *Flavobacterium* (1.01%).

In the aerobic period (A3 group), 153 genera were identified, with nine genera exceeding 1% abundance and accounting for 88.65% of total abundance. The main genera were *Lactobacillus* (45.34%), *Sporolactobacillus* (28.46%), *Klebsiella* (5.79%), *Pediococcus* (2.90%), *Stenotrophomonas* (1.43%), *Rahnella* (1.43%), *Sphingomonas* (1.16%), *Pantoea* (1.07%), and *Clostridium* (1.07%).

Lactobacillus abundances in early fermentation, late fermentation, and aerobic period were 49.78%, 64.46%, and 45.34%, respectively, maintaining absolute dominance. Its abundance increased during ensilage, peaking at day 40, then decreasing after bag opening. In contrast, *Sporolactobacillus* was absent in early fermentation, increased to 7.79% in late fermentation, and further increased to 28.46% after three days of air exposure, indicating that air exposure favored its growth. Other genera remained relatively stable and continuously present, with *Klebsiella* maintaining relatively high abundances of 9.08%, 5.48%, and 5.79% across the three stages. *Clostridium* was undetectable during ensilage but increased to 1.07% after three days of air exposure. Additionally, low-abundance genera such as *Pantoea* and *Sphingomonas* were consistently present. Thus, *Lactobacillus* was the dominant fermentation genus, beneficial for silage quality improvement, while air exposure altered the microbial structure and promoted the growth of harmful bacteria.

2.6 Alpha Diversity Analysis of Microbial Communities

Table 3 presents the alpha diversity indices of whole-plant maize during ensilage and after air exposure. Alpha diversity represents within-sample microbial community diversity. Chao and Ace indices measure community richness, with higher values indicating greater richness. Simpson and Shannon indices measure

community diversity, with higher Shannon values indicating lower diversity and higher Simpson values indicating greater diversity.

Alpha diversity analysis revealed changes in microbial quantity and composition during ensilage. Overall, the three stages showed large sequence numbers and high levels of OTU numbers, Chao values, and Ace values, indicating substantial microbial populations and relatively high diversity levels. Compared with early fermentation, late fermentation and aerobic period showed increases in sequence numbers, OTU numbers, Chao values, and Ace values, while Shannon values decreased, suggesting that fermentation promoted microbial proliferation and development of many species, thereby increasing both microbial diversity and richness.

For short-term air exposure, the increases in sequence numbers, OTU numbers, Chao values, and Ace values relative to late fermentation were minimal, and the decrease in Shannon values was also small, indicating that microbial growth and species diversity did not change substantially in the new gaseous and temperature environment after short-term bag opening.

2.7 Venn Diagram and Heatmap Analysis of Microbial Communities

The Venn diagram (Figure 5 [Figure 5: see original paper]-A) showed that among the 239 OTUs across three samples, 173 core OTUs were shared, accounting for 73.49%, indicating a large core microbiome during ensilage and after air exposure. Between early and late fermentation stages, 180 core OTUs were shared among 223 total OTUs (80.72%). Between late fermentation and aerobic period, 198 core OTUs were shared among 238 total OTUs (83.19%). Unique OTUs were scarce in the F5, F40, and A3 groups (1, 9, and 16, respectively). Shared species between F5 and F40 or A3 groups were few (7 and 8, respectively), while F40 and A3 groups shared more species (25), indicating greater similarity between these two stages. Overall, Venn analysis demonstrated that the F40 and A3 groups were more similar in microbial community composition, suggesting that although three days of air exposure altered the microbial community, it maintained substantial similarity with late fermentation, thereby preserving silage quality to some extent.

The Heatmap (Figure 5-B) displayed the abundances of the top 20 genera, where color intensity represents the abundance of each genus in each sample, with numerical values indicating sequence numbers. *Lactobacillus* maintained high abundance (deep color) across all three stages. *Klebsiella* also showed good continuity and relatively high abundance, while *Sporolactobacillus* showed intensified color in late fermentation and aerobic period, particularly during the aerobic period. *Clostridium* appeared suddenly during the aerobic period with relatively high abundance. The Heatmap also revealed that most genera showed good continuity across the three samples, with only a few showing interruption (green blocks), indicating that beneficial bacteria such as lactic acid bacteria main-

tained dominance throughout fermentation and three days after bag opening, although some genera showed reciprocal changes in abundance with prolonged fermentation and altered conditions after opening.

Compared with corn stover silage, whole-plant maize silage offers higher nutritional value, better palatability, higher digestibility, and higher energy value, enabling long-term preservation and year-round balanced supply, making it the most effective approach to providing roughage for ruminants such as cattle and sheep. In this study, sensory evaluation during sampling at day 5, day 40, and day 3 after opening revealed good quality, with colors of yellow-green, yellow-brown, and yellow-brown, respectively, no mold or stickiness, and a pleasant acidic alcoholic aroma without off-odors. Regarding fermentation quality, after 40 days of ensilage and after short-term bag opening, ammonia nitrogen/total nitrogen was below 9%, pH was below 4.00, lactic acid content exceeded 9%, and acetic acid content was below 3%, similar to quality reported by Zhou et al. [20] for whole-plant maize silage at 20 or 25 °C. Considering these fermentation quality parameters, whole-plant maize silage after 40 days demonstrated good fermentation quality. Fermentation reduced NDF and ADF contents in whole-plant maize, but consumption of large amounts of soluble carbohydrates by dominant lactic acid bacteria significantly decreased WSC content, which was confirmed in this study. Therefore, to further reduce NDF and ADF contents and minimize WSC consumption, some studies have added carbohydrates, cellulase, or silage additives [21-23] to improve nutritional value.

Many European and American countries have incorporated whole-plant maize silage as an indispensable roughage component in total mixed rations for dairy and beef cattle [24]. Microbial community composition and abundance change substantially during and after ensilage, affecting silage nutritional value and quality [20,25]. Dolci et al. [26] reported that silage fermentation quality largely depends on the quantity and proportion of excellent fermentation strains during early fermentation. This study used MiSeq high-throughput sequencing to analyze microbial community structure during whole-plant maize ensilage and after air exposure, revealing that *Lactobacillus* rapidly became dominant by day 5, reaching 49.78% abundance and establishing the microbial community trajectory, while suppressing other strains. With prolonged fermentation, *Lactobacillus* further expanded its dominance, reaching 64.46% abundance at day 40 and securing an absolute advantage. This provides microbial assurance for improved silage quality. As a lactic acid bacteria (LAB), *Lactobacillus* produces lactic acid, reduces silage pH, inhibits mold and other harmful microorganisms, and enhances silage flavor [27]. In this study, *Lactobacillus* abundance during whole-plant maize ensilage was substantially higher than the 9.73% reported by Tao et al. [14] in corn stover silage. Due to oxygen depletion during ensilage, harmful aerobic bacteria such as *Pseudomonas* and molds were suppressed, with aerobic genera abundances remaining below 1%. Besides the dominant *Lactobacillus*, other typical but low-abundance LAB species commonly found in silage were present, including *Lactococcus*, *Pediococcus*, *Enterococcus*, and *Weissella*, though none became dominant.

This study demonstrated that air exposure substantially affected microbial community composition in whole-plant maize silage. After three days of bag opening, *Lactobacillus* abundance decreased dramatically from 64.46% in late fermentation to 45.34%, as increased oxygen content inhibited LAB growth while favoring aerobic or facultative aerobic bacteria such as *Sporolactobacillus*, which increased from 7.79% in late fermentation to 28.46%. Few studies have reported *Sporolactobacillus* in silage. Smoker [28] found that *Sporolactobacillus* could ferment various carbohydrates to produce lactic acid under laboratory natural silo conditions, suggesting its potential as a silage inoculant. Kharazian et al. [29] reported that adding *Sporolactobacillus terrae* to whole-plant maize silage inhibited some plant pathogenic fungi. Liu et al. [30] found that adding 100 mg/kg *Sporolactobacillus* to broiler diets promoted intestinal development, improved digestion and absorption, and inhibited *Escherichia coli* proliferation in the jejunum. However, the mechanisms by which *Sporolactobacillus* as a silage inoculant affects microbial communities, fermentation quality, and ruminant health require further investigation.

Whole-plant maize ensilage requires strict anaerobic conditions. Air leakage can lead to mold proliferation, causing silage spoilage, nutrient degradation, and production of harmful mycotoxins that endanger livestock [31-32]. Vissers et al. [33] reported that Gram-positive *Bacillus* is the main spoilage organism in silage, primarily degrading proteins and amino acids and playing an important role in aerobic deterioration. *Bacillus* also rapidly proliferates after silo opening. However, *Bacillus* was not detected in this study during ensilage or three days after bag opening. Notably, the harmful genus *Clostridium* reached 1.07% abundance on day 3 after opening, potentially adversely affecting silage quality. McDonald et al. [34] detected *Clostridium* in poorly fermented silage, where it can decompose sugars, organic acids, and proteins under anaerobic or microaerophilic conditions, making it a harmful silage microorganism. This aligns with our detection of *Clostridium* after air exposure.

This study also found that *Klebsiella* maintained relatively high abundance across all three sampling periods, potentially playing an important role in whole-plant maize silage, though few studies have reported this. Its specific mechanisms and functions require further investigation. Although *Pantoea* and *Sphingomonas* had low abundances, their presence throughout all three sampling periods suggests continuous roles in maize ensilage. *Enterobacter* appeared only during early fermentation, while *Stenotrophomonas* appeared during late fermentation and aerobic period, and *Rahnella* appeared during early fermentation and aerobic period, indicating that ensilage and air exposure influenced the occurrence of certain genera. Tao et al. [14] and Dunière [35] both reported the presence of *Stenotrophomonas* and *Sphingomonas* in corn silage, consistent with our findings and suggesting these genera are common in corn silage.

Conclusions

1. Forty days of whole-plant maize ensilage significantly decreased pH and NDF and ADF contents while significantly increasing lactic acid content, resulting in good fermentation quality and nutritional value. Short-term air exposure after 40 days of ensilage had no significant effect on fermentation quality or nutritional value.

2. Principal component analysis revealed substantial differences in microbial diversity among the three fermentation stages, representing three distinct groups, indicating significant changes in microbial community composition during early fermentation, late fermentation, and aerobic period.

3. Across the three fermentation stages, 16 phyla and 163 genera of bacteria were identified. At the phylum level, Firmicutes and Proteobacteria were consistently dominant. At the genus level, the dominant community in early fermentation consisted mainly of *Lactobacillus*, *Klebsiella*, and Cyanobacteria; late fermentation was dominated by *Lactobacillus*, *Sporolactobacillus*, and *Klebsiella*; and short-term air exposure resulted in dominance by *Lactobacillus*, *Sporolactobacillus*, and *Klebsiella*.

References

[1] BERTHIAUME R, MANDELL I, FAUCITANO L, et al. Comparison of alternative beef production systems based on forage finishing or grain-forage diets with and without promotants: 1. Feedlot performance, carcass quality, and production costs[J]. Journal of Animal Science, 2006, 84(8): 2168–2177.

[2] HATEW B, BANNINK A, VAN LAAR H, et al. Increasing harvest maturity of whole-plant corn silage reduces methane emission of lactating dairy cows[J]. Journal of Dairy Science, 2016, 99(1): 354–368.

[3] ELLIS J L, BANNINK I K, HINDRICHSEN R D, et al. The effect of lactic acid bacteria included as a probiotic or silage inoculant on in vitro rumen digestibility, total gas and methane production[J]. Animal Feed Science and Technology, 2016, 211: 61–74.

[4] CARRILLO J A, HE Y H, LI Y K, et al. Integrated metabolomic and transcriptome analyses reveal finishing forage affects metabolic pathways related to beef quality and animal welfare[J]. Scientific Reports, 2016, 6: 25948, doi:10.1038/srep25948.

[5] LIN C J, BOLSEN K K, BRENT B E, et al. Epiphytic lactic acid bacteria succession during the pre-ensiling and ensiling periods of alfalfa and maize[J]. Journal of Applied Microbiology, 2010, 73(5): 375–387.

[6] MUYZER G, DE WAAL E C, UITTERLINDEN A G. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified encoding rRNA[J]. Applied Environmental Microbiology, 1993, 59(3): 695–700.

[7] HAN J Y, HOU X Z, YANG K, et al. Analysis of lactic acid bacteria diversity in silage corn from different regions of Inner Mongolia by PCR-DGGE[J]. Chinese Journal of Animal Nutrition, 2009, 21(6): 974-981.

[8] WANG H M, SUN Q Z, TU Y, et al. Isolation and identification of excellent lactic acid bacteria from wild forage silage in Hulunbuir grassland[J]. Acta Prataculturae Sinica, 2016, 25(8): 189-196.

[9] CAPORASO J G, LAUBER C L, WALTERS W A, et al. Ultra-high-throughput microbial community analysis on Illumina HiSeq and MiSeq platforms[J]. The Journal, 2012, 6(8): 1621-1624.

[10] ZHANG H P, YU J. New progress in lactic acid bacteria genomics research[J]. Journal of Chinese Institute of Food Science and Technology, 2016, 16(2): 1-8.

[11] JIANG Y, OGUNADE I M, QI S, et al. Effects of the dose and viability of Saccharomyces cerevisiae. 1. Diversity of ruminal microbes as analyzed by Illumina MiSeq sequencing and quantitative PCR[J]. Journal of Dairy Science, 2017, 100(1): 325-342.

[12] LI L H, SUN Y M, YUAN Z H, et al. Effect of microalgae supplementation on the silage quality anaerobic digestion performance of Manyflower silvergrass[J]. Bioresource Technology, 2015, 189: 334-340.

[13] LIU J J. Study on the effect and mechanism of biological additives on switchgrass silage[D]. PhD thesis. Beijing: China Agricultural University, 2015.

[14] TAO L, DIAO Q Y. Effect of ensiling fermentation on the quality and microbial community composition of corn stover[J]. Chinese Journal of Animal Nutrition, 2016, 28(1): 198-207.

[15] BAO W C, MI Z H, XU H Y, et al. Assessing quality of Medicago sativa silage by monitoring bacterial composition with single molecule, real-time sequencing technology and various physiological parameters[J]. Scientific Reports, 2016, 6: 28358, doi:10.1038/srep28358.

[16] WAN C Y, NIU Y X, HUANG F H, et al. Study on color reaction conditions for lactic acid determination by p-hydroxybiphenyl colorimetric method[J]. Science and Technology of Food Industry, 2013, 34(7): 322-324, 353.

[17] ZHANG L Y. Feed analysis and feed quality detection technology[M]. 2nd ed. Beijing: China Agricultural University Press, 2003: 53-56, 70-74.

[18] OWENS V N, ALBRECHT K A, MUCK R E, et al. Protein degradation and fermentation characteristics of red clover and alfalfa silage harvested with varying levels of total nonstructural carbohydrates[J]. Crop Science, 1999, 39(6): 1873-1880.

[19] DENNIS K L, WANG Y W, BLATNER N R, et al. Adenomatous polyps are driven by microbe-instigated focal inflammation and are controlled by IL-10-producing T cells[J]. Cancer Research, 2013, 73(19): 5905-5913.

[20] ZHOU Y, DROUIN P, LAFRENIÈRE C. Effect of temperature (5–25°C) on epiphytic lactic acid bacteria populations and fermentation of whole-plant corn silage[J]. Journal of Applied Microbiology, 2016, 121(3): 657–671.

[21] XING L, HAN L J, LIU X, et al. Effects of lactic acid bacteria and cellulase on fermentation quality and microbial community of whole-plant corn silage[J]. Journal of China Agricultural University, 2004, 9(5): 38–41.

[22] XU Q F, ZHANG X, CUI Z W, et al. Effects of different additives on the quality of whole-plant corn silage[J]. Acta Agrestia Sinica, 2009, 17(2): 157–161.

[23] SANTOS A O, ÁVILA C L S, SCHWAN R F. Selection of tropical lactic acid bacteria for enhancing the quality of maize silage[J]. Journal of Dairy Science, 2013, 96(12): 7777–7789.

[24] MUCK R E. Recent advances silage microbiology[J]. Agriculture Food Science, 2013, 22(1): 3–15.

[25] RANJIT N K, TAYLOR C C, KUNG L, Jr. Effect of Lactobacillus buchneri 40788 on the fermentation, aerobic stability and nutritive value of maize silage[J]. Grass Forage Science, 2002, 57(2): 73–81.

[26] DOLCI P, TABACCO E, COCOLIN L, et al. Microbial dynamics during aerobic exposure of corn silage stored under oxygen barrier or polyethylene films[J]. Applied and Environmental Microbiology, 2011, 77(21): 7499–7507.

[27] TOHNO M, KOBAYASHI H, NOMURA M, et al. Identification and characterization of lactic acid bacteria isolated from mixed pasture of timothy and orchardgrass, and its badly preserved silage[J]. Journal of Animal Science, 2002, 83(4): 318–330.

[28] SMOKER A N. Screening of Sporolactobacillus and Bacillus Strains for Use in Silage Inoculation[D]. Master Thesis. Corvallis: Oregon State University, 1999.

[29] KHARAZIANA Z A, JOUZANIA G S, AGHDAS M, et al. Biocontrol potential of Lactobacillus strains isolated from corn silage against some plant pathogenic fungi[J]. Biological Control, 2017, 110(1): 33–43.

[30] LIU L, ZHU L X. Effects of Sporolactobacillus on growth performance, intestinal development and microbial flora of broilers[J]. Chinese Journal of Animal Nutrition, 2011, 23(12): 2136–2142.

[31] DRIEHUIS F, ELFERINK S J W H O. The impact of the quality of silage on animal health and food safety: a review[J]. Veterinary Quarterly, 2000, 22(4): 212–216.

[32] WAMBACQ E, VANHOUTTE I, AUDENAERT K, et al. Occurrence, prevention and remediation of toxigenic fungi and mycotoxins in silage: a review[J]. Journal of the Science of Food and Agriculture, 2016, 96(7): 2284–2302.

[33] VISSERS M M M, TE GIFFEL M C, DRIEHUIS F, et al. Minimizing the level of Bacillus cereus spores in farm tank milk[J]. Journal of Dairy Science, 2007, 90(7): 3286-3293.

[34] MCDONALD P, HENDERSON A R, HERON S J E. The biochemistry of silage[M]. 2nd ed. Marlow: Chalcombe Publications, 1991: 81-151.

[35] DUNIÈRE L, SINDOU J, CHAUCHEYRAS-DURAND F, et al. Silage processing and strategies to prevent persistence of undesirable microorganisms[J]. Animal Feed Science and Technology, 2013, 182(1/2/3/4): 1-15.

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