Effects of Silage Fermentation on Corn Stover Quality and Microbial Community Composition (Postprint)

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Abstract

This study aimed to investigate the effects of silage fermentation on the sensory characteristics, fermentation quality, nutritional composition, and bacterial community composition and abundance at five taxonomic levels (phylum, class, order, family, and genus) of corn stalks. Wax-ripe corn stalks that remained green after ear removal were chopped and processed to 1–2 cm, and moisture content was adjusted to 65%–70%. Pre-ensiling samples (3 replicates) and post-ensiling samples (3 replicates) were vacuum-packed in polyethylene bags, stored at room temperature for 45 days before opening for sampling. Laboratory detection methods and MiSeq high-throughput sequencing technology were employed to analyze changes in corn stalk quality and microbial community structure before and after ensiling. The results showed: 1) After 45 days of silage fermentation, the corn stalk silage exhibited a yellow-green color, good texture, and acidic aroma. Silage fermentation rapidly reduced the pH of corn stalks ($P < 0.05$), significantly increased lactic acid content ($P < 0.05$), and showed a decreasing trend in neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents ($P > 0.05$). 2) Following silage fermentation, the abundance of Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, and Weissella was significantly decreased ($P < 0.05$); silage fermentation significantly increased the abundance of Firmicutes, Bacilli, Lactobacillales, Lactobacillaceae, Pediococcus, and Lactobacillus ($P < 0.05$). 3) While improving the sensory characteristics, fermentation quality, and nutritional composition of corn stalks, silage fermentation effectively reduced the number of harmful bacteria and increased the number of beneficial bacteria, thereby decreasing the potential risk of pathogenic bacteria to livestock health. In summary, the combination of laboratory detection methods and MiSeq high-throughput sequencing technology can provide information on the entire microbial community composition and abundance changes before and after ensiling while analyzing silage quality, thereby offering a basis for the regulation of the fermentation process.

Full Text

Effects of Ensiling on Quality and Bacterial Community Composition of Corn Stalk

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Abstract

This study investigated the effects of ensiling on sensory indicators, fermentation quality, nutritional composition, and bacterial community composition and abundance at the phylum, class, order, family, and genus levels in corn stalk silage. Corn stalks at the dough stage, still green after ear removal, were chopped to 1-2 cm length and adjusted to 65-70% moisture. Samples were collected before ensiling (3 replicates) and after ensiling (3 replicates), vacuum-packed in polyethylene bags, stored at room temperature for 45 days, then sampled. Laboratory testing methods and MiSeq high-throughput sequencing were used to analyze changes in silage quality and bacterial community structure. The results showed: (1) After 45 days of ensiling, corn stalk silage exhibited yellow-green color, good texture, and acidic aroma. Ensiling rapidly decreased pH ($P < 0.05$) and significantly increased lactic acid content ($P < 0.05$), while neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents showed downward trends ($P > 0.05$). (2) After ensiling, the abundances of Proteobacteria (phylum), Gammaproteobacteria (class), Enterobacteriales (order), Enterobacteriaceae (family), and Weissella (genus) were significantly reduced ($P < 0.05$), while the abundances of Firmicutes (phylum), Bacilli (class), Lactobacillales (order), Lactobacillaceae (family), Pediococcus (genus), and Lactobacillus (genus) were significantly increased ($P < 0.05$). (3) Ensiling improved sensory indicators, fermentation quality, and nutritional composition while effectively reducing harmful bacteria and increasing beneficial bacteria, thereby decreasing potential health risks to livestock from pathogenic bacteria. In conclusion, combining laboratory testing methods with MiSeq high-throughput sequencing provides comprehensive information on silage quality and changes in bacterial community composition and abundance, offering a basis for fermentation process regulation.

Keywords: MiSeq; corn stalk; ensiling; quality; bacteria

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Introduction

Silage is often considered a product of lactic acid bacteria fermentation, but it is actually an extremely complex microbial symbiotic system. Numerous microorganisms participate in the ensiling process, including microbes attached to raw materials, those causing fermentation, and those causing spoilage [1]. During ensiling, bacterial community composition and abundance continuously change, with a few dominant populations such as lactic acid bacteria playing a decisive role [2]. To regulate the ensiling process, promote growth of beneficial bacteria, rapidly stabilize silage, inhibit harmful bacteria, reduce losses of dry matter, protein, and soluble carbohydrates, and improve fermentation quality, it is essential to understand microbial activity patterns, particularly those of key bacterial groups before and after ensiling [3].

Recent research on microbial species and abundance during ensiling has been a hot topic both domestically and internationally, focusing on: (1) dynamic changes of microorganisms at different ensiling stages, such as lactic acid bacteria, E. coli, molds, and yeasts [1-2,4]; (2) effects of different additives, raw materials, and conditions on microbial communities [5-6]. China has abundant corn stalk resources, and corn stalk silage is widely used. After ensiling, organic matter digestibility increases by 4.8-15.4%, digestible energy increases by 0.23-0.62 MJ, and metabolizable energy increases by 0.19-0.56 MJ [5-6]. However, most studies have focused on silage quality, with limited research on microbial community changes during fermentation and correlations between quality and bacterial communities.

Traditional methods for studying silage microorganisms include plate culture, microplate analysis, phospholipid fatty acid analysis, and molecular biology techniques [DGGE/TGGE/TTGE, T-RFLP, SSCP, FISH, blot hybridization, quantitative PCR, gene chips], which only reveal information about a few dominant bacteria and cannot provide absolute quantification or comprehensive evolutionary relationships [7-8]. The second-generation MiSeq high-throughput sequencing platform uses sequencing-by-synthesis technology, enabling parallel sequencing of hundreds of thousands to millions of DNA molecules with improved speed and throughput. It can determine microbial composition and abundance at all taxonomic levels (phylum to genus) with high sequencing depth, facilitating identification of low-abundance species. It has become the preferred method for studying bacterial and fungal community diversity [7-8] and is widely recognized by scholars.

However, few studies have used MiSeq high-throughput whole-genome sequencing to investigate bacterial communities in corn stalk silage. This study systematically analyzed corn stalk quality and bacterial communities before and

after ensiling to elucidate effects of fermentation on quality and bacterial communities and their relationships, providing a theoretical basis for developing microbial inoculants and biologically regulating the ensiling process.

Materials and Methods

1.1 Experimental Materials

Corn stalks (variety: Sanbei 21) were harvested in Baoding, Hebei Province, on September 30, 2014, at the dough stage when some leaves remained green after ear removal.

1.2 Experimental Design

Two groups were established: pre-ensiling and post-ensiling, each with three replicates. Sample numbers are shown in Table 1 . After harvesting, corn stalks were chopped to 1–2 cm using a silage chopper. Distilled water was sprayed evenly to adjust moisture to 65–70%. Raw material samples were collected, and additional samples were packed in polyethylene bags (24 cm × 40 cm, 1 kg per bag), vacuum-sealed using a DZ-280/2SD vacuum packager. Raw material samples were stored at -20°C in an ice box, while silage samples were stored at room temperature (25–37°C) for 45 days before opening and immediately stored at -80°C for analysis.

1.3 Methods

1.3.1 Sensory Evaluation Comprehensive evaluation was conducted according to the German Agricultural Association (DLG) sensory scoring standards and grading methods [9–10].

1.3.2 Determination of Fermentation Quality and Nutritional Composition Twenty grams of corn stalk samples (before and after ensiling) were mixed with 180 mL distilled water, homogenized for 1 min, and filtered through four layers of gauze and qualitative filter paper. The pH of the extract was measured using a pH meter [11]. Ammonia nitrogen (NH3-N) content was determined by the phenol-hypochlorite colorimetric method [12]. Dry matter (DM) content was measured by oven drying. Crude protein (CP) and total nitrogen (TN) were determined by the Kjeldahl method [13]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were measured by the Van Soest method [13]. Water-soluble carbohydrates (WSC) were determined by anthrone spectrophotometry [14]. Lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA) were analyzed using a SHIMADZE-10A high-performance liquid chromatograph with a Shodex Rspak KC-811 S-DVB gel column (30 mm × 8 mm), SPD-M10AVP detector, 3 mmol/L perchloric acid mobile phase at 1 mL/min flow rate, column temperature 50°C, detection wavelength 210 nm, and injection volume 5 L [10].

1.3.3 Determination of Microbial Species and Abundance 1.3.3.1 DNA Extraction

DNA was extracted from raw materials and silage samples using the sodium dodecyl sulfate (SDS) method [15]. Briefly, 0.5 g sample was mixed with 3 mL phosphate buffer saline (PBS) (8 g NaCl, 0.2 g KCl, 2.8 g Na2HPO4·12H2O, 0.2 g KH2PO4, pH 7.0–7.4), vortexed for 15 min, and centrifuged at 1,790×g for 5 min. The supernatant was discarded, and the pellet was washed twice with 3 mL PBS. One milliliter extraction buffer (50 mmol/L Tris-HCl, 5 mmol/L EDTA, 3% SDS, pH 8.0) was added, centrifuged at 16,000×g for 5 min at 4°C, and the supernatant was mixed with an equal volume of isopropanol for precipitation at –20°C for 1 h. After centrifugation at 12,000 r/min for 20 min at 4°C, the pellet was washed with 70% ethanol, dried for 5 min, and resuspended in 30 L distilled water. DNA purity and concentration were checked by agarose gel electrophoresis, and samples were diluted to 1 ng/ L with sterile water.

1.3.3.2 Quantitative PCR Detection

PCR amplification: The V4 region of the 16S rRNA gene was amplified using primers 515F (5’ -GTGCCAGCMGCCGCGG-3’) and 806R (5’ -GGACTACHVGGGTWTCTAAT-3’) [16]. The 30 L reaction mixture contained 15 L Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 mol/L each primer, and 10 ng DNA template [17]. PCR conditions: 98°C for 1 min; 30 cycles of 98°C for 10 s, 50°C for 30 s, 72°C for 60 s; final extension at 72°C for 5 min.

PCR product pooling and purification: PCR products were detected by 2% agarose gel electrophoresis. Equal concentrations of PCR products were pooled, mixed thoroughly, and purified using the Thermo Scientific Gene JET Gel Extraction Kit [18].

1.3.3.3 Library Construction and Sequencing

Conducted by Beijing Novogene Bioinformatics Technology Co., Ltd. Libraries were constructed using the NEB Next® Ultra™ DNA Library Prep Kit for Illumina, quantified by Qubit, and sequenced using MiSeq high-throughput sequencing technology [7–8].

1.3.3.4 Bioinformatics Analysis

Sequences with >97% similarity were defined as the same operational taxonomic unit (OTU) using CLUSTALW software. Raw data were filtered and assembled to obtain clean data. OTU clustering and taxonomic classification were performed, followed by abundance and diversity index analyses. Community structure was statistically analyzed at all taxonomic levels [8].

1.4 Statistical Analysis

Data were analyzed using SPSS 19.0 software. Dissimilarity coefficients among samples were analyzed by ANOVA. Bacterial abundance at phylum, class, order,

family, and genus levels, as well as fermentation quality and nutritional composition, were analyzed by independent samples t-test. $P < 0.05$ was considered statistically significant.

Results

2.1 Sensory Evaluation of Corn Stalk Silage

After 45 days of ensiling, all three replicates of corn stalk silage exhibited yellow-green color with no significant differences in appearance, no mold, good texture, no stickiness, and acidic aroma. All samples achieved excellent quality grades according to sensory evaluation.

2.2 Fermentation Quality and Nutritional Composition Before and After Ensiling

As shown in Table 2 , ensiling rapidly decreased pH ($P < 0.05$) and significantly increased lactic acid content ($P < 0.05$). NDF, ADF, and CP contents showed downward trends ($P > 0.05$), while DM content did not differ significantly ($P > 0.05$). Ensiling also consumed WSC in corn stalks.

2.3 Microbial Community Analysis

2.3.1 Sequencing Quality After filtering, a total of 490,251 tags were obtained from six samples, with 476,217 taxon tags (97.14% effective tags). At >97% similarity, 7,258 OTUs were detected. As shown in Figure 1 [Figure 1: see original paper], rarefaction curves plateaued, indicating adequate sequencing depth. Bacterial diversity was higher in pre-ensiling samples than post-ensiling samples at the same sequencing depth, suggesting that ensiling reduces bacterial diversity.

2.3.2 Species Diversity and Similarity Analysis As shown in Figure 2 [Figure 2: see original paper], pre- and post-ensiling samples shared 575 OTUs, with 832 unique OTUs in pre-ensiling and 161 unique OTUs in post-ensiling samples, indicating both specificity and similarity in bacterial composition. Table 3 shows small within-group dissimilarity coefficients but large between-group coefficients, demonstrating good sample repeatability and significant differences in bacterial diversity between pre- and post-ensiling samples.

2.3.3 Dominant Bacterial Groups at Different Taxonomic Levels Phylum Level

As shown in Table 4 , Proteobacteria and Firmicutes dominated pre-ensiling corn stalks. After 45 days, Proteobacteria significantly decreased ($P < 0.05$) from 78.08% to 50.90%, while Firmicutes significantly increased ($P < 0.05$) from 18.76% to 44.06%.

Class Level

As shown in Table 5 , Gammaproteobacteria, Bacilli, and Alphaproteobacteria were the top three classes in pre-ensiling samples. After 45 days, Gammaproteobacteria significantly decreased ($P < 0.05$) from 67.72% to 35.66%, Bacilli significantly increased ($P < 0.05$) from 18.68% to 43.94%, and Alphaproteobacteria significantly increased ($P < 0.05$) from 8.98% to 13.39%.

Order Level

As shown in Table 6 , Enterobacteriales and Lactobacillales dominated pre-ensiling samples. After 45 days, Enterobacteriales significantly decreased ($P < 0.05$), while Lactobacillales significantly increased ($P < 0.05$).

Family Level

As shown in Table 7 , the top three families in pre-ensiling samples were Enterobacteriaceae, Leuconostocaceae, and Pseudomonadaceae. After 45 days, the top three were Lactobacillaceae, Enterobacteriaceae, and Leuconostocaceae. Enterobacteriaceae and Leuconostocaceae significantly decreased ($P < 0.05$), while Lactobacillaceae significantly increased ($P < 0.05$).

Genus Level

As shown in Table 8 , the top three genera in pre-ensiling samples were Weissella (16.92%), Sphingomonas (2.26%), and Swaminathania (2.22%). After 45 days, the top three were Pediococcus (22.65%), Weissella (9.98%), and Lactobacillus (9.74%). Pediococcus and Lactobacillus significantly increased ($P < 0.05$), while Weissella significantly decreased ($P < 0.05$).

Discussion

China is one of the world' s largest producers of crop straw, with theoretical resources of 840 million tons in 2010, of which approximately 700 million tons were collectible. Corn stalk was the most abundant, accounting for 243 million tons (34.7% of collectible straw resources) [19]. Previous studies reported that ensiled corn stalk maintains fresh green feed status with sweet-sour aroma, increases crude protein by 3.3–16.4%, and decreases crude fiber by 5–15% compared to raw corn stalk [20–21]. Our study found that 45-day ensiled corn stalk silage exhibited yellow-green color, no mold, good texture, no stickiness, and acidic aroma, consistent with previous reports. NDF and ADF contents showed downward trends, also in agreement with literature. However, CP content showed a downward trend in our study, contrary to previous findings, possibly because NH3-N increased from 14.98 to 61.19 g/kg TN, consuming some crude protein.

MiSeq sequencing revealed that post-ensiling bacterial species were only 52.31% of pre-ensiling levels, with shared species accounting for only 40.87% of pre-ensiling species. Ensiling alters community structure and abundance, likely because the anaerobic environment created by vacuum sealing and substrates like soluble carbohydrates promote rapid proliferation of beneficial bacteria, decreasing pH and affecting bacterial community composition [22]. The simultaneous

changes in quality and bacterial composition suggest strong correlations.

The two dominant phyla in corn stalk were Firmicutes and Proteobacteria. After ensiling, Firmicutes increased from 18.76% to 44.06%. Firmicutes are low-GC Gram-positive bacteria including spore-forming, non-spore-forming, and mycoplasma groups. Many Bacillus species can degrade macromolecules like cellulose, starch, and protein [2]. The decreased NDF, ADF, and CP contents may be partially attributed to Firmicutes activity. Proteobacteria are Gram-negative bacteria including many pathogens like E. coli, Salmonella, Vibrio, and Helicobacter [23]. Although enteric bacteria in silage are not considered pathogenic, they compete with lactic acid bacteria for carbohydrates, degrade protein, produce toxic biogenic amines affecting flavor, and hinder pH reduction. They can also convert nitrate to nitrite [24]. The significant decrease in Proteobacteria suggests they consumed some WSC and contributed to CP reduction and NH3-N increase. At class, order, and family levels, pathogen-containing Gammaproteobacteria, Enterobacteriales, and Enterobacteriaceae significantly decreased, while beneficial Bacilli, Lactobacillales, and Lactobacillaceae increased. This demonstrates that ensiling improves quality while reducing harmful bacteria and increasing beneficial bacteria, decreasing potential health risks to livestock.

Cai et al. [25] isolated 161 lactic acid bacteria strains from rice silage at various stages, identifying them as Lactococcus, Lactobacillus, Leuconostoc, Enterococcus, Pediococcus, and Weissella, with homofermentative lactic acid bacteria accounting for 66%. Dunière et al. [3] identified Paenibacillus, Flavobacteriaceae, Sphingomonas, Exiguobacterium, Rhizobium, Acinetobacter, and Buchnera as dominant in corn silage. In our study, the top 10 genera after ensiling were Pediococcus, Weissella, Lactobacillus, Sphingomonas, Stenotrophomonas, Swaminathania, Acinetobacter, Pseudomonas, Hymenobacter, and Erwinia. Differences may be due to detection methods, sample types, fermentation time, and conditions. Previous studies found Pediococcus plays an important role in early fermentation, followed by Streptococcus faecalis and Leuconostoc mesenteroides, then replaced by acid-tolerant strains like Lactobacillus plantarum, Lactobacillus brevis, and Lactobacillus buchneri [26]. Zhang et al. [27] reported Lactobacillus dominates in late fermentation, while Yang et al. [28] suggested Weissella is important in early fermentation but contributes little to quality improvement. Our study found Pediococcus increased from 1.05% to 22.65%, Weissella decreased from 16.92% to 9.98%, and Lactobacillus increased from 0.07% to 9.74% after 45 days, indicating Pediococcus and Lactobacillus proliferated rapidly and played important roles, while Weissella was more important in early fermentation, consistent with Yang et al. [28]. Compared with traditional methods, MiSeq sequencing provides comprehensive community composition, overcoming limitations of identifying only dominant bacteria and inability to absolutely quantify microorganisms. This study provides preliminary exploration of MiSeq technology for analyzing bacterial community structure and abundance in corn stalk silage. Further research is ongoing in our group to investigate effects of different treatments, varieties, and ensiling times.

Conclusion

1. After ensiling, corn stalk silage exhibited yellow-green color, good quality, and acidic aroma; pH rapidly decreased, lactic acid content significantly increased, and NDF and ADF contents showed downward trends.
2. Ensiling significantly decreased the abundances of Proteobacteria, Gammaproteobacteria, Enterobacteriales, Enterobacteriaceae, and Weissella, while significantly increasing Firmicutes, Bacilli, Lactobacillales, Lactobacillaceae, Pediococcus, and Lactobacillus.
3. Combining laboratory testing methods with MiSeq high-throughput sequencing technology can simultaneously analyze silage quality and provide comprehensive information on bacterial community composition and abundance changes, offering a basis for fermentation process regulation.

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Note: Figure translations are in progress. See original paper for figures.