Changes of the fermentation quality and microbial community during re-ensiling of sweet corn stalk silage

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ABSTRACT
In order to investigate the effects of re-ensiling on the fermentation quality and microbial community, the present work simulated the conditions of silage transfer and re-ensiling to check for possible effects of exposure to air on the fermentation quality and microbial communities of the sweet corn stalk (SCS) silage. The SCS harvested at the milky stage was ensiled for 90 d and 180 d, respectively. The silage ensiled for 90 d was opened and exposed to the air for 0, 6, 12, 24, 36 and 48 h, respectively, then re-ensiled for 90 d. The SCS silage directly ensiled for 180 d was the control. Results showed that the SCS silage ensiled for 90 d had good fermentation quality and kept well within 48 h aerobic exposure. There were no significant differences \((p > 0.05)\) in pH values, organic acids and \(NH_3-N\) contents between re-ensiled and control silages. Ensiling for 90 d and 180 d significantly decreased \((p < 0.05)\) the counts of microorganisms and increased the abundance of \(Lactobacillus\) and \(Bacillus\) in silage. In the re-ensiled silage ensiled after 48 h exposure, the relative abundance of \(Lactobacillus\) decreased, and that of undesirable bacteria like \(Paenibacillus\), \(Acetobacter\), \(Methylobacterium\), \(Clostridium\), \(Pleurocapsa\) and \(Bacillus\) increased, compared to the conventional silage. In summary, ensiling SCS was an effective approach to preserve nutrition, and re-ensiling within 48 h of aerobic exposure affected the bacteria community but did not affect the fermentation quality of SCS silage.

HIGHLIGHTS
- Sweet corn stalk (SCS) silage had good fermentation quality and kept well within 48 h aerobic exposure.
- Ensiling increased the relative abundance of desirable bacteria \(Lactobacillus\).
- Re-ensiling within 48 h exposure did not affect the fermentation quality of SCS silage.

Introduction
There has been an increasing demand for quality silage with the expansion of livestock industry in China and elsewhere (Doi et al. 2013). The production of sweet corn (\(Zea mays\) var. Rugosa) has recently experienced a sharp rise in China (NBSC 2020); however, most of sweet corn stalk (SCS) are left unused or being burnt directly in the field, which wastes resources and produces greenhouse gases and air pollutants (Warman and Havard 1998). SCS can be used as feed for livestock, and contain higher starch and water-soluble carbohydrate (WSC) contents compared to common corn stalk (Liu et al. 2013; Zhang et al. 2018). Utilisation of SCS as silage can provide a low-cost feed source for livestocks and reduce the environmental impact caused by burning the stalk (Tang et al. 2011).

Ensiling is a commonly used method for preservation of seasonally harvested crops, providing a stable supply of feed with good nutritional value for livestocks (McDonald et al. 1991). In the tropics and sub-tropics, achieving high fermentation quality of silage is difficult due to hot and humid climate and climatic variations during the rainy season, especially for small-scale farms that do not have sufficient manpower and machineries to make silage (Namihira et al. 2010), while nearly one fifth of the world population are smallholding livestock keepers (McDermott et al. 2010). For those farmers, instead of making silage themselves, the common practice is to purchase
quality silage from large-scale silage producers and compacted and sealed again for future use. However, the transferring of silage from the silage producer to those farms may expose silage to air for several hours to 1–2 d. Even for round bale silage, due to lacking labour during the crop harvesting, the harvested forage may need to be temporarily stored in the bunker silos for making round bales later on (Chen and Weinberg 2014). On the other hand, round bale silage may need to be opened and moved to bunker silos for preserving in some cases (Kawamoto et al. 2003). All the situations mentioned above are equivalent to re-ensiling, which are very common in countries like China, Brazil and Japan. During these re-ensiling processes, possible detrimental effects of silage exposure may occur, for example, the aerobic microorganisms may deteriorate the silage quality (Ashbell et al. 1990). Although it is a very common practice, there is very limited information available about re-ensiling, particularly relating to microbial community. dos Anjos et al. (2018) reported that re-ensiling did not significantly affect the fermentation quality of sorghum (Sorghum bicolor). Similar result was also obtained from common corn by de Lima et al. (2016). However, these studies have not investigated why re-ensiling did not affect the fermentation quality from microorganism perspectives. High-throughput sequencing can detect the microbial community in sample materials, including bacteria like lactic acid bacteria (LAB), enterobacteria, clostridia, bacilli, acetic acid bacteria and other microorganisms (yeasts and moulds). This technique has been applied to silage (Wang et al. 2019b). Yin et al. (2021) found unwilted napier grass (Pennisetum purpureum) silage contained higher relative abundance of Paenibacillus, and the fermentation quality of unwilted napier grass silage re-ensiled after over 24 h of aerobic exposure significantly decreased. Moreover, re-ensiling increased relative abundance of Lactobacillus, and decreased relative abundance of Klebsiella, Bacillus and Paenibacillus. However, SCS has more nutrition than sorghum and napier grass, which might promote the growth of aerobic microorganisms involved in silage deterioration (dos Anjos et al. 2018; Yin et al. 2021). Additionally, the studies on the fermentation quality and microbial community of re-ensiled silage are rare.

The main purpose of this study was to evaluate the effects of re-ensiling on the fermentation quality and bacterial community of SCS silage. The insights obtained from this study could provide practical guidelines for re-ensiling and prevent potential deterioration of silage quality during re-ensiling.

Materials and methods

Planting, harvesting and ensiling

Sweet corn was planted on the experimental field of South China Agricultural University (Guangzhou, China), which is located at 23°N and 113°E with a typical subtropical climate. SCS was collected after ears were removed at the milky stage, and then chopped into 2–3 cm in length, mixed and filled into six 5-L plastic laboratory silos. The six silos were filled at an initial density of 700 kg/m³ FM (230 kg/m³ DM) and were stored at the ambient temperature (21.0–37.0°C). Three silos were opened after stored for 90 d (S90) and silo caps were replaced with two layers of clean cheesecloth for aerobic exposure. During the aerobic exposure period, the air temperature varied from 22.6 to 27.2°C. After exposure for 0, 6, 12, 24, 36 and 48 h, the exposed silages were then sampled and re-ensiled using plastic film bags (200 × 300 mm) in three replications. The exposure periods were designed based upon the fact that in most cases re-ensiling time required 1–2 d (Chen and Weinberg 2014). Each bag was filled with 300 g silage, degassed, sealed using a vacuum sealer (Mingkang Packing Co., Ltd., Zhongshan, China) and stored for 90 d. The exposed silages were then sampled and re-ensiled using plastic film bags (200 × 300 mm) in three replications. The exposure periods were designed based upon the fact that in most cases re-ensiling time required 1–2 d (Chen and Weinberg 2014). Each bag was filled with 300 g silage, degassed, sealed using a vacuum sealer (Mingkang Packing Co., Ltd., Zhongshan, China) and stored for 90 d. The silage re-ensiled after exposed to air for 48 h was RS90. The other three silos were not opened and continued to be stored for further 90 d as the control (S180) (Figure 1). All samples were taken and analysed for fermentation quality. The F, S90, RS90 and S180 samples were analysed for 16S rRNA gene amplicons sequencing.
Analysis of microbial population and chemical composition

Ten grams of fresh material were shaken well with 90 mL of sterilised water, and diluted to solutions with concentration from $10^{-1}$ to $10^{-5}$ using sterilised water. Lactic acid bacteria were counted on de Man, Rogosa and Sharp (MRS) medium agar (Huan Kai Microbiology Co., Ltd., Guangzhou, China) after incubation in an anaerobic incubator at 37°C for 2 d. Aerobic bacteria were counted on nutrient agar, while yeasts and moulds were counted on potato dextrose agar acidified with sterilised tartaric acid solution (to pH 3.5) after the agar plates being incubated at 37°C for 3 d.

Twenty grams of samples were shaken well with 80 mL of distilled water, and stored in a refrigerator at 4°C for 18 h. The filtered material was then used for the measurements of pH and buffering capacity. The pH value was measured by a glass electrode pH metre. The buffering capacity was determined using the method described by Playne and McDonald (1966).

About 200 g fresh matter (FM) were measured for dry matter (DM) content by oven drying at 70°C for 48 h (Zhang et al. 2011) and then ground to pass a 1.0 mm screen by a laboratory knife mill. Crude protein and ether extract were analysed following the methods of AOAC (1990). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and crude fibre contents were measured following the procedures described by van Soest et al. (1991). WSC content was determined using the anthrone method (Murphy 1958).

Analyses of fermentation quality

The pH value of fermented materials was measured using the same method for measuring the fresh material. The material filtrate was used for the measurements of ammonia nitrogen (NH₃-N) and organic acids contents. The NH₃-N content was determined by colorimetry (Broderick and Kang 1980), and the organic acids (lactic acid, acetic acid, propionic acid and butyric acid) contents were measured by the HPLC method (column: Shodex Ionpak KC-811, Showa Denko K.K., Kawasaki, Akashi, Japan; detector: DAD, 210 nm, SPD-20A, Shimadzu Co., Ltd., Kyoto, Japan; eluent: 0.1% HP₃O₅/H₂O, 1.0 mL/min; temperature: 40°C).

DNA extraction of the microbial community

Twenty grams of fresh SCS and silages were homogenised with 80 mL distilled water, filtered through two layers of medical gauze and centrifuged for 15 min at 10,000 × g at 4°C. Triplicate samples of centrifuge production were dissolved and blended together. DNA was then extracted using the MOBIO PowerSoil™ DNA Isolation Kit for bacteria (MOBIO Laboratories, Carlsbad, CA). The DNA concentration and purity were measured using the NanoDrop One (Thermo Fisher Scientific, Waltham, MA). Both Primers 515F (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGGGTWTCTAAT) with 12 bps barcodes were used to amplify the V3–V4 hyper-variable region of the bacterial 16S rRNA gene. 16S rRNA genes of distinct regions were amplified using specific primer with 12 bps barcodes. Primers were synthesised by Invitrogen (Invitrogen, Carlsbad, CA). The PCR products were then detected by 1% agarose gel electrophoresis (BioRad S1000, Bio-Rad Laboratory, Hercules, CA) and mixed in equidensity ratios according to the Gene Tools Analysis Software (Version4.0 3.0 5.0, Syn Gene, Bangalore, India). The mixed PCR products were purified with EZNA Gel Extraction Kit (Omega, Doraville, GA). Sequencing on an Illumina Hiseq 2500 platform and 250 bps paired-end reads were generated (Guangdong Magigene Biotechnology Co., Ltd., Guangzhou, China).

Statistical analyses

The data of silage fermentation were analysed by one-way analysis of variance to evaluate the effects of ensiling, re-ensiling and exposure time on the fermentation quality and microorganism compositions. The means were then compared for significance by Duncan’s multiple range method. All statistical procedures were performed using the statistical packages for the social sciences (SPSS 19.0 for Windows; SPSS Inc., Chicago, IL).

Results

Chemical characteristics of fresh SCS before ensiling

The chemical composition and microbial population of the fresh SCS before ensiling are shown in Table 1. The DM content of SCS was 33.12%. The crude protein and WSC contents exceeded 8% and 13% DM, respectively. The crude fibre and ADF contents were below 40% DM. The buffering capacity was less than
100 mE/kg DM. In addition, aerobic bacteria count approaching 8 log_{10} cfu/g FM was more than LAB, yeasts and moulds counts detected in fresh SCS.

**Fermentation quality of silage and re-ensiled silage**

As shown in Table 2, the SCS silage ensiled for 90 d (exposure for 0 h) had low pH (3.82), high lactic acid content (10.71% DM) and low volatile fatty acid content. The pH values and organic acid contents had no significant differences (p > 0.05) among the silages with different exposure time, while the NH₃-N content increased gradually with exposure time. The LAB and aerobic bacteria counts were 4.5–5.5 log_{10} cfu/g FM, while yeasts counts were below 2 log_{10} cfu/g FM in the silages at different exposure time.

As shown in Table 3, the fermentation quality did not significantly differ between re-ensiled and control silages, indicated by the equal pH value, organic acids and NH₃-N contents. However, LAB and aerobic bacteria counts of re-ensiled silages were significantly lower (p < 0.05) than those of the control silage.

**Microbial diversity of fresh stalk and silage**

A total of 501,876 valid reads were obtained from the four merged samples (F: fresh SCS; S90: silage ensiled for 90 d; RS90: silage re-ensiled after exposure to the air for 48 h; S180: silage directly ensiled for 180 d), with the lowest reads of 122,094 obtained from the F sample and the highest reads of 129,819 obtained from the RS90 sample. The coverage of all samples was approximately 0.99, indicating the data of samples were large to represent all of the bacterial communities in the materials. The Chao 1, Shannon and OTUs index of S90 or RS90 increased but that of S180 decreased slightly compared to fresh SCS, indicating that the bacterial diversity varied considerably, which might have been caused by aerobic exposure and fermentation periods (Table 4).

As shown in Figure 2, the phylum of fresh SCS and S180 was dominated by Proteobacteria (91.45% and 72.45%), and S90 and RS90 were dominated by Firmicutes (55.15% and 48.69%). The family of fresh SCS and S180 was dominated by Enterobacteriaceae (58.04% and 64.54%), and S90 and RS90 were dominated by Lactobacillaceae (21.37%) and Lachnospiraceae (14.61%), respectively. The relative abundance of bacterial communities at genus level in SCS before and after ensiling is shown in Figures 3 and 4. The genera in fresh SCS, S90, RS90 and S180 were dominated by *Methylobacterium* (3.05%), *Lactobacillus* (22.03%), *Paenibacillus* (12.30%) and *Bacillus* (8.22%), respectively (Figure 3).

**Discussion**

**Chemical composition of the sweet corn stalk silage**

The crude protein (8.88% DM) and WSC (13.26% DM) contents of SCS in the present study were lower than those of fresh corn stalk. The chemical composition and microbial population of sweet corn stalk prior to ensiling is shown in Table 1.

**Table 1. Chemical composition and microbial population of sweet corn stalk prior to ensiling.**

| Parameter                        | Value                  |
|----------------------------------|------------------------|
| Dry matter, % FM                 | 33.12                  |
| Crude protein, % DM              | 8.88                   |
| Ether extract, % DM              | 2.83                   |
| Crude fibre, % DM                | 38.05                  |
| Neutral detergent fibre, % DM    | 61.69                  |
| Acid detergent fibre, % DM       | 32.70                  |
| Water soluble carbohydrates, % DM| 13.26                  |
| Buffering capacity, mE/kg DM     | 94.94                  |
| pH                               | 5.51                   |
| Lactic acid bacteria, log_{10} cfu/g FM | 5.12      |
| Aerobic bacteria, log_{10} cfu/g FM | 7.99      |
| Yeasts, log_{10} cfu/g FM        | 4.87                   |
| Moulds, log_{10} cfu/g FM        | 4.63                   |

FM: fresh matter; DM: dry matter; cfu: colony forming units.

**Table 2. Fermentation quality and microbial population of sweet corn stalk silages exposed to air for different time.**

| Items                     | Exposure time, h | SEM (n = 3) | Significant |
|---------------------------|------------------|-------------|-------------|
| pH                        | 3.82             | 0.000       | ns          |
| Lactic acid, % DM         | 10.71            | 0.451       | ns          |
| Acetic acid, % DM         | 1.58             | 0.014       | ns          |
| Propionic acid, % DM      | 0.04             | 0.001       | ns          |
| Butyric acid, % DM        | 0.56             | 0.011       | ns          |
| NH₃-N, % TN               | 5.49 d           | 0.563       | **          |
| Lactic acid bacteria, log_{10} cfu/g FM | 4.56 e       |
| Aerobic bacteria, log_{10} cfu/g FM | 4.89 b      |
| Yeasts, log_{10} cfu/g FM | <2               | 0.231       | **          |
| Moulds, log_{10} cfu/g FM | 2.57 a           | 0.330       | **          |

1DM: dry matter; FM: fresh matter; TN: total nitrogen; NH₃-N: ammonia nitrogen; cfu: colony forming units.
2SEM: standard error of means.
3ns: no significant at 0.05.
**Significant at 0.01.
Means with different superscripts (a–d) in each row differ significantly (p < 0.05).
those (11.20% DM and 14.43% DM) in the report of Wang et al. (2019b). These differences in chemical compositions might be caused by different harvest time (Cui et al. 2011) and varieties (van Soest et al. 1978). Cui et al. (2011) reported that SCS had the best nutrient quality when harvested within 6 d after ear harvest in winter and within 9 d in summer, while the collected time of SCS in this study was later than above mentioned time. The WSC content was approximately twice of 6–8% DM, which is considered as the theoretical requirement to obtain well preserved silage (Smith 1962). The count of LAB in fresh material reached to 10^5 cfu/g, which is considered to be at effective amount for silage fermentation (McDonald et al. 1991). High population of the undesirable aerobic bacteria, yeasts and moulds in the SCS were similar with the results reported by Liu et al. (2013).

Fermentation quality and microbial population of silage and re-ensiled silage

The pH value and acetic acid content were similar to SCS reported by Zhou et al. (2019), but the lactic acid content of SCS silage in this study was higher. The pH value and organic acid contents of SCS silage ensiled for 90 d are consistent with the results of corn silage obtained by de Lima et al. (2016). In addition, Zhang et al. (2018) found that lactic acid content of corn stalk was about 5% of DM and pH value was 4.3, and silage deteriorated gradually after initial exposure (0–48 h). However, there were no significant changes in chemical and microbial compositions of SCS silages exposed for 0–48 h. This might be attributed to lacking of yeasts in SCS silages, which is responsible for the aerobic deterioration of most silages (Pitt et al. 1991). The NH3-N content (approximately 5–11% TN) in this study was higher than that of SCS silage reported by Wang et al. (2019b), and thereafter increased with exposure time, indicating that more proteolysis occurred. This might be because several aerobic bacteria like Enterobacter competed with LAB for the substrate and metabolised protein into NH3-N (McDonald et al. 1991). Indeed, Clostridium, resulting in proteolysis (Kung et al. 2018), were detected in these silages (Figure 3). The re-ensiled SCS silages were well preserved, which was consistent about the results of corn silage (de Lima et al. 2016). This might be attributed to less aerobic microorganisms in the silages before re-ensiling (Tabacco et al. 2009).

Microbial diversity of fresh stalk and silage

In general, the complex microbial communities of the raw materials are gradually replaced by LAB after anaerobic fermentation, the microbial diversity will sharply reduce after successful fermentation.
In the present study, the relative abundance of *Lactobacillus* increased from 1.69% to 22.03% after SCS was ensiled for 90 d, whereas the Chao 1, Shannon and OTUs indexes indicating bacterial diversity increased (Table 4). This phenomenon is difficult to explain, but similar results were also reported in the ensiling experiments of corn (Guan et al. 2018) and mixture of corn stalk and Italian ryegrass (Yan et al. 2019). The bacterial diversity of RS90 decreased compared to S90, but was still higher than that of fresh SCS.

(Aerobic microorganisms (like *Enterobacteriaceae*) grow and consume oxygen during the early stage of fermentation, and then anaerobes start to grow. LAB become dominant in the silage and play an important role in inhibiting the growth of harmful microorganisms through producing lactic acid and reducing the silage pH, so that the nutrients of the forage can be preserved (Dunière et al. 2013). In this study, the most dominant phylum in fresh SCS was Proteobacteria. Differently, for the S90 or RS90, Firmicutes was the most dominant phylum. This is because LAB belong to Firmicutes. At genus level, *Methylobacterium* was the most abundant bacteria in fresh SCS, followed by *Pantoea*. It was reported that *Methylobacterium*, strictly aerobic bacteria, was commonly found at >5% of the total population in fresh whole crop corn and Italian ryegrass, and was also dominant genera in fresh alfalfa.
The relative abundance of desirable bacteria *Lactobacillus* significantly increased and became the dominant microbes in S90, but the relative abundance of other undesirable bacteria like *Bacillus*, *Pantoea*, *Paenibacillus*, *Methylobacterium*, *Clostridium*, *Streptomyces* and *Achromobacter* did not decrease. The presence or increase of these bacteria in the silage is undesirable as they may compete with LAB for nutrients, and several bacteria like *Pantoea* and *Clostridium* produce high level of alcohol and NH$_3$-N during ensiling (Queiroz et al. 2018; Wang et al. 2019a; Avila and Carvalho 2020). This might be the reason for the high NH$_3$-N content of SCS silage in this study.

There were higher relative abundance of undesirable bacteria (*Paenibacillus*, *Acetobacter*, *Methylobacterium*, *Clostridium* and *Anaerocolumna*) in RS90 than in S180 (Figure 3), indicating re-ensiling affected the bacterial community due to air exposure. This might be that air exposure at re-ensiling promoted the growth of obligate aerobic bacteria like *Acetobacter* or facultative anaerobic organisms like *Paenibacillus* (Pahlow et al. 2003). *Paenibacillus* grow in large numbers during the aerobic exposure, causing the silage to be more prone to aerobic deterioration (Jobim et al. 2007). Yin et al. (2021) reported the fermentation quality of napier grass silage re-ensiled after over 24 h of aerobic exposure significantly decreased, owing to more *Paenibacillus*. *Acetobacter*, obligate aerobic bacteria, is commonly found in corn silage and can initiate the aerobic deterioration process, and usually simultaneously with yeasts (Spoelstra et al. 1988). Generally, *Clostridium* spp. are strictly anaerobic and prefer high moisture. The phenomenon that more *Clostridium* was observed in RS90 might be from the sharply decrease in relative abundance of some genera like *Lactobacillus* and *Pantoea* in RS90. However, the data of microbial population showed that re-ensiling reduced the counts of aerobic bacteria compared to S180 (Table 3), which seemed to be contrary to the result of re-ensiled silage with higher abundance of undesirable bacteria. This might be because several uncultured bacteria like some species in *Acetobacter* and *Bacillus* (Zouache et al. 2009) were not counted. Besides, S90, S180 and RS90 had different microbial communities but had similar fermentation quality. This might be because the activities of most microorganisms (except for spore-forming bacteria) were inhibited and the fermentation quality tended to be stable in the later stage of ensiling. The reason that RS90 had similar fermentation quality to S90 and S180 might be attributed to limited exposure time and low yeasts counts. Therefore, it is necessary to further study the effect of re-ensiling on the microbial diversity (especially yeasts) and aerobic stability of re-ensiled silage, and probiotics might be added to inhibit the growth of undesirable microorganisms in re-ensiled silage (dos Anjos et al. 2018).
Conclusions

This study revealed that SCS silage had good fermentation quality and kept well within 48 h aerobic exposure. Re-ensiling had no significant effect on the fermentation quality. Ensiling for 90 d increased the relative abundance of *Lactobacillus* and *Bacillus*. Re-ensiling within 48 h of aerobic exposure decreased the relative abundance of *Lactobacillus*, and mainly increased that of *Paenibacillus*, *Acetobacter* and *Methylobacterium* compared to the conventional silage. These results indicated that re-ensiling SCS silage within 48 h was feasible.

Ethical approval

All research reported in this research has been conducted in an ethical and responsible manner, and is in full compliance with all relevant codes of experimentation and legislation.

Disclosure statement

The authors have no conflict of interest to this work. The authors are responsible for the content and writing of this article.

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Data availability statement

All data generated or analysed during this study are included in this published article. The raw sequence data reported in this study are available from the corresponding author, Jianguo Zhang.

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