Influence of Various Additives On The Fermentation Quality And Bacterial Community of High-Moisture Whole-Plant Quinoa Silage

Zhihao Dong
Nanjing Agricultural University https://orcid.org/0000-0001-6532-4784
Junfeng Li
Nanjing Agricultural University
Siran Wang
Nanjing Agricultural University
Jie Zhao
Nanjing Agricultural University
Dong Dong
Nanjing Agricultural University
Tao Shao (taoshaolan@163.com)
Nanjing Agricultural University https://orcid.org/0000-0002-3218-046X

Research Article

Keywords: Additive, Bacterial community, Fermentation quality, Whole-plant quinoa silage.

Posted Date: January 28th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1231549/v1

Abstract

To explore the potential of whole-plant quinoa (WPQ) as a high-protein source for livestock feed, this study evaluated the effects of additives on the fermentation quality and bacterial community of high-moisture WPQ silage. High-moisture WPQ was ensiled either untreated (control) or treated with cellulase (E), molasses (M), LAB inoculant (L), a combination of cellulase and LAB inoculant (EL), and a combination of molasses and LAB inoculant (ML). The fermentation quality and bacterial community after 60 days of ensiling were analyzed. Naturally fermented WPQ exhibited acetic acid-type fermentation dominated by enterobacteria, with low lactic acid content (21.1 g/kg DM), and high pH value (6.43) and NH_3-N production (182 g/kg TN). Adding molasses alone or combined with LAB inoculant shifted the fermentation patterns toward increased intensity of lactic acid fermentation, lowering the pH value (<4.60), NH_3-N content (<130 g/kg TN) and total abundance of enterobacteria (<19%), and increasing the lactic acid content (>61.5 g/kg DM), lactic/acetic acid ratio (>1.42) and the relative abundance of *Lactobacillus* (>70.5%). The results suggested that the lack of fermentable sugar could be the main factor of restricting extensive lactic acid fermentation in WPQ silage. Supplementing fermentable sugar or co-ensiling with materials with high WSC content and low moisture content could be beneficial strategies for producing high-quality WPQ silage.

Introduction

Quinoa (*Chenopodium quinoa* Willd.) is native to the Andean region of South America and is a traditional food for the indigenous peoples. In most recent years, quinoa seed has received much attention because of its exceptional nutritional value and potential health benefits (Vega-Gálvez et al. 2010). Quinoa is rich in proteins, lipids, fibres, vitamins and minerals, and contains significant amounts of bioactive compounds such as phytosterols, phytoecdysteroids, bioactive peptides and phenolic compounds (Zhang et al. 2020). Besides high nutritional value, quinoa can survive under harsh conditions, withstanding a wide range of temperatures (-4～38℃) and pH (6.0~8.5), low rainfall (50 mm/year) and high salinity (40 ms/cm) (Liu et al. 2021). These characteristics allow this plant to grow well in many marginal conditions. The vegetative part of quinoa plant is rich in protein and has far more biomass accumulation compared with the grain (Basra et al. 2014). Therefore, whole-plant quinoa (WPQ) is believed to have great potential for feeding animals. In some developed regions of livestock husbandry, fresh WPQ has been used to feed animals (Liu et al. 2021). However, the WPQ biomass is largely accumulated in very short time during harvest. The main stem axis and high moisture content make quick drying of a large biomass of WPQ difficult, limiting its use as a dried forage. Based on economical and practical feasibility, ensiling could be the best option for WPQ preservation. This technique depends on epiphytic lactic acid bacteria (LAB) converting water soluble carbohydrates (WSC) to organic acids mainly lactic acid under anaerobic conditions, which creates an acid environment to inhibit spoilage organisms and achieves the goal of maintaining the original quality of the moist forage as much as possible. However, the WPQ is lower in WSC and extremely higher in moisture content compared with the traditional forage crops, such as Italian ryegrass and whole-crop maize (Li et al. 2013). These traits may

be the restriction factors for the success of ensiling under natural fermentation conditions. Hence, silage additives or appropriate measures are required at the time of WPQ ensiling.

Homofermentative LAB is the most common biological additive in silage preservation. Previous studies have shown that LAB inoculation successfully directed the fermentation, reduced the dry matter (DM) loss and improved the fermentation quality of alfalfa silage (Guo et al. 2018; Zheng et al. 2017). Chemical additives, such as cellulase and molasses, are also often used as fermentation stimulants to increase the amount of readily fermentable sugars for LAB. Adding cellulase produced more WSC and improved the organic matter digestibility of silage by the degradation of plant cell walls (Mu et al. 2020; Tian et al. 2014). Adding molasses to king grass silage enhanced the fermentation by promoting the lactic acid production during the early stages of ensiling (Li et al. 2014). However, the possible beneficial effects of additives on silage fermentation depend on the properties of the forage crops being ensiled (Dong et al. 2020). To our knowledge, few or no studies have identified the effects of the additives on WPQ silage. The information is valuable in terms of producing high-quality WPQ silage.

Silage fermentation is a dynamic process of microbial community succession and metabolite changes. Recent advances in culture-independent analyses, such as high-throughput sequencing technology, have enabled microbial communities to be defined with a degree of detail that is impossible using classical microbiology (Dong et al. 2019b). Understanding of the microbial communities involved in the ensiling process would provide an insight into approaches to improve the forage conservation (Tian et al. 2021; Zhao et al. 2021). Therefore, the objective of this study was to investigate the effects of different additives on the fermentation quality and bacterial community of high-moisture WPQ silage.

Materials And Methods

Plant Materials and silage preparation

Quinoa was grown at Xinyang Agricultural Experiment Station of Yancheng City (N33°52', E120°44', Yancheng, China). The quinoa was planted in three experimental blocks with same tillage, irrigation and fertilization practices. These experimental blocks were kept as replicates throughout the whole experiment. After nine weeks of growth, the entire plant was harvested at the early blooming stage 10 cm above ground level and then was chopped into a theoretical length of 1~2 cm using a forage cutter. The chopped WPQ from each experimental block was used for ensiling after 20 h of wilting on a plastic film placed at a ventilated lobby.

The wilted WPQ was divided into six groups. The six groups were randomly assigned to one of the following treatments: (1) control; (2) cellulase (E); (3) molasses (M); (4) LAB inoculant (L); (5) a combination of cellulase and LAB inoculant (EL); (6) a combination of molasses and LAB inoculant (ML). The application rate of molasses was 1% on fresh matter (FM). The cellulase was a mixture of cellulase and a hemicellulose (supplied by Oddfoni Biological Technology Co., Ltd., Nanjing, China) and applied at a rate of 0.05 mg/g FM as suggested by Chen et al. (2019). The LAB inoculant was supplied by Institute

of Grass Ensiling and Processing of Nanjing Agricultural University, mainly consisting of *Lactobacillus plantarum*. The application rate of LAB was 1.0×10^6 colony forming units (cfu)/g of FM, according to the manufacturer's specification. The additives were all diluted in deionized water and applied in liquid forms. The control silage was received with equal volume of deionized water. The treated WPQ (about 200 g) were packed into vacuum-sealing polyethylene plastic bags (20×30 cm) and heat-sealed after vacuum completed in the bag. A total of 18 bags (6 treatments× 3 replicates) were prepared and stored at room temperature (20~25℃). These bags were opened after 60 days of ensiling and sampled for further analysis.

Chemical and microbial analysis

The pre-ensiling and ensiled WPQ were sampled for chemical composition and microbial population analysis. Approximately 100g sample was oven-dried for 48 h at 60°C for DM measurement and ground to pass 1-mm screen with a laboratory pulverizer (FW100, Taisite Instrument Co., Ltd., Tianjin, China) for chemical composition analysis. Total nitrogen was measured with the method of the Association of Official Analytical Chemists (AOAC, 2000) and multiply 6.25 to obtain crude protein content. The water-soluble carbohydrates (WSC) content was measured by colorimetry after reaction with anthrone reagent (Dong et al. 2019a). The neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined by the procedures of Van Soest et al. (1991), with heat stable amylase and sodium sulphite being used for neutral detergent fibre procedure.

To determine ensiling traits of WPQ material and fermentation parameters of WPQ silage, about 35 grams of sample was blended with 60 mL distilled water and macerated for 24 h at 4 ºC. The extract was filtered through 2 layers of cheesecloth and a filter paper (Xinhua Co, China). The filtrate was used for pH, organic acids and ammonia nitrogen (NH_3-N) determinations. The pH was measured with a HANNA HI 2221 pH meter (Hanna Instruments Italia Srl, Villafranca Padovana, Italy). The NH_3-N was determined using the phenol-hypochlorite reaction method (Dong et al. 2019a). The buffering capacity of WPQ was determined according to the method of Liu et al. (2019). The organic acids (including lactic, acetic, propionic and butyric acids) and ethanol were quantified using an Agilent 1260 HPLC system equipped with a refractive index detector (Carbomix® H-NP5 column, 2.5mM H_2SO_4, 0.5 mL/min).

For microbial population analysis, ten grams of sample was thoroughly mixed with 90 mL of sterilized saline solution on a shaker at 120 rpm for 2 h. After that, one hundred microliters of solution were used and serially diluted with sterilized saline solution to 10^{-2}~10^{-5} for culture-medium plating. The LAB and Enterobacteriaceae were, respectively, counted on de Man, Rogosa, Sharpe and Violet Red Bile Glucose Agar mediums after anaerobically incubated at 37°C for 48 h. Aerobic bacteria were counted on nutrient agar under aerobic conditions at 37°C for 24 h. Yeasts were determined on potato dextrose agar under aerobic conditions at 30°C for 3 days. The remaining solution was filtered into a 50-mL centrifuge tube with 4 layers of medical gauze and stored at -80°C for DNA extraction.

Bacterial community analysis

The frozen solution for DNA extraction were thawed at 4°C and then centrifuged at 12,000×g for 30 min to obtain a pellet for subsequent DNA extraction. The DNA extraction was conducted using the FastDNA® SPIN Kit and the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA, U.S.) according to the manufacture's protocols. The quantity and quality of obtained DNA were determined by NanoDrop 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA). The universal primers 338F and 806R were used for the Polymerase Chain Reaction (PCR) amplification with the target of V3-V4 region of the bacterial 16S ribosomal RNA gene (Wang et al. 2020). The PCR products were purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantified using QuantiFluor™-ST (Promega, USA) according to the manufacturer's protocol. The DNA were paired-end sequenced (2 × 300 bp) on an Illumina MiSeq PE300 platform (Illumina Inc., San Diego, CA) at Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China.

Raw sequences were processed using FLASH (version 1.2.11). The QIIME quality control process (version 1.7.0) was used to discard low-quality sequences (quality scores <20). Chimeric sequences were identified and removed using UCHIME (version 1.7.0). Only sequences at least 200 bp long after quality filtering were grouped into operational taxonomic units (OTUs) at 97% similarity level. The alpha-diversity (Shannon, ACE, Chao1 and Coverage indices) and beta-diversity were analyzed using QIIME. Community structures of bacteria was analyzed from phylum to genus levels using the Silva database with a confidence threshold of 70%. Principal coordinate analysis (PCoA) was constructed to visualize the variation in microbial communities between samples. To illuminate the interactions among microbes, correlations of microbes were analyzed by Spearman's rank correlations between the abundant genera (top 10) and a network was created with Ctytoscape (version 3.9.0) to visualize the correlations. Spearman correlation heatmap was constructed by the R software (version 3.3.1) to show the relationships of bacterial community and fermentation parameters. The sequences data reported in this study was archived in the Sequence Read Archive (SRA) with the accession number PRJNA795324.

Statistical Analyses

Data on chemical and microbial compositions of fresh and ensiled WPQ were tested to one-way analysis of variance (ANOVA) using the SPSS 22. Tukey's multiple comparison was used for the means separation. Significant differences were declared when $P < 0.05$.

Results

The WPQ characteristics before ensiling

The DM, WSC, crude protein, buffering capacity, neutral detergent fiber, acid detergent fiber contents in WPQ were 142 g/kg FM, 29.2 g/kg DM, 191 g/kg DM, 566 mEq/kg DM, 488 g/kg DM and 424 g/kg DM, respectively (Table 1). The LAB, aerobic bacteria, Enterobacteriaceae and yeasts number was 5.44, 6.50, 6.63 and 6.71 $\log_{10}$ cfu/g FM, respectively.

Effects of additives on fermentation characteristics and chemical composition of WPQ silage

The effects of additives on the fermentation characterstics of WPQ silage are given in Table 2. Additive affected (P<0.05) fermentation parameters except for propionic acid and ethanol content. Naturally fermented WPQ exhibited acetic acid-type fermentation, with high pH value (6.34) and acetic acid content (85.4 g/kg DM) as well as low lactic/acetic acid ratio (0.25). Among the additives, addition of M and ML to WPQ silage increased the intensity of lactic acid production, indicated by increases (P<0.05) in lactic acid content and lactic/acetic acid ratio, and decreases (P<0.05) in pH value and acetic acid contents. Furthermore, M and ML addition decreased (P<0.05) the propionic acid and NH_3-N contents, whereas E and EL addition increased (P<0.05) the NH_3-N contents. The effects of additives on the chemical composition of WPQ silage are shown in Table 3. The DM, WSC and CP contents were affected (P<0.05) by the additive. Compared with other silages, the DM and CP content was higher (P<0.05) in the M-treated silages (M and ML silages). Addition of ML increased (P<0.05) the residual WSC content compared with control silage.

Effects of additives on bacterial community of WPQ silage

A total of 2,576,159 high-quality sequences with an average read length of 428 bp were obtained. Based on a 97% sequence identity threshold, these reads were clustered into 315 OTUs. Table 4 shows the alpha diversity indices for fresh WPQ and WPQ silages treated with different additives. The coverage of all samples was > 0.99. Shannon indice was comparable between fresh WPQ and silage samples. The Chao 1, ACE and OTUs number decreased in silage samples compared with those in fresh WPQ. The PCoA analysis of bacterial communities on OTU level in fresh and ensiled WPQ is displayed in Fig.1. All fresh samples were clearly separated from the silage samples. Among the silage samples, M and ML silage samples were separated from the control silage samples.

On the phylum level (Fig.2A), Proteobacteria (91.6%) was most abundant phylum in fresh WPQ, followed by Firmicutes (4.38%). After 60 days of ensiling, the dominant phyla in control and L silage was Proteobacteria (67.5 % for control and 56.9 % for L), while in E, M, EL and ML silage was Firmicutes (55.2%, 75.6%, 53.5% and 81.0%). On the genus level (Fig. 2B), there were 15 genera with a relative abundance greater than 1%. In fresh WPQ, the top 3 abundant genera were *Pantoea* (75.4%), *Serratia* (9.92%) and *Pesudomonas* (3.88%). After 60 days of ensiling, the most abundant genera in all silages was *Lactobacillus* (26.3%, 52.3%, 70.5%, 40.8%, 47.4% and 80.8% for control, E, M, L, EL and ML silage, respectively). The subdominant genera in control silage were *Morganella* (21.9%) and *Citrobacter* (20.4%); in E silage were *Pantoea* (14.4%) and *Proteus* (11.6%); in M silage were *Pantoea* (18.5%) and *Pediococcus* (3.26%); in L silage were *Citrobacter* (28.4%) and *Proteus* (6.50%); in EL silage were *Citrobacter* (22.6%) and *Pantoea* (12.7%); in ML silage were *Pantoea* (15.6%). Statistical comparison indicated that seven genera showed significant difference among the silages (Fig. 3). Compared with the control silage, higher (P<0.001) abundance of *Lactobacillus*, and lower (P<0.05) abundances of *Morganella* and *Providencia* were dectected in the M and ML silages compared with the control silage. To illuminate the interactions among microbes during WPQ fermentation, we explored the correlations of

microbes based on Spearman's rank correlations (|p|>0.5 and *P*<0.05). In total, 13 pairs of positive correlations and 6 pairs of negative correlations were identified from the top 10 genera (Fig. 4). The network showed that *Morganella*, *Citrobacter*, *Hafnia-Obesumbacterium*, *Providencia* and *Enterococcus* were negatively correlated with the *Lactobacillus*.

Correlation analysis of bacterial community and silage characteristics

Spearman correlations between bacterial community and silage characteristics are presented in Fig. 5. *Lactobacillus* statistically positively correlated with lactic acid, lactic/acetic acid ratio and WSC, while negatively correlated with pH, acetic acid and propionic acid. *Citrobacter*, *Providencia*, *Proteus* and *Enterococcus* were grouped together. They were positively correlated with acetic acid, propionic acid and pH, whereas negatively correlated with WSC, CP, lactic acid and lactic/acetic acid ratio. In addition, *Proteasu* also showed statistically positive correlation with NH_3-N.

Discussion

The WPQ characteristics before ensiling

The crude protein content of WPQ was as high as 191 g/kg DM, a level comparable with that of alfalfa harvested at squaring stage (Wang and Yu, 2020). High crude protein content, coupled with the high biomass production, suggests that WPQ can be used as a high-protein source for livestock feed. It is generally considered that the ideal moisture range for ensiling is 600-700 g/kg FM since such a moisture can maintain the vigorous growth of LAB as well as prevent the undesirable clostridial fermentation (Du et al. 2021). However, Moser (1995) reported that the stems dried 10 to 15 times slower than the grass leaves. Large amount of stem-stored water increases the difficulty of wilting WPQ to an ideal moisture in a short time. After 20 h of wilting, the moisture content of WPQ was still as high as 858 g/kg DM, which could be the great challenge for making high-quality WPQ silage. The WSC content and epiphytic LAB count of the material are another two determinants of fermentation quality (Dong et al. 2020). Zhang et al. (2015) suggested that the minimum WSC level for successful fermentation is 60 g/kg DM. Based on criterion, fresh WPQ failed to meet the requirement. However, the LAB number was adequate, exceeding the recommended level (5 $\log_{10}$ cfu/g FM) as suggested by Mu et al. (2020).

Effects of additives on fermentation characterstics and chemical composition of WPQ silage

The lactic acid is the desired fermentation product as it is the main driver of lowering the silage pH. However, naturally fermented WPQ (control silage) exhibited acetic acid-type fermentation indicated by high pH value (6.43) and acetic acid content (85.4 g/kg DM) as well as low lactic/acetic acid ratio (0.25). Moderate content of acetic acid (30~40 g/kg DM) in silage can be beneficial, because they inhibit yeasts, resulting in improved stability when silage is exposed to air (Kung et al. 2018). However, as acetic acid is a weaker acid than lactic acid, excessively high production of acetic acid (>60 g/kg DM) represents

inefficient fermention and is accompanied by poor DM recovery in silage (Wang et al. 2019). In particular, poorly fermented silages with high acetic acid contents also undergo substantial protein degradation and result in accumulations of detrimental compounds (e.g., biogenic amine) that decrease the intake and negatively affect the animal production (Muck, 2010). High acetic acid contents is frequently observed in extremely wet silages dominated by enterobacteria, clostridia or heterolactic acid bacteria (Kung et al. 2018). This is because high level of moisture dilutes the fermentation acids and more lactic acid production by LAB is required to inhibit the activity of acetic acid-producing microbes. In the experiment, high moisture, together with the low WSC content and relatively high buffering capacity, may hamper the pH decline and allow the acetic acid-producing microbes to be active for long time during WPQ ensiling. Among the additives, adding molasses (M and ML) successfully shifted the fermentation patterns toward increased intensity of lactic acid production, indicated by lower pH values (<4.56), higher lactic acid content (>60.5 g/kg DM) and lactic/acetic acid ratio (>1.40) than the control silage, suggesting the improvement of fermentation quality. Cellulase addition failed to affect the fermentaion in WPQ silage. Commercial cellulases are known to have the optimum activity at pH 4.5-5.4 (Henderson and McDonald, 1977). The marginal effect of cellulase addition on silage fermentation was probably because the high silage pH (>5.4) was not within the optimum range for cellulase activity. This may weaken the function of cellulase in the silage. Similarly, Kung et al. (1991) have observed that if pH optima have not been achieved in the silage, cellulase effect will be partially negated. LAB inoculation also did not elicit the significant effects on silage fermentation. The LAB used in the experiment belongs to lactobacilli and is capable of quickly producing large amounts of LA by fermenting a wide variety of substrates. Little value of LAB inoculation to the WPQ silage reflects that LAB number or activity may be not the restricting factor for lactic acid fermentation. Overall, the responses of fermentation to the additives suggest that the lack of fermentable sugar could be the main factor of restricting extensive lactic acid fermentaion in WPQ silage.

The presence of propionic acid, butyric acid and ethanol in silages are unacceptable given that their generation is an energy-waste metabolism. The results showed the propionic acid contents (<9.00 g/kg DM) in all WPQ silages were within the acceptable range, as suggested by Kung et al. (2018). Moist forage that is poorly preserved often contains high concentrations of butyric acid due to the clostridial activity (Zheng et al. 2017). In the study, trace quantities of butyric acid (< 5g/kg DM) suggested that there was little or no clostridial activity in the WPQ silages. Ethanol has little preservation effect during ensiling, and it causes extremely higher DM and energy losses. According to Kung et al. (2018), over 30-40 g/kg DM of ethanol production in silage is associated with the action of yeast. The results showed that ethanol contents in all WPQ silages were < 20 g/kg DM, suggesting that ethanol was mainly produced by microbes such as heterolactic acid bacteria and enterobacteria.

The proteolysis by plant and microbial enzymes lowers the nutritive value of ensiled forage by degrading forage protein into non-protein fractions, such as peptides, free amino acid and NH_3 (Guo et al. 2008). As an end-product, NH_3-N can be taken as an indicator of protein degradation in silage. Generally, over 100-150 g/kg TN of NH_3-N indicates extensive protein degradation occurred during ensiling (Kung et al.

2018). High NH_3-N content (162 g/kg TN) in the control silage indicated that WPQ protein was badly preserved under natural fermentaion conditions. Adding molasses (M and ML) decreased the propionic acid and NH_3-N contents. It indicates that molasses addition is beneficial for suppressing inefficient fermentations and improving the protein preservation of WPQ silage. By contrast, cellulase increased the NH_3-N contents compared with control silage. Similar phenomenon has been reported by Kung et al. (1991). They ascribed it to the N contributions from the enzyme complex.

The molasses-treated silage had higher DM content than other silages, due to the addition of molasses. Higher residual WSC is nutritionally desirable because it is rapidly digestible in the rumen (McDonald et al. 1991). Addition of ML to WPQ silage increased the residual WSC contents, similar to the findings of Guo et al. (2014) and Ebrahimi et al. (2014). It was presumably because more fermentable sugars plus high-activity LAB resulted in faster rates of lactic acid production and pH decline, quickly suppressing the growth of undesirable microbes and thus retaining more WSC in the silage. The CP content increased with the molasses addition, which may be linked with the decreased extent of protein degradation. Compared fresh WPQ, ensiled WPQ had lower NDF and ADF contents. Similarly, Kung et al. (1991) reported that fiber degraded naturally during ensiling as a result of some silage microflora producing extracellular cellulases and hemicellulases. NDF and ADF represent the less digestible fiber portions for animals. The decreased NDF and ADF contents in ensiled WPQ silage suggested the improvement of nutritional value.

Effects of additives on bacterial community of WPQ silage

Ensiling is a bacterial-driven process in which the types and abundances of bacteria involved play a critical role in fermentation quality (Guan et al. 2018; He et al. 2020). As far as we know, this is the first report concerning the bacterial community of WPQ silage. Good's coverage (>99%) indicated that sequencing depth had adequately captured most of the bacterial communities in the samples. Alpha diversity reflects the bacterial diversity and species richness in a single sample. The Shannon indice are used to measure bacterial diversity, whereas Chao1, ACE indices and OTUs number are measures of species richness. Generally, considerable reductions in bacterial diversity and species richness are expected to occur during successful ensiling because complex microbial communities in fresh materials will be replaced by the development of LAB (Du et al. 2021). However, ensiled WPQ had comparable or increased bacterial diversity compared with the fresh WPQ sample. It indicated high abundances of non-LAB microbes present in the WPQ silages. All silage samples had lower species richness than did fresh WPQ sample. This might be attributed to the decline in relative abundance of epiphytic aerobic bacteria, which are unable to survive under the anaerobic conditions (Yuan et al. 2020).

PCoA analysis is a method to explore and to visualize similarities or dissimilarities of the bacterial communities. The clear separation between fresh WPQ samples and silage samples suggests that microbial communities greatly changed during ensiling process. Among the silage samples, M and ML silage samples were clearly separated from the control silage samples, suggesting that these treatments significantly affected the microbial community in WPQ silage.

Proteobacteria is a major phylum of Gram-negative bacteria that includes a wide variety of pathogenic genera, such as *Escherichia*, *Salmonella*, *Vibrio*, *Helicobacter*, *Yersinia* and *Legionellales*. On the phylum level, the dominance of Proteobacteria suggested the large amount of undesirable microbes present in fresh WPQ. After ensiling, *Firmicutes* increased dramatically and became abundant in the WPQ silage. As all LAB belong to *Firmicutes*, the increased abundances of *Firmicutes* are beneficial to the silage fermentation. However, for control and L-treated silages, Proteobacteria remained to be most abundant phylum, suggesting the failure of LAB to dominate the microbita during ensiling.

The LAB are desirable bacteria contributing to the fermentation quality of silage. Members of the *Leuconostocs*, *Lactococcus*, *Pediococcus* and *Weissella* genera are the LAB most frequently detected on standing plants and contributes to the initial decline in silage pH (Dong et al. 2019b; Wang et al. 2020). Similar to those reported in forage (Ali et al. 2020; Cai, 1999), LAB genera represent only a very small fraction of the epiphytic microbiota in the fresh WPQ. Silage fermentation is an anaerobic, closed, solid-fermentation system. High abundance of *Lactobacillus* is frequently observed in this system because of high acid-resistant nature. *Lactobacillus* can quickly ferment a wide variety of substrates to produce large amount of lactic acid. Therefore, they are crucial bacteria responsible for the largest amount of lactic acid production and pH decline in silage (Costa et al. 2021). In the study, adding M and ML increased the relative abundance of *Lactobacillus*, explaining why lactic acid contents increased in these WPQ silages. However, it was observed that cellulase addition (E and EL) also promoted the development of *Lactobacillus* to some extents but did not result in a significant increment of lactic acid production. Such a discrepancy might be explained by the fact that sugars released from cellulose hydrolysis did not reach the significant level to affect the fermentation patterns in WPQ silage. Dong et al. (2020) stated that under WSC-deficient conditions, facultatively heterofermentative strains of *Lactobacillus* (e.g. *Lactobacillus plantarum*) process heterofermentative activity rather than homofermentative activity.

Silage fermentation is a complex process involving interactions among many factors. Ideal fermentation is not always obtained, and sometimes undesirable bacteria may dominate the silage. Enterobacteria represent a major group of undesirable bacteria in silage. They compete with LAB for the available sugars, and in addition they can degrade protein and result in production of toxic compounds such as biogenic amines and branched fatty acids (Muck, 2010). In this study, over 5 genera of Enterobacteria were detected in large quantities in the WPQ silage, and the total abundance was > 50% in the control silages. The principal fermentation product of Enterobacteria is acetic acid, not lactic. It explained the high acetic acid production and substantial protein degradation in the WPQ silage. Enterobacteria will not proliferate at low pH, and their population will be controlled by acidification (McDonald et al. 1991). Therefore, Enterobacteria was suppressed with the increased lactic acid production by *Lactobacillus* (Fig. 4). Among the Enterobacteria, *Citrobacter*, *Morganella* and *Providencia* were particularly suppressed. *Citrobacter* share all the general properties and biochemical characteristics of the family Enterobacteriaceae. They are found in a variety of environmental sources, including soil and water, and in the human intestines. *Citrobacter* has been found to associate with the losses of polyunsaturated fatty acid, α-tocopherol and β-carotene in alfalfa silage (Zong et al. 2021). *Morganella* previously belongs to family Enterobacteriaceae. They conduct anaerobic respiration and found to associate with the increase

in acetic acid content in high-moisture Italian ryegrass silage (Li and Nishino, 2013). *Providencia* is closely related to the *Morganella*. These bacteria have been abundantly dectected in oat and barley silages (Liu et al 2019; Jia et al. 2021). Some members of *Providencia* were described to associate the diseases in animals (Jia et al. 2021).

Correlation analysis of bacterial community and silage characteristics

Studying the correlations between bacterial community and silage characteristics would give us a deep understanding of the key bacteria to silage quality. *Lactobacillus* positively correlated with lactic acid, lactic/acetic acid ratio and WSC, while negatively correlated with pH, acetic acid and propionic acid. It confirmed that *Lactobacillus* have played a crucial role in improving the fermentation quality and nutrients preservation of WPQ silage. *Citrobacter*, *Providencia* and *Morganella* are known to be able to produce acetic acid as the main product. Positive correlations of acetic acid with them suggests that they are main contributors to the acetic acid production in WPQ silage. *Proteus* has been identified as NH_3-producing bacteria in fermented skate (*Raja kenojei*). Positive correlation between *Proteus* and NH_3-N, suggests that their important role in producing NH_3-N during WPQ ensiling. *Enterococcus* are cocci LAB and can only grow in pH > 4.5 (Cai, 1999). Weak acid- resistance of this LAB may explain their positive correlation with pH, acetic acid, propionic acid and butyric acid, and negative correlations with lactic acid and WSC.

Conclusion

In conclusion, naturally ensiled WPQ with high moisture content was prone to acetic acid-type fermentation dominated by enterobacteria, resulting in poor fermentation quality and substantial protein degradation. Adding molasses alone or combined with LAB successfully shifted the fermentation patterns toward increased intensity of lactic acid production by promoting *Lactobacillus* and decreasing the abundance of enterobacteria. The results reveal that the lack of fermentable sugar could be the main factor of restricting extensive lactic acid fermentaion in WPQ silage. Supplementing fermentable sugar or co-ensiling with materials with high WSC content and low moisture content could be beneficial for producing high-quality WPQ silage.

Declarations

Acknowledgements

This work was financially supported by National Natural Science Foundation of China (Grant No. 32001398), and China Postdoctoral Science Foundation (Grant No. 2020M671523).

Author Contributions

Conceptualization: Tao Shao; Methodology: Zhihao Dong; Formal analysis: Junfeng Li and Siran Wang; Writing-original draft preparation: Zhihao Dong; Project administration: Dong Dong, Jie Zhao and Zhihao Dong. Writing-review and editing: Tao Shao

Declaration of Competing Interest

The authors declare no conflict of interest regarding this work.

References

1. Ali N, Wang S, Zhao J, Dong Z, Li J, Nazar M, Shao T (2020) Microbial diversity and fermentation profile of red clover silage inoculated with reconstituted indigenous and exogenous epiphytic microbiota. Bioresour Technol 123606. https://doi.org/10.1016/j.biortech.2020.123606
2. AOAC International (2000) Official Methods of Analysis, 17th edn. Association of Official Analytical Chemists, Arlington, VA
3. Basra SMA, Iqbal S, Afzal I (2014) Evaluating the response of nitrogen application on growth, development and yield of quinoa genotypes. Int J Agric Biol 16:886–892
4. Cai Y (2019) Identification and characterization of *Enterococcus* species isolated from forage crops and their influence on silage fermentation. J Dairy Sci 82:2466–2471. https://doi.org/10.3168/jds.S0022-0302(99)75498-6
5. Chen L, Li J, Dong Z, Shao T (2019) Effects of lactic acid bacteria inoculants and fibrolytic enzymes on the fermentation quality, *in vitro* degradability, ruminal variables and microbial communities of high-moisture alfalfa silage. Grassl Sci 65:216–225. https://doi.org/10.1111/grs.12240
6. Costa DM, Carvalho BF, Bernardes TF, Schwan RF, Vila C (2021) New epiphytic strains of lactic acid bacteria improve the conservation of corn silage harvested at late maturity. Anim Feed Sci Tech 274:114852. https://doi.org/10.1016/j.anifeedsci.2021.114852
7. Dong Z, Li J, Chen L, Wang S, Liu Q, Shao T (2019) Using proteomics to decipher the effect of tissue damage on the fate of red clover proteins during ensiling. Anim Feed Sci Tech 257:114271. https://doi.org/10.1016/j.anifeedsci.2019.114271
8. Dong Z, Li J, Chen L, Wang S, Shao T (2019) Effects of freeze-thaw event on microbial community dynamics during red clover ensiling. Front Microbiol 10:1559. https://doi.org/10.3389/fmicb.2019.01559
9. Dong Z, Wang S, Zhao J, Li J, Shao T (2020) Effects of additives on the fermentation quality, *in vitro* digestibility and aerobic stability of mulberry (*Morus alba* L.) leaves silage. Asian Austral J Anim 33:1292–1300. https://doi.org/10.5713/ajas.19.0420
10. Du Z, Lin Y, Sun L, Yang F, Cai Y (2021) Microbial community structure, co-occurrence network and fermentation characteristics of woody plant silage. J Sci Food Agr. https://doi.org/10.1002/jsfa.11457

11. Ebrahimi M, Ali Rajion M, Goh Y, Soleimani farjam A, Sazili AQ, Schonewille J (2014) The effects of adding lactic acid bacteria and cellulase in oil palm (*Elais guineensis* Jacq.) frond silages on fermentation quality, chemical composition and *in vitro* digestibility. Ital J Anim Sci 13:3358. https://doi.org/10.4081/ijas.2014.3358
12. Guan H, Yan Y, Li X, Li X, Shuai Y, Feng G, Ran Q, Cai Y, Li Y, Zhang X (2018) Microbial communities and natural fermentation of corn silages prepared with farm bunker-silo in Southwest China. Bioresour Technol 265:282–290. https://doi.org/10.1016/j.biortech.2018.06.018
13. Guo G, Y XJ, Li L, Wen A, Sha T (2014) Effects of fibrolytic enzymes, molasses and lactic acid bacteria on fermentation quality of mixed silage of corn and hulless-barely straw in the Tibetan Plateau. Grassl Sci 60:240–246. https://doi.org/10.1111/grs.12060
14. Guo XS, Ding WR, Han JG, Zhou H (2008) Characterization of protein fractions and amino acids in ensiled alfalfa treated with different chemical additives. Anim Feed Sci Tech 142:89–98. https://doi.org/10.1016/j.anifeedsci.2007.07.005
15. Guo XS, Ke WC, Ding WR, Ding LM, Xu DM, Wang WW, Zhang P, Yang FY (2018) Profiling of metabolome and bacterial community dynamics in ensiled Medicago sativa inoculated without or with *Lactobacillus plantarum* or *Lactobacillus buchneri*. Sci Rep 8:357. https://doi.org/10.1038/s41598-017-18348-0
16. He L, Wang C, Xing Y, Zhou W, Pian R, Chen X, Zhang Q (2020) Ensiling characteristics, proteolysis and bacterial community of high-moisture corn stalk and stylo silage prepared with Bauhinia variegate flower. Bioresour Technol 296:122336. https://doi.org/10.1016/j.biortech.2019.122336
17. Henderson AR, McDonald P (1977) The effect of cellulase preparations on the chemical changes during the ensilage of grass in laboratory silos. J Sci Food Agr 28:486–490. https://doi.org/10.1002/jsfa.2740280604
18. Jia T, Yun Y, Yu Z (2021) Propionic acid and sodium benzoate affected biogenic amine formation, microbial community, and quality of oat silage. 12:3263. https://doi.org/10.3389/fmicb.2021.750920
19. Kung L, Shaver RD, Grant RJ, Schmidt RJ (2018) Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. J Dairy Sci 101:4020–4033. https://doi.org/10.1002/jsfa.2740280604
20. Kung L, Tung RS, Maciorowski KG, Buffum K, Knutsen K, Aimutis WR (1991) Effects of plant cell-wall-degrading enzymes and lactic acid bacteria on silage fermentation and composition. J Dairy Sci 74:4284–4296. https://doi.org/10.3168/jds.S0022-0302(91)78623-2
21. Li M, Zi X, Zhou H, Hou G, Cai Y (2014) Effects of sucrose, glucose, molasses and cellulase on fermentation quality and *in vitro* gas production of king grass silage. Anim Feed Sci Tech 197:206–212. https://doi.org/10.1016/j.anifeedsci.2014.06.016
22. Li Y, Nishino N (2013) Effects of ensiling fermentation and aerobic deterioration on the bacterial community in italian ryegrass, guinea grass, and whole-crop maize silages stored at high moisture content. Asian Austral J Anim 26:1304–1312. https://doi.org/10.5713/ajas.2013.13175

23. Liu B, Huan H, Gu H, Xu N, Shen Q, Ding C (2019) Dynamics of a microbial community during ensiling and upon aerobic exposure in lactic acid bacteria inoculation-treated and untreated barley silages. Bioresour Technol 273:212–219. https://doi.org/10.1016/j.biortech.2018.10.041
24. Liu M, Yang M, Yang H (2021) Biomass production and nutritional characteristics of quinoa subjected to cutting and sowing date in the midwestern China. Grassl Sci 67:215–224. https://doi.org/10.1111/grs.12307
25. McDonald P, Henderson AR, Heron SJE (1991) Microorganisms. In: McDonald P, Henderson AR, Heron SJE (eds) The Biochemistry of Silage, 2nd edn. Chalcombe Publications, Abersytwyth, pp 81–151
26. Moser LE (1995) Post-harvest physiology and preservation of forages.. In: In: Kenneth J, Moore, Michael A (eds) Peterson Post-harvest physiological changes in forage plants, 2rd edn. CSSA Special Publications, Madison, WI, pp 1–19
27. Mu L, Xie Z, Hu L, Chen G, Zhang Z (2020) Cellulase interacts with *Lactobacillus plantarum* to affect chemical composition, bacterial communities, and aerobic stability in mixed silage of high-moisture amaranth and rice straw. Bioresour Technol 315:123772. https://doi.org/10.1016/j.biortech.2020.123772
28. Muck RE (2010) Silage microbiology and its control through additives. Rev Bras Zootecn 39:183–191
29. Tian H, Wang Y, Liu Z, Hu Z, Guo Y, Deng M, Liu G, Sun B (2021) Effects of malic acid and sucrose on the fermentation parameters, CNCPS nitrogen fractions, and bacterial community of moringa oleifera leaves silage. Microorganisms 9:2102. https://doi.org/10.3390/microorganisms9102102
30. Tian J, Yu Y, Yu Z, Shao T, Na R, Zhao M (2014) Effects of lactic acid bacteria inoculants and cellulase on fermentation quality and *in vitro* digestibility of *Leymus chinensis* silage. Grassl Sci 60:199–205. https://doi.org/10.1111/grs.12059
31. Van Soest PJ, Robertson JB, Lewis BA (1991) Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J Dairy Sci 74:3583. https://doi.org/10.3168/jds.S0022-0302(91)78551-2
32. Vega-Gálvez A, Miranda M, Vergara J, Uribe E, Puente L, Martínez EA (2010) Nutrition facts and functional potential of quinoa (*Chenopodium quinoa* willd.), an ancient Andean grain: a review. J Sci Food Agr 90:2541–2547. https://doi.org/10.1002/jsfa.4158
33. Wang B, Yu Z (2020) Effects of moisture content and additives on the ensiling quality and vitamins changes of alfalfa silage with or without rain damage. Anim Sci J 91:e13379. https://doi.org/10.1111/asj.13379
34. Wang S, Zhao J, Dong Z, Li J, Kaka NA, Shao T (2020) Sequencing and microbiota transplantation to determine the role of microbiota on the fermentation type of oat silage. Bioresour Technol 309:123371. https://doi.org/10.1016/j.biortech.2020.123371
35. Wang Y, He L, Xing Y, Zhou W, Pian R, Yang F, Chen X, Zhang Q (2019) Bacterial diversity and fermentation quality of *Moringa oleifera* leaves silage prepared with lactic acid bacteria inoculants

and stored at different temperatures. Bioresour Technol 284:349–358. https://doi.org/10.1016/j.biortech.2019.03.139

36. Yuan X, Dong Z, Li J, Shao T (2020) Microbial community dynamics and their contributions to organic acid production during the early stage of the ensiling of Napier grass (*Pennisetum purpureum*). Grass Forage Sci 75:37–44. https://doi.org/10.1111/gfs.12455
37. Zhang Q, Wu B, Nishino N, Wang X, Yu Z (2016) Fermentation and microbial population dynamics during the ensiling of native grass and subsequent exposure to air. Anim Sci J 87:389–397. https://doi.org/10.1111/asj.12427
38. Zhang Q, Xing B, Sun M, Zhou B, Ren G, Qin P (2020) Changes in bioaccessibility, polyphenol profile and antioxidants of quinoa and djulis sprouts during *in vitro* simulated gastrointestinal digestion. Food Sci Nutr 8:4232–4241. https://doi.org/10.1002/fsn3.1718
39. Zhao S, Yang F, Wang Y, Fan X, Feng C, Wang Y (2021) Dynamics of fermentation parameters and bacterial community in high-moisture alfalfa silage with or without lactic acid bacteria. Microorganisms 9:1225. https://doi.org/10.3390/microorganisms9061225
40. Zheng ML, Niu DZ, Jiang D, Zuo SS, Xu CC (2017) Dynamics of microbial community during ensiling direct-cut alfalfa with and without LAB inoculant and sugar. J Appl Microbiol 122:1456–1470. https://doi.org/10.1111/jam.13456
41. Zong C, Wu Q, Wu A, Chen S, Dong D, Zhao J, Shao T, Liu Q (2021) Exploring the diversity mechanism of fatty acids and the loss mechanisms of polyunsaturated fatty acids and fat-soluble vitamins in alfalfa silage using different additives. Anim Feed Sci Tech 280:115044. https://doi.org/10.1016/j.anifeedsci.2021.115044

Tables

Table 1. The WPQ characteristics before ensiling

Item	WPQ	SEM
Dry matter (g/kg FM)	142	0.258
WSC (g/kg DM)	29.2	1.044
Crude protein (g/kg DM)	191	0.856
Buffering capacity (mEq/kg DM)	566	8.961
Neutral detergent fiber (g/kg DM)	488	4.523
Acid detergent fiber (g/kg DM)	424	9.560
LAB (log_{10} cfu/g FM)	5.44	0.053
Aerobic bacteria (log_{10} cfu/g FM)	6.50	0.049
Enterobacteriaceae (log_{10} cfu/g FM)	6.63	0.026
Yeast (log_{10} cfu/g FM)	6.71	0.053

FM, fresh matter; WSC, water-soluble carbohydrates; cfu, colony forming units. SEM, standard error of means.

Table 2. Effects of additives on the fermentation characteristics of WPQ silage

Item	Control	E	M	L	EL	ML	SEM	*P*-value
pH value	6.43^{A}	5.85^{B}	4.59^{C}	6.52^{A}	6.12^{AB}	4.43^{C}	0.141	<0.001
Lactic acid (g/kg DM)	21.1^{B}	18.9^{B}	61.5^{A}	19.2^{B}	25.9^{B}	66.1^{A}	4.190	0.008
Acetic acid (g/kg DM)	85.4^{A}	90.1^{A}	43.7^{B}	93.6^{A}	89.1^{A}	38.1^{B}	5.146	0.013
Lactic acid/acetic acid	0.25^{B}	0.21^{B}	1.42^{A}	0.21^{B}	0.32^{B}	1.74^{A}	0.128	0.007
Propionic acid (g/kg DM)	8.52^{A}	5.64^{AB}	4.00^{B}	9.62^{A}	7.37^{AB}	4.13^{B}	0.417	0.003
Butyric acid (g/kg DM)	3.79	3.91	3.44	3.98	4.06	3.55	0.111	0.009
Ethanol (g/kg DM)	15.5	16.4	15.7	15.4	14.6	13.9	0.292	0.096
NH_3-N (g/kg TN)	182^{B}	231^{A}	110^{C}	200^{B}	225^{A}	127^{C}	10.31	0.021

Means with different letters in the same row (A-C) differed ($P < 0.05$).DM, dry matter; TN, total nitrogen; E, cellulase; M, molasses; L, LAB inoculant; EL, combination of cellulase and LAB inoculant; ML, combination of molasses and LAB inoculant; SEM, standard error of means.

Table 3. Effects of additives on the chemical composition of WPQ silage

Item	Control	E	M	L	EL	ML	SEM	*P*-value
Dry matter (g/kg FM)	129^{AB}	128^{AB}	138^{AB}	124^{AB}	123^{B}	144^{A}	2.541	0.015
WSC (g/kg DM)	14.8^{B}	16.8^{B}	20.1^{AB}	14.9^{B}	15.6^{B}	23.7^{A}	0.970	0.013
Crude protein (g/kg DM)	151^{BC}	168^{AB}	179^{A}	152^{BC}	142^{C}	184^{A}	1.288	0.023
NDF (g/kg DM)	355	311	303	351	353	300	10.39	0.126
ADF (g/kg DM)	244	221	217	268	264	200	5.129	0.099

Means with different letters in the same row (A-C) differed ($P < 0.05$). FM, fresh matter; DM, dry matter; WSC, water-soluble carbohydrates; NDF, neutral detergent fiber; ADF, acid detergent fiber; E, cellulase; M, molasses; L, LAB inoculant; EL, combination of cellulase and LAB inoculant; ML, combination of molasses and LAB inoculant; SEM, standard error of means.

Table 4. Alpha diversity of bacterial community for fresh WPQ and WPQ silages treated with different additives

Treatment	Shannon	Chao1	ACE	OTUs number	Coverage
FM	1.95	194	179	88.5	0.9985
Control	2.09	47.7	59.0	35.9	1.0000
E	1.96	67.6	80.0	41.9	1.0000
M	1.85	82.1	130	44.6	0.9990
L	1.76	50.8	59.0	35.7	1.0000
EL	1.90	56.0	55.0	33.9	1.0000
ML	1.46	68.1	71.0	41.8	0.9990

FM, fresh material; E, silage treated with cellulase; M, silage treated with molasses; L, silage treated with LAB inoculant; EL, silage treated with combination of cellulase and LAB inoculant; ML, silage treated with combination of molasses and LAB inoculant.

Figures

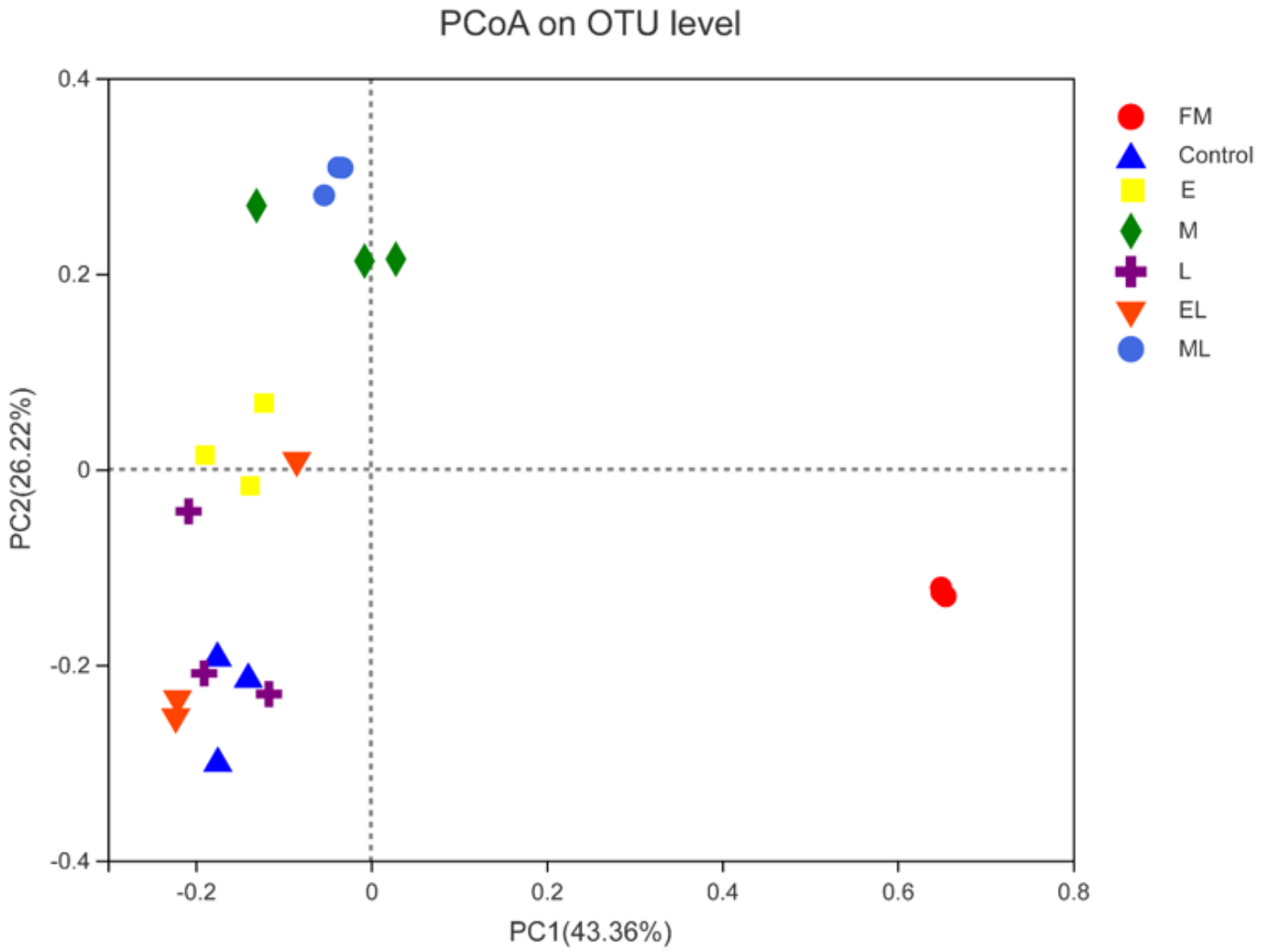

Figure 1

Principal co-ordinates analysis (PCoA) of bacterial communities on OTU level in fresh and ensiled WPQ. FM, fresh material; E, cellulase; M, molasses; L, LAB inoculant; EL, combination of cellulase and LAB inoculant; ML, combination of molasses and LAB inoculant.

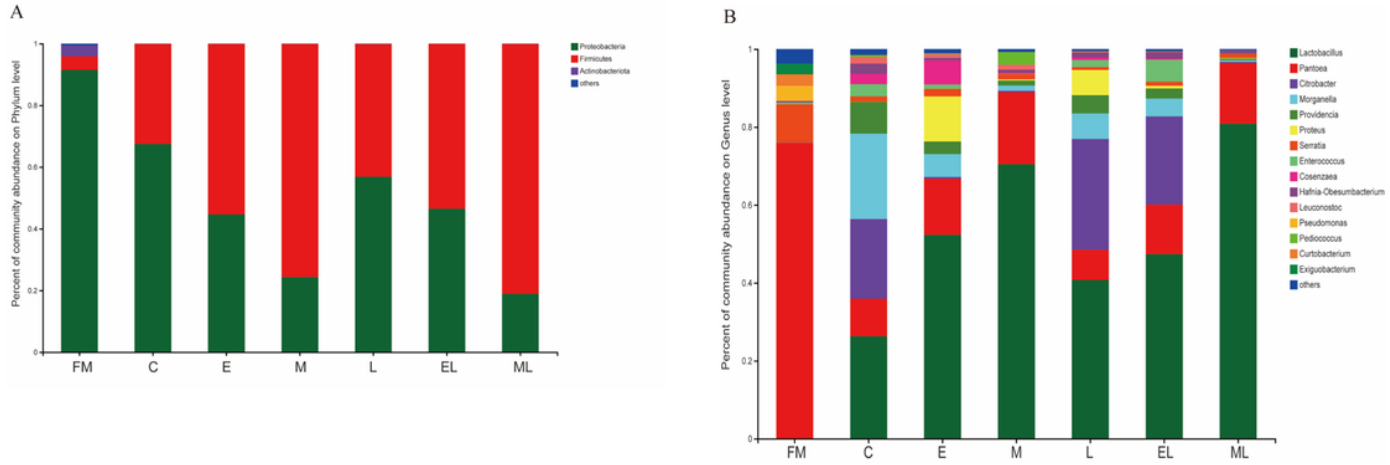

Figure 2

The bacterial community structure on phylum (A) and genus level (B) level of in fresh and ensiled WPQ silages (n=3). Phyla and genera detected at less than 1.0% of total sequence reads are not included. E, cellulase; M, molasses; L, LAB inoculant; EL, combination of cellulase and LAB inoculant; ML, combination of molasses and LAB inoculant.

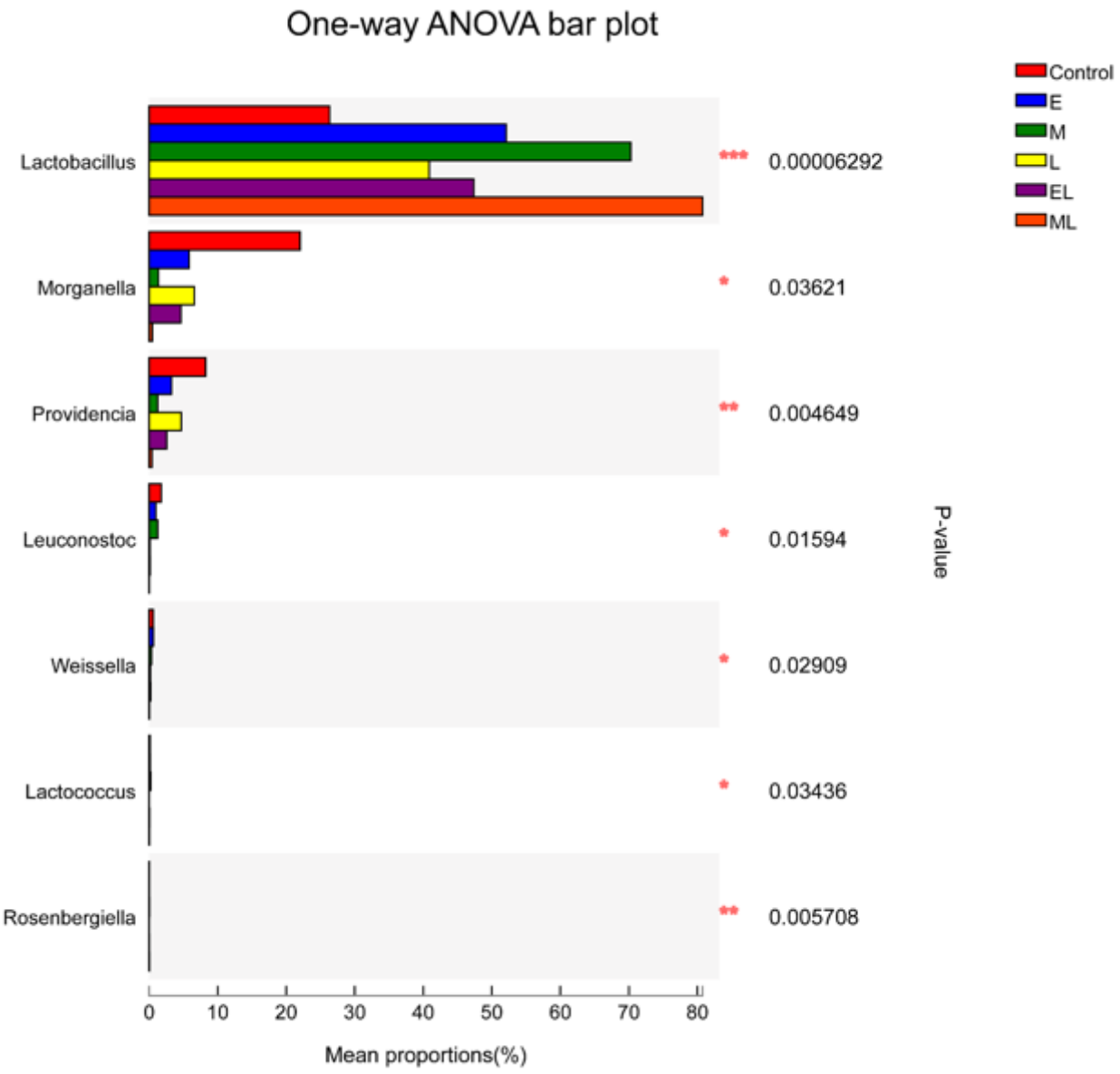

Figure 3

Statistical comparison of the relative abundance of bacterial communities among WPQ silages treated with different additives. E, cellulase; M, molasses; L, LAB inoculant; EL, combination of cellulase and LAB inoculant; ML, combination of molasses and LAB inoculant. A $P < 0.05$ was considered statistically significant.

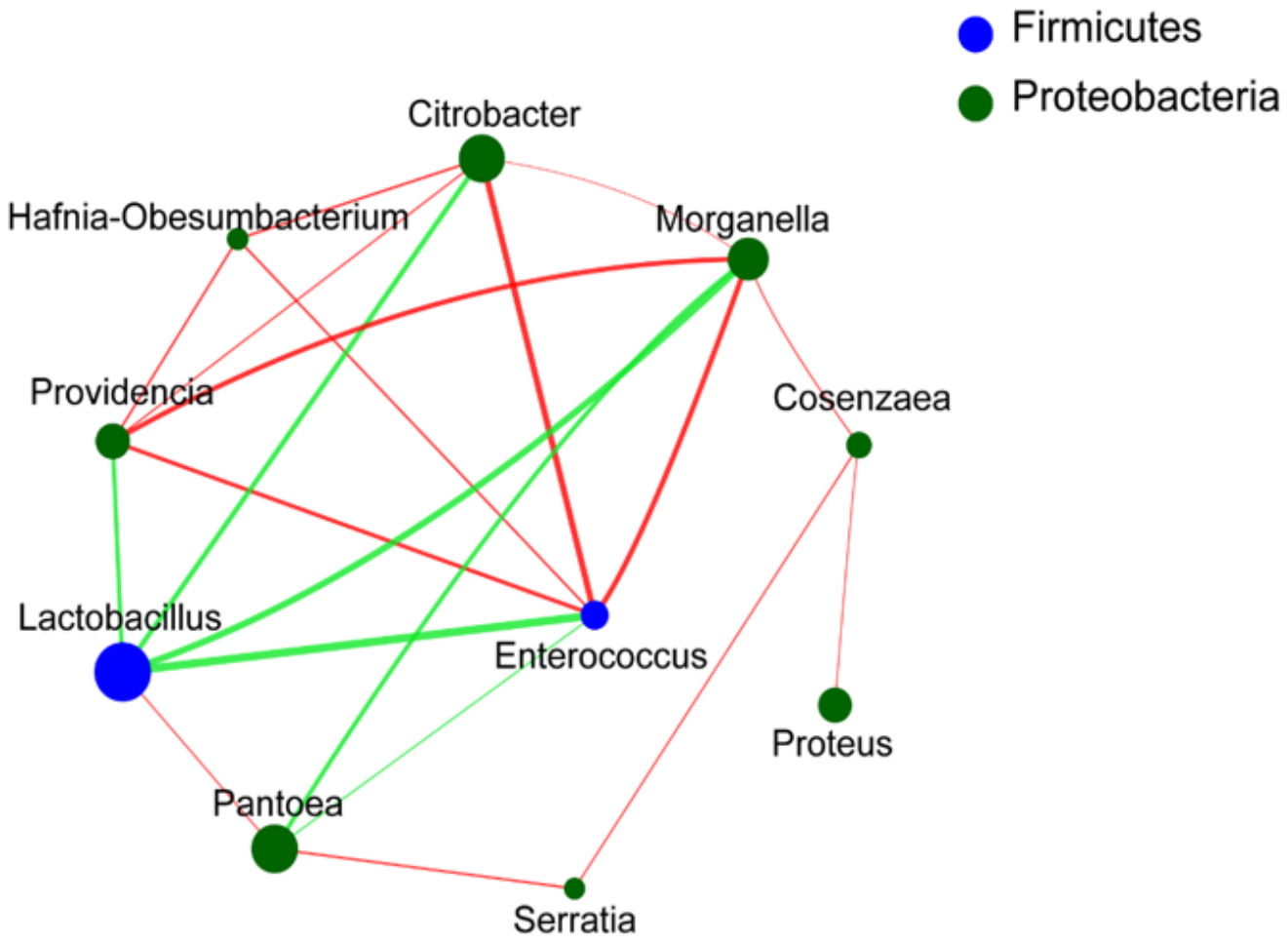

Figure 4

Relationships among top 10 genera during WPQ ensiling. A connection stands for a significant ($P < 0.05$) and strong (Spearman's |p| >0.5) correlation. Size of each node is proportional to the relative abundance, and the nodes are colored by phylum. The thickness of each connection (edge) between two nodes is proportional to the value of Spearman's correlation coefficient (p). The color of the edges corresponds to a positive (red) or negative (blue) relationship.

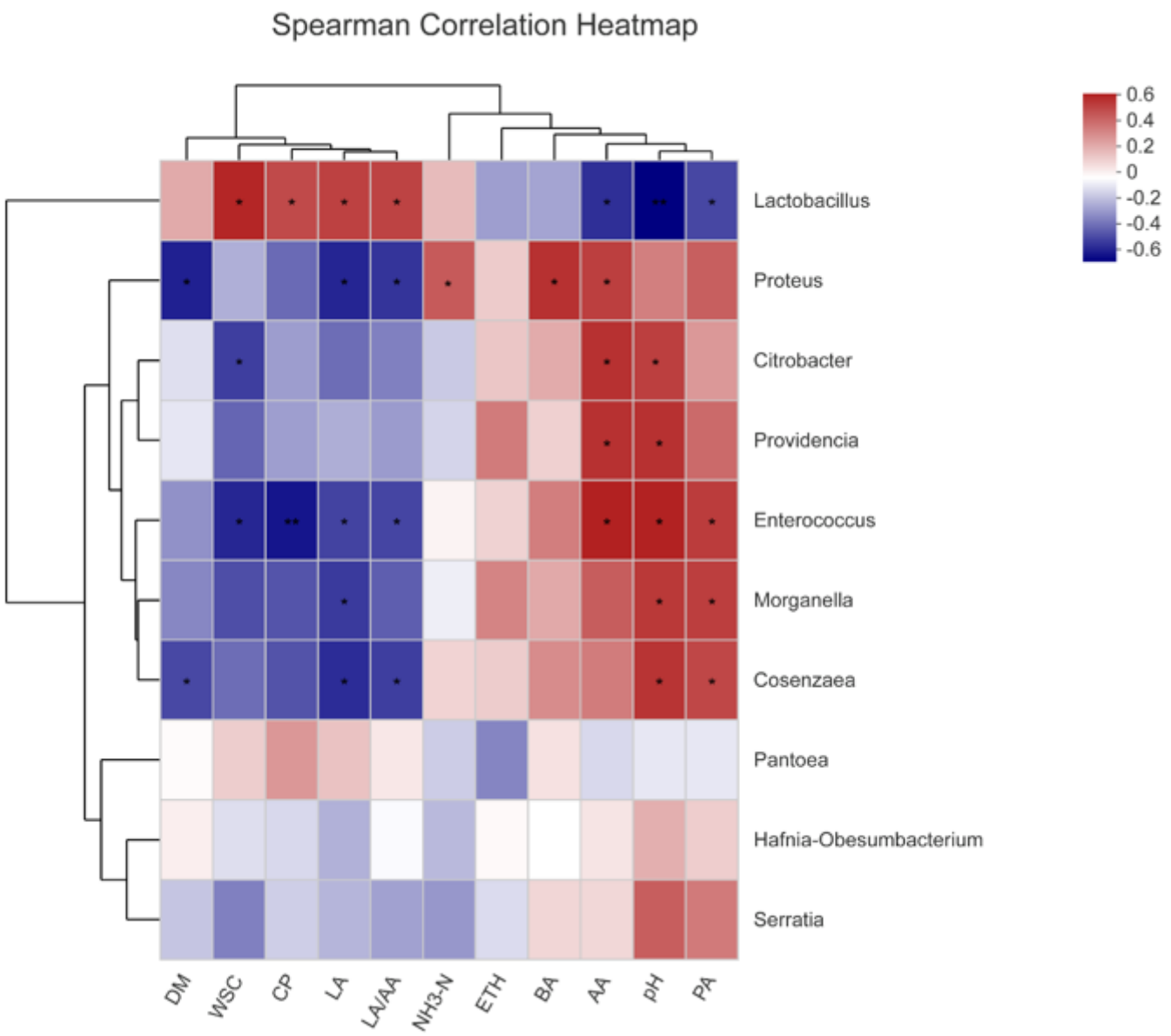

Figure 5

Correlation analysis of the top 10 genera with WPQ silage characteristics. X axis are silage characteristics. Y axis are genera. The corresponding value of the middle heat map is the Spearman correlation coefficient r, which ranges between −1 and 1, r < 0 indicates a negative correlation (blue), r > 0 indicates a positive correlation (red), and '*', '**' and '***' represent $P < 0.05$, $P<0.01$ and $P < 0.001$, respectively. WSC, water-soluble carbohydrates; LA, lactic acid; LA/AA, lactic/acetic acid ratio; DM, dry matter; CP, crude protein; ETH, ethanol; AA, acetic acid; BA, butyric acid; PA, propionic acid.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Authorchecklist.docx