BMC Microbiology

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Effects of cellulase or *Lactobacillus plantarum* on ensiling performance and bacterial community of sorghum straw

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Abstract

This study aimed to evaluate the effects of cellulase or *Lactobacillus plantarum* (*L. plantarum*) on the fermentation characteristics and microbial community structure of the sorghum straw silage. Sorghum straw was treated with the following four experimental conditions: distilled water (control, CK), cellulase (CEL), *Lactobacillus plantarum* (LP), and a combined treatment of *Lactobacillus plantarum* with cellulase (LPCEL). These results indicated that the LP treatment could markedly ($p < 0.05$) preserve the crude protein content compared to that in other treatments, whereas the CEL significantly ($p < 0.05$) reduced the acid detergent fiber content, while the LPCEL had the highest lactic acid content and lowest pH value. Proteobacteria and *Pantoea* were identified as the dominant phylum and genus in fresh materials, respectively. This phylum level dominance transitioned to Firmicutes post-treatment, while at the genus level, the community shifted from *Pantoea* to co-dominance of *Lactobacillus* and *Prevotella*, with *Lactobacillus* being the most abundant in both the CEL and LPCEL treatments. In conclusion, adding *L. plantarum* and cellulase to sorghum straw can significantly improve the fermentation quality of sorghum straw silage, and improve the nutritional value of silage by affecting the microbial community structure and metabolic pathways.

Highlights

- The physicochemical properties of sorghum straw silage were improved by *L. plantarum* or cellulase.
- Microbiomic analyses revealed a progressive reduction after ensiling progress.
- The ensiling parameters were influenced by the microbial communities and functions.

Keywords Bacteria-enzyme synergy, Fermentation, Inoculant, Sorghum straw silage

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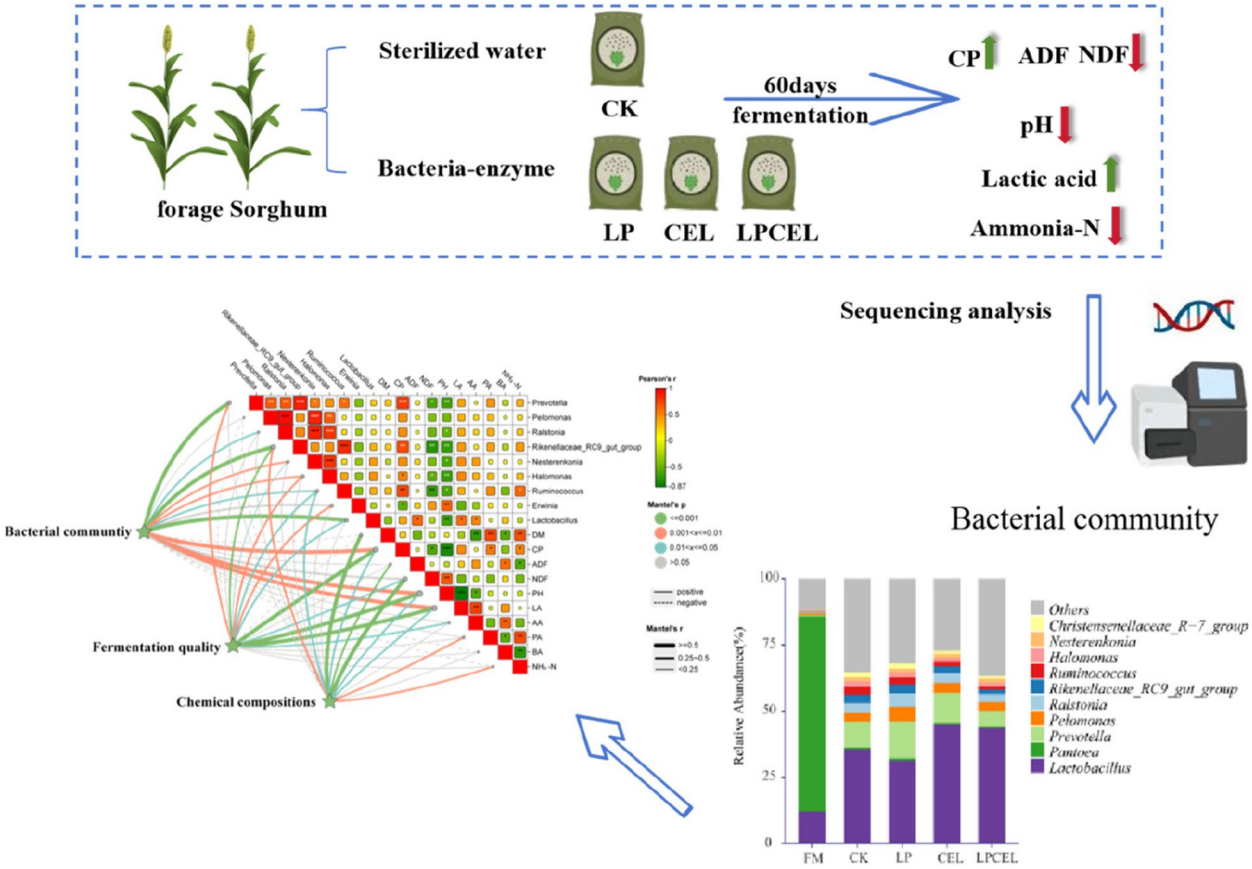
Graphical Abstract



Introduction

The world produces 1.14 billion tons of crop straw every year [27]. As a big agricultural country, China produces a large amount of crop straw every year. From 2018 to 2020, the average output of straw in China was 600 million tons [29]. Sorghum (*Sorghum bicolor* (L.) Moench) an annual economic or forage crop, belongs to the genus Sorghum in the family Poaceae [11], Sorghum straw is an attractive raw material because up to 40% of the dry weight is composed of easily fermented sucrose, glucose and fructose, and proper treatment, such as silage or ammonification, can enhance its feed value, therefore, sorghum straw as a feed in China's livestock production has great potential [20, 24, 38].

Silage is an important means to improve the feed quality of sweet sorghum straw. By microbial fermentation, the fiber content of sorghum straw is reduced, the protein and vitamin contents are increased, so as to improve its nutritional value [10, 29]. Silage additives are used to improve the quality of silage [7]. At present, researchers at home and abroad have studied various additives, lactic acid bacteria (LAB) preparations, enzyme preparations, organic acids, sugars and so on. Previous report showed that additives played a vital role in promoting the growth of beneficial microorganisms, inhibiting the reproduction of harmful microorganisms, reducing pH value, and improving the nutritional value of silage [14]. *Lactobacillus plantarum* (*L. plantarum*) produces lactic acid (LA) through fermentation, which reduces the pH value of silage, thus inhibiting the growth of harmful microorganisms and improving the quality of silage [4, 50]. Cellulase can decompose cellulose in sorghum straw, release soluble sugar, provide energy source for microorganisms, and further promote the growth of LAB [15, 39]. In contrast, the effects of inoculants, enzymes and inoculant-enzyme mixtures on the fermentation and nutritional value of sorghum straw silage were less studied.

In this study, the effects of *L. plantarum*, cellulase and combination of *L. plantarum* and cellulase on the quality and microbial community of sorghum straw

silage were studied to provide theoretical basis for rational use of additives in practical production.

Materials and methods

Substrate and silage preparation

Sorghum straw was collected from Hulunbuir Grassland Ecosystem National Observation and Research Station, Chinese Academy of Agricultural Sciences (49°23′13 "N, 120°02′47" E;627–635 m above sea level; The annual temperature is -2.4°C). On August 20, 2023, Forage sorghum 3701 was harvested and threshed at the later stage of wax-ripening, spread 3 h after harvest (China Linyi Fulida Tools Co., LTD.), and cut into 30 mm lengths. A total of 400 g of freshly collected sorghum straw samples were stored in a freezer and sent to the laboratory for raw material analysis. Sorghum straw composition is listed in Table 1. The two additives used in this study are *L. plantarum* (MTD-1, Jiangsu Luc Biotechnology Company) and a commercial cellulase (AC, Acremonium cellulase, Meiji Essence Pharmaceutical Company, Tokyo, Japan).

The sorghum straw was inoculated with distilled water, *L. plantarum*, cellulase, and cellulase combined with *L. plantarum* as the CK, LP, CEL, and LPCEL treatments, respectively. The LAB inoculants were added at 1.0×10^5 colony forming unit (cfu)/g fresh matter (FM). The cellulase was added at 0.01% of the FM. The experiment employed a completely randomized design with three replicates per treatment. Each replicate containing approximately 250 g of chopped sorghum stalks was aseptically packaged in polyethylene bags (32 cm × 26 cm), followed by vacuum-sealing. The samples were equally distributed and kept at ambient (25 °C) for 60 days of fermentation process.

Table 1 Chemical composition of sorghum straw for feed before silage

Item	Sorghum straw
Dry matter (%)	42.35
Crude protein (%DM)	2.32
Water-soluble carbohydrates (% DM)	12.88
Neutral detergent fibre (% DM)	62.94
Acid detergent fibre (% DM)	36.96
LAB (lg10 cfu/g FM)	4.59
Coliform bacteria (log10 cfu/g FM)	7.87
Aerobic bacteria (log10 cfu/g FM)	8.26
Yeast (lg10 cfu/g FM)	7.70
Molds (lg10 cfu/g FM)	7.58

FM fresh matter, *DM* dry matter, *cfu* colony-forming units, *LAB* lactic acid bacteria

Analysis of chemical composition and fermentation quality

Fresh sorghum straw and silage samples after 60 days of fermentation were placed in a forced air-drying oven and dried at 65°C for 48 h to determine their dry matter (DM) content. The specimens were ground through a 1 mm screen (FW100, Taisite Instrument, China) for the further chemical composition analysis. The crude protein (CP) content was determined by the method of Kjeldahl [47]. The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were determined according to the method descried by Van Soest [52]. The ammonia nitrogen (NH_3-N) was detected as described in a previous report [6].

The organic acid contents of the filtrate were analyzed by high-performance liquid chromatography (HPLC) with a UV detector (210 nm) and 3 mmol/L of $HClO_4$ was the mobile phase at a flow rate of 1.0 mL min^{-1} at 50°C [58]. Determination of water-soluble carbohydrate (WSC) was by anthrone method [3].

The microbial population in the fresh materials (FMs) was counted by the plate count method and expressed on colony-forming units (cfu)/g of FW. The numbers of LAB and coliform bacteria were counted on de Man, Rogosa, Sharpe agar (Difco Laboratories, Detroit, MI, USA) and blue light broth agar (Nissui Ltd., Tokyo, Japan) incubated at 30 ℃ for 48 h, the numbers of mold and yeast, and aerobic bacteria were counted on potato dextrose agar (Nissui Ltd., Tokyo, Japan) and nutrient agar Nissui Ltd., Tokyo, Japan) incubated at 30 ℃ for 24 h, respectively [58].

Bacterial community analysis

Ten-gram silage samples were immediately put into 90 mL of sterile water for collecting microorganisms and treated with a shaker at 160 rpm for 2 h (4℃). Then, they were filtered through two layers of sterile gauze and rinsed for several times with sterile water to recover residual microorganisms. The filtrates were centrifuged at 10,000 × g, 4 ℃, for 15 min, then the bacterial cells were kept at − 80°C. The total microbial DNA were extracted using the TIANamp Bacteria DNA isolation kit (DP302-02, Tiangen, Beijing, China) following the manufacturer's protocol. Then, the extracted DNA samples were used for amplifying the V3-V4 hypervariable region of 16S rRNA gene with the following universal primer (forward, 5′-ACTCCTACGGGAGGCAGCA-3′; reverse, 5′-GGACTACHVGGGTWTCTAAT-3′). The procedures of PCR amplification were followed as described by previous method [35]. The diversity index and microbial community of the sorghum straw silage were calculated via bioinformatics cloud analytics platform (https://www.omicsmart.com.accessed on May 30, 2024).

Statistical analysis

All reported results are averages of three replicates. Nutrition data and the fermentation data was by SPSS software (Version28.0: IBMCorp. Armonk, NY) on the single factor analysis of variance. Microbial data analysis using base dior online platform (https://www.omicshare.com/tools/home/report/reportvenn.html). Bacteria functional analysis using origin is analyzed. Correlation analysis was conducted to determine the relationship between bacterial classification characteristics and silage quality variables by calculating spearman correlation coefficient, And plotted by online analysis software (https://chiplot.online/). The significance was compared at the probability level of 0.05.

Results

Effects of inoculants on fermentation quality and chemical composition

The fermentation characteristics of the sorghum straw silage are shown in Table 2. After 60 days of ensiling process, the CP content in the LP treatment was significantly ($p < 0.05$) higher than that in other treatments. Compared with CK, ADF content in the additive treatment was decreased, and the lowest ADF content in the CEL treatment (35.92) was significantly ($p < 0.05$) lower than that in the CK treatment. The pH value of the LPCEL treatment was significantly ($p < 0.05$) lower than that of other treatments. Compared with the CK treatment, LA content in the CEL treatment was decreased, while that in the LP treatment and the LPCEL treatment was increased, with the highest content in the LPCEL treatment (40.59). The highest acetic acid (AA) content in the LPCEL treatment was significantly ($p < 0.05$) different from other treatments. The content of propanoic acid (PA) in the LPCEL treatment was the lowest (0.04), followed by the CEL treatment (0.29). The highest BA content was in the LPCEL treatment, followed by the CEL treatment. The lowest NH_3-N content in LP treatment (5.25) was significantly ($p < 0.05$) lower than that in other treatments.

Bacterial community analysis of forage sorghum straw silage

The α diversity of bacterial communities in fresh feed and silage samples was analyzed in Table 3. It showed the diversity of microbial communities under different treatments. Good's coverage of bacteria in all treatments reached 0.99, indicating that the sequencing depth was

Table 2 Research on fermentation quality of sorghum straw

Item	CK	LP	CEL	LPCEL	SEM	*p*-value
DM (%FM)	42.74a	41.31a	40.03a	40.98a	0.38	0.26
CP (%DM)	5.81b	8.11a	5.57b	4.64b	0.32	0.02
ADF (%DM)	39.27a	37.01ab	35.92b	38.48ab	0.75	0.04
NDF (%DM)	59.96a	57.78a	57.20a	56.19a	0.49	0.36
pH	3.68a	3.70a	3.63a	3.50b	0.04	< 0.001
Lactic acid (g/kg)	22.26c	25.11b	22.15c	40.59a	4.41	< 0.001
Acetic acid (g/kg)	4.65b	4.30b	5.45b	6.72a	0.54	0.01
Propionic acid (g/kg)	0.47b	0.79a	0.29c	0.04d	0.16	< 0.001
Butyric acid (g/kg)	0.59c	0.84c	1.18b	1.86a	0.28	< 0.001
NH_3-N/TN (%)	7.99a	5.25b	7.85a	8.30a	2.02	0.005

DM dry matter, *CP* crude protein, *ADF* acid detergent fibre, *NDF* neutral detergent fibre, *NH_3-N* ammonia nitrogen, *CK* control treatment, *LP L. plantarum* inoculated treatment, *CEL* cellulase inoculated treatment, *LPCEL* mixed additive treatment

The numbers; SEM, standard error of the mean.a, b, c indicating significant differences at $p < 0.05$ level

Table 3 Bacterial community diversity index of sorghum straw silage

Item	FM	CK	LP	CEL	LPCEL	SEM	*p*-value
Ace	676 ± 97.14c	1103 ± 452b	1127 ± 440.44b	1410 ± 99.73a	1309 ± 162.44a	93.605	0.015
Chao1	647 ± 82.43c	1022 ± 402.3b	1099 ± 435.29b	1351 ± 93.9a	1244 ± 134.88a	73.749	< 0.001
Simpson	0.58 ± 0.18b	0.91 ± 0.03a	0.92 ± 0.06a	0.86 ± 0.07a	0.84 ± 0.12a	0.041	0.014
Shannon	2.25 ± 0.56c	6.13 ± 0.30a	6.13 ± 0.80a	5.63 ± 0.66b	5.35 ± 0.9b	0.416	< 0.001
Coverage	> 0.99	> 0.99	> 0.99	> 0.99	> 0.99		

CK control treatment, *LP L. plantarum* inoculated treatment, *CEL* cellulase inoculated treatment, *LPCEL* mixed additive treatment. The numbers; SEM, standard error of the mean. a, b, c indicating significant differences at $p < 0.05$ level

suitable for bacterial community analysis in all treatments. Different additives had significant ($p < 0.05$) effects on Ace, Chao1 and Shannon indices. Ace, Chao1, Simpson and Shannon indexes in silage treatments were significantly ($p < 0.05$) higher than those in the FM treatments. The Shannon index of the LP and the CK treatment was significantly ($p < 0.05$) higher than that of the CEL and the LPCEL treatment. The Chao1 and Ace indices of the CEL and the LPCEL treatments were significantly ($p < 0.05$) higher than those of the CK and the LP treatments.

A partial least square discriminant analysis was used to compare the differences in β diversity of microbial communities between different treatments, and significant separations and differences were observed among the FM, CK, CEL, LP, and LPCEL sample sites (Fig. 1B). The figure shows the phylum level composition of the microbiome in FM and forage sorghum straw, with a total of 10 phyla detected (Fig. 1C). Firmicutes, Proteobacteria, Bacteroidota, Cyanobacteria, Actinobacteriota, Patescibacteria, Euryarchaeota, Acidobacteriota, Chloroflexi, Gemmatimonadota. Firmicutes, Proteobacteria, Bacteroidota, Cyanobacteria and Actinobacteriota were relatively abundant among different treatments. The dominant phylum of the FM treatment is Proteobacteria (77.71%). After the fermentation process, the level of primary bacteria shifted from Proteobacteria to Firmicutes, and Firmicutes dominated in the fermentation process. In addition, the relative abundance of Bacteroidota and Actinobacteriota increased, while that of Cyanobacteria decreased. Figure 1D shows the generic-level composition of bacterial communities in the FM and sorghum straw. There were 10 genera with relative abundance greater than 1% in the bacterial communities between different treatments. The relative abundance of *Lactobacillus* was the highest, followed by *Pantoea*. In the FM treatment, *Panthenium* was dominant. After 60 days of silage, *Pantoea* was significantly decreased, while *Lactobacillus* was significantly increased and dominated.

The LEfSe data reveal different classification features among different processes (Fig. 1E). Cyanobacteria and Proteobacteria were mainly concentrated in the FM. After the fermentation process, Firmicutes were enriched by the CEL treatment, actinobacteria and Bacteroidetes by the LP treatment, *Erwinia* and *Pantoea* by the FM. After the fermentation process, the *Prevotella* and *Lactobacillus* were enriched under the CEL treatment. A Venn diagram depicts the common and unique OTUs in different treatments. Totally 551 OTUs, as core genera, were shared by all treatments, at the same time, 99, 69, 51, 147 and 138 OTUs belong to the FM, CK, LP, CEL and LPCEL treatments, respectively (Fig. 1A).

Relationships between fermentation parameters and bacterial community

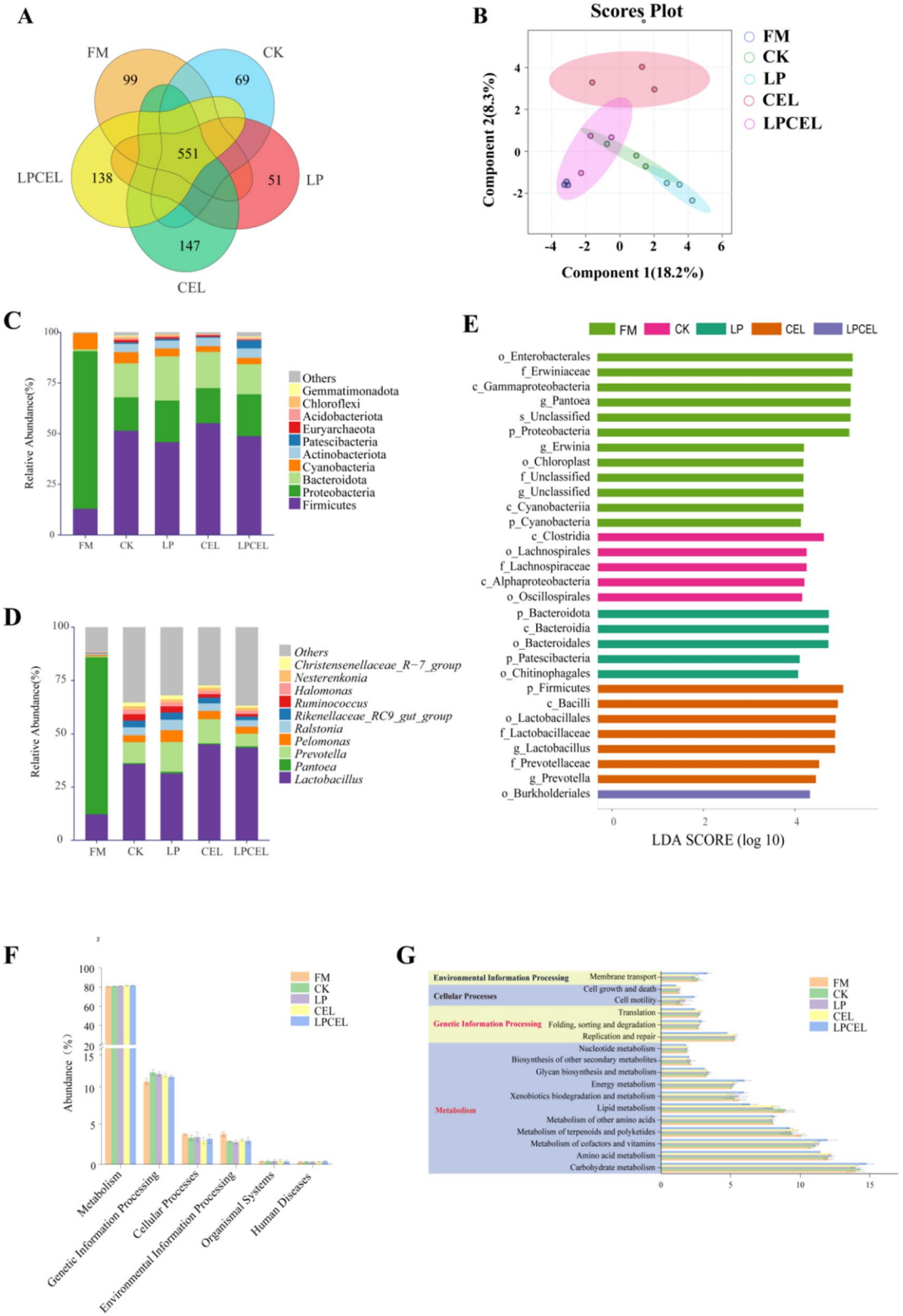
In the current study, the heatmap was used to evaluate the correlations between the microbial genus (Top 10) and fermentation profile based on Spearman analysis (Fig. 2). *Lactobacillus* showed a positive correlation with the content of LA, AA, and ADF (LA: rho = 0.593, $p = 0.020$; AA: rho = 0.517, $p = 0.048$; ADF: rho = 0.628, $p = 0.012$) and a negative correlation with the pH value (rho = -0.642, $p = 0.01$). *Erwinia* was positively correlated with pH (rho = 0.649, $p = 0.009$) and negatively correlated with CP (rho = -0.523, $p = 0.046$). *Ruminococcus* is negatively correlated with pH value and ADF content (pH: rho = -0.610, $p = 0.016$; ADF: rho = -0.677, $p = 0.006$) and positively correlated with NH_3-N and CP content (NH_3-N: rho = 0.629, $p = 0.012$; CP: rho = 0.736, $p = 0.002$). *Prevotella* (CP: rho = 0.778, $p = 0.001$; NDF: rho = -0.606, $p = 0.017$; pH: rho = -0.645, $p = 0.009$), *Rikenellaceae*_RC9_gut_group (CP: rho = 0.724, $p = 0.002$; NDF: rho = -0.736, $p = 0.002$; pH: rho = -0.689, $p = 0.004$), and *Halomonas* (CP: rho = 0.517, $p = 0.048$; NDF: rho = -0.536, $p = 0.039$; pH: rho = -0.672, $p = 0.006$) show a positive correlation with CP content and a negative correlation with NDF and pH.

Prediction of KEGG function in bacterial communities

The 16S rRNA gene predictive functional profiles describe the first (Fig. 1F), and second pathway levels (Fig. 1G). As shown in the Fig. 1F, the KEGG functional spectrum of sorghum stalk and KEGG in different treatments showed 6 different metabolic pathways. Mainly predicted genes and Metabolism, accounting for about 80% in FM and silage. Followed by Genetic Information Processing, Cellular Processes, Environmental Information Processing. The FM and silage samples 60 days after the second pathway level are shown in Fig. 1G. The top 17 abundant pathways were Environmental Information

(See figure on next page.)

Fig. 1 Effects of different additives on microbial community structure and functional prediction of sorghum straw. **A** Venn diagram representing the common and unique OTU found at fresh materials and silages. **B** Sparse partial least squares discriminant analysis (sPLS-DA). **C** Relative abundance at the level of the phyla. **D** Relative abundance at the bacterial genus level. **E** Comparison of microbial variation in mixed silage using LEfSe online tool. **F** Level 1 metabolic pathways; **G** Level 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog functional predictions of the relative abundances of the top 17 metabolic functions



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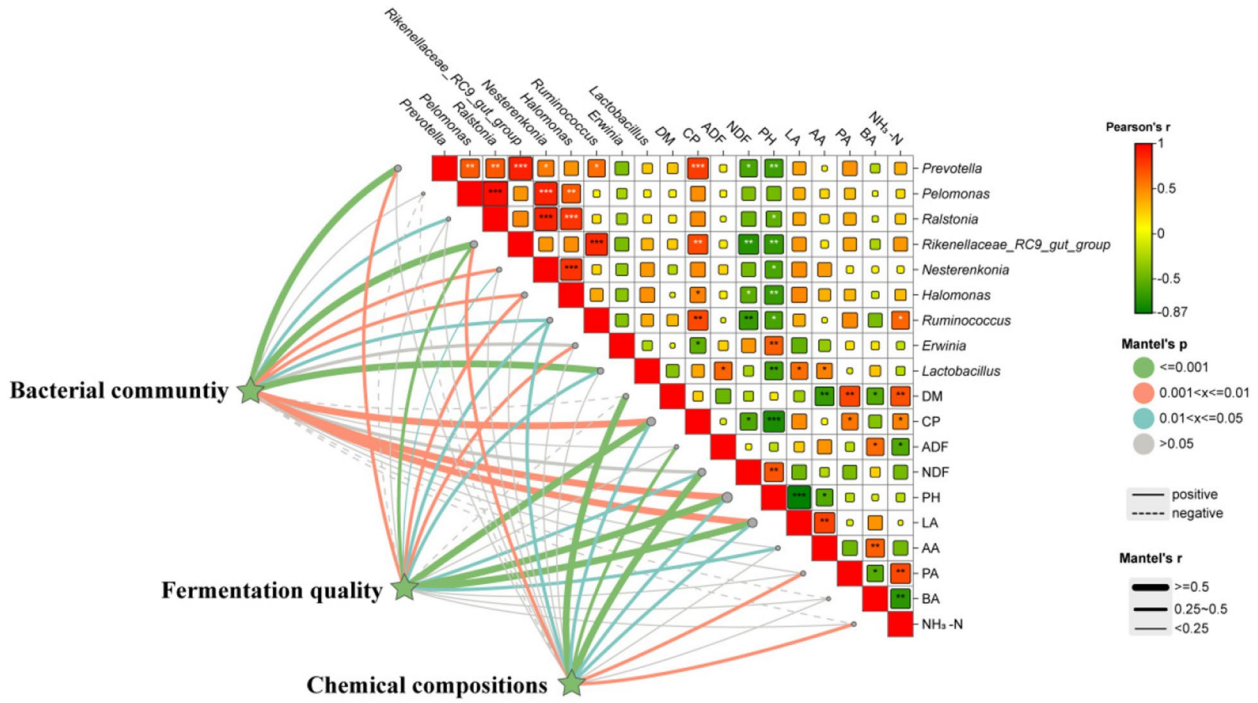

Fig. 2 Mantel analysis of nutrient and fermentation indexes of sorghum straw silage and bacteria levels. The positive correlation was indicated by red color, and negative correlation was indicated by green color. * Indicates significant at $p < 0.05$, ** indicates significant at $p < 0.01$ and *** indicates significant at $p < 0.001$. The square plot indicates the magnitude of the correlation coefficient, and the shade of the color indicates the importance of the correlation coefficient

Processing (1 pathway), Cellular Processes (2 pathways), and Genetic Information Processing (3 pathways) and Metabolism (11 pathways). Metabolism is obviously much more common than other pathways. Compared to other metabolic pathways, Carbohydrate metabolism, Amino acid metabolism, Metabolism of cofactors and vitamins, Metabolism of terpenoids and Terpenoids. The Metabolism of polyketides, Metabolism of other amino acids, and Lipid metabolism are the main metabolic pathways with high relative abundance.

Discussion

This experiment was to investigate the effects of *L. plantarum*, cellulase and their combination on fermentation quality and microbial community structure of sorghum straw silage, and to analyze the relationship between fermentation quality and microbial community dynamics, in order to provide a supplement for related fields.

Chemical and microbial compositions of sorghum straw

The crucial role in lactic acid fermentation is played by the WSC content in fresh feed. For optimal silage fermentation to be achieved, it is essential that the WSC content exceeds 5% DM [26]. The requirement is met by the WSC content of fresh sorghum feed, which is measured at 12.88%DM. However, compared to the previous findings [57], the WSC content observed in this study is found to be lower. This discrepancy may be attributed to the variability in soil salinity, which can influence the growth and metabolism of sorghum, and subsequently affect its soluble sugar content [62]. Furthermore, in comparison to the results presented by Sun et al. [48], the DM content in this study is higher, while the NDF and ADF contents are lower. This variation may be related to genetic differences among sorghum varieties, which can impact the composition of dry matter and fiber content [61]. The fermentation process was influenced by the number of LAB, with the LAB count being crucial to exceed 5.0 lg cfu/g FW in fresh materials for optimal fermentation [8]. In this study, a lower count of LAB (4.59 lg cfu/g FW) was observed, alongside higher levels of harmful microorganisms which exceeded 5.0 lg cfu/g FW, resulting in undesirable fermentation and end products. It is deemed essential that LAB be incorporated to clarify their role in silage fermentation and the shifts in bacterial populations that occur.

Effects of additives on the quality of sorghum straw silage

Parameters such as pH value, short-chain fatty acids and ammonia content of silage are commonly used as

indicators of silage quality. It is generally believed that in well-preserved silages, the pH value should be < 4.2 [31], and in this study all silages ended up with a pH between 3.50–3.70. This is due to the abundance of water-soluble carbohydrates and the number of epiphytic LAB and the low buffering energy in fresh grass. The final pH of LPCEL silage was lower than that of LP and CEL silage ($p < 0.05$). This may be due to a rapid increase in lactic acid. In addition, according to the previous report who found the ensiling treatment with cellulase combined with LAB was more successful in reducing pH than treatment with LAB or cellulase alone [25].

LAB additive can significantly increase the lactic acid concentration of silage feed [31]. LAB are fast and efficient producers of lactic acid. The addition of cellulase degrades silage cell walls, thereby increasing the availability of WSC as a lactic acid substrate. In this study, the lactic acid concentration of the mixed additive treatment was significantly increased [16, 57]. Ebrahimi et al. [16] research shows that after adding cellulase and cellulase plus *Lactobacillus*, pH value of oil palm leaves decreased, lactic acid content and IVDMD increased. This is consistent with the results of this study. It was concluded that the addition of cellulase effectively increased the substrate required for fermentation of LAB, thus promoting the metabolic activity of LAB [40, 44, 46]. This effect allows *L. plantarum* to more efficiently convert soluble sugars into lactic acid, resulting in the highest lactic acid concentration in the mixed additive treatment.

The concentrations of AA acid, propionic acid, butyric acid and NH_3-N in silages can reflect nutrient loss to a certain extent, and can be used as an indicator of fermentation quality of silages [24]. High concentrations of acetic acid may result in low energy and dry matter recovery [23]. Low acetic acid concentration is not conducive for aerobic stability of silages [9]. In this study, acetic acid concentrations in all treated silage ranged from 4.30 g/kg to 6.72 g/kg, which was sufficient to maintain aerobic stability of silage. The ratio of lactic acid to acetic acid in silages is greater than 3.0 for homofermentative fermentation and less than 3.0 for heterofermentative fermentation [2]. During silages, the ratio of lactic acid to acetic acid was always higher than 3.00, indicating that homogeneous fermentation was strong. Agarussi et al. [1] showed that the propionic acid content (< 6.00 g kg^{-1} in inoculated silage) at the end of fermentation was within the acceptable range of this acid, and the propionic acid content in this experiment was all lower than 1 g kg^{-1}, among which the propionic acid content in LP treatment was higher. At the same time, the relative abundance of *Prevotella* in LP treatment was also the highest. According to Russell and Rychlik [42] the fermentation products of *Prevotella* include succinate, acetate, formate and propionate. Therefore, the high relative abundance of *Prevotella* may be one of the reasons for the higher propionic acid content in the LP treatment.

The NH_3-N content in silage is an index reflecting the degree of protein degradation. In general, the NH_3-N content in high-quality silage should be less than 100 g kg^{-1} TN [31]. In this experiment, NH_3-N content met this requirement, and LP treatment had the lowest NH_3-N content in this experiment, which may be due to the fact that *L. plantarum* is a homologous fermentation LAB, which can rapidly increase LA in the early stage of silage, resulting in a sharp decline in pH value, rapidly inhibiting aerobic microorganisms and plant enzymes, resulting in reduced protein degradation in the fermentation process. Similarly, the study by Zahiroddini et al. [60] also showed that the rapid decline in the pH value of silage inhibits aerobic microorganisms and reduces the proteolytic activity of plant enzymes. The concentration of DM in silages treated with cellulase (CEL and LPCEL) was lower than that in silages treated with *L. plantarum*, which may be due to the fact that cellulase reduces structural carbohydrates in silages, promotes the production of lactic acid and increases the loss of DM. Wang et al. showed [53] that compared with silages supplemented with *L. plantarum*, the addition of cellulase reduced the content of DM in the whole corn and peanut vine mixed feed. The LP treatment has the highest CP content because *L. plantarum* is a homofermentative lactic acid bacterium that can produce lactic acid from glucose, pentose and 3-xylose through a variety of metabolic pathways, including the Emden-Meyerhoff pathway, phosphoketoolase pathway and pentose phosphate pathway [18, 34, 51]. This leads to a rapid increase in lactic acid content, which inhibits the growth and metabolism of harmful microorganisms such as *Clostridium* and *Aspergillus*, resulting in reduced protein degradation. This is consistent with previous studies [24, 58]. After ensiling for 60 days, the CP content of LPCEL treatment was the lowest, indicating that protein degradation occurred. This degradation may be the result of the combined action of cellulase and microbial metabolism [56]. Silages treated with cellulase (CEL) and cellulase (LPCEL) reduced structural carbohydrates (NDF, ADF), probably due to the co-hydrolysis of enzymes and acids. Mu et al. [33] found that the contents of NDF, ADF and HC were reduced, and the low pH value accelerated the hydrolysis of structural carbohydrates.

Effects of additives on bacterial community structure of sorghum straw silage

In essence, silage fermentation is a competitive process between LAB and undesirable microorganisms, so the bacterial community succession that determines

the quality of silage is worthy of attention [19]. In the fermentation process of sorghum straw silage, the 16S rRNA sequencing was used to analyze the diversity and composition of bacteria to comprehensively understand the changes in the whole process. All sequenced samples in this study achieved coverage of more than 0.99, indicating that most bacteria were detected by high-throughput sequencing techniques. Silage decreased α diversity of bacterial community of sorghum stalk, in which Shannon index was an index to determine species richness and evenness, and the influencing factor was species richness and evenness [13]. The higher the value, the higher the richness and evenness, and the lower the vice versa. In this experiment, the Shannon of the CEL and LPCEL treatments was significantly lower than that of the LP and CK treatments. The reason may be that cellulase degrades cellulose in sorghum straw and provides a large amount of fermentation substrate, resulting in the disappearance of anaerobic microorganisms that are not acid resistant [12], and the bacterial species are gradually occupied by beneficial bacteria such as *Lactobacillus*, which is consistent with the research results [28]. At the same time, Mendez-Garcia et al. showed that low pH conditions were the main factor in reducing microbial community diversity in acidic environments [32].

The β-diversity analysis was used to determine the difference of bacterial communities among different samples, and it was found that silages treatments with different additives had a significant impact on the microbial community structure of sorghum straw silages. In this study, we did not use traditional PCA analysis, but sparse partial least squares discriminant analysis (sPLS-DA). sPLS-DA combines sparsity and partial least squares regression (PLS) for discriminant analysis and is more effective than traditional PCA in identifying biological significance variables. By introducing sparsity constraints, sPLS-DA can simultaneously perform variable selection and model construction, thus ensuring better interpretability and stability of the model. The sPLS-DA map clearly showed the difference between the bacterial communities of the three treatments, and the results showed that the use of additives had a significant effect on the sorghum straw silage flora. The application of a single additive can effectively isolate the bacterial community, which is consistent with the previous report [27]. The inactivation of aerobic and acid-resistant apigenic bacteria during silage may be the main reason for the local separation of silage samples from fresh ones. The obvious change of bacterial community from Proteobacteria to Firmicutes before and after silage. This phenomenon is consistent with the study of Romero et al. [41]. Ma et al. believe that Proteus plays a key role in the degradation of organic matter, nitrogen and carbon cycles during anaerobic digestion, and is a phylum that is unfavorable to silage fermentation [30]. Firmicutes are acid-hydrolytic microorganisms that are critical in anaerobic environments, producing a variety of enzymes such as proteases, lipases, and cellulases, and the acidic and anaerobic conditions of silage are conducive to Firmicutes growth [21]. In this study, Firmicute became the dominant phyla after 60 days of silage, especially in the CEL treatment with cellulase added, its relative abundance was the highest, reaching 55.16%. This is consistent with the findings of Pankratov et al., who suggested that Firmicutes play a major role in the degradation of lignocellulose [37]. In addition, the study of Ma et al. found that the relative abundance of Firmicutes reached a maximum on day 21 in both membrane-covered and non-membrane-covered conditions, which was consistent with the high degradation rate of cellulose, indicating that the degradation rate of cellulose was proportional to the abundance of Firmicutes [30]. This view is further proved.

At the genus level, the microbial quantity of fresh feed and sorghum straw silage changed significantly. *Pantoea* is the dominant strain in FM, which is gradually replaced by *Lactobacillus* after silage. *Pantoea* is the most common facultative aerobic genus in FM. *Pantoea* has been observed in fresh native grasses [58], alfalfa [43], and soybeans [35]. *Pantoea* abundance decreased after additive treatment, which may be due to their high sensitivity to pH decline [36]. This study found that compared with CK treatment, the abundance of *Lactobacillus* in LP treatment decreased, while CEL and LPCEL increased in value. It may be that the increased consumption of certain substrates by *Pelomonas* and *Prevotella* in pre-silage slowed down the relative abundance of *Lactobacillus* in pre-silage. However, sorghum straw is rich in a large amount of fiber, and the addition of cellulase can destroy the cellulose cell wall and release a large amount of contents in the cell, resulting in more adequate fermentation substrate for *L. plantarum* under anaerobic conditions. In turn, the relative abundance of *Lactobacillus* increases, leading to an increase in LA content. In addition, silage can increase the relative abundance of *Prevotella*. *Prevotella* can produce organic acids such as lactic acid and acetic acid through glycolysis, and reduce the pH value of silage. Witzig studies have shown that the abundance of *Prevotella* increases as the proportion of forage silage increases, which may be due to the higher concentration of crude protein in these feeds [55]. In this study, CP content was positively correlated with the relative abundance of *Prevotella* among all treatments, which was consistent with the study results.

Therefore, this experiment once again confirmed the mechanism of action of lactic acid bacteria: Lactic acid bacteria grow and reproduce rapidly in a closed

environment, producing a large amount of lactic acid. After an acidic anaerobic environment is formed, aerobic bacteria such as *Pantoea* are gradually replaced by lactic acid bacteria. Subsequently, lactic acid bacteria form a dominant flora, converting more carbohydrates into lactic acid and other organic acids (such as acetic acid and propionic acid), lowering the pH of silage to a greater extent. This achieves the purpose of inhibiting the growth and reproduction of more anaerobic spoilage microorganisms and minimizing the loss of nutrients, thus enabling long-term preservation of the feed.

To illustrate the relationship between the identified microorganisms and the measured silage products, heat maps were used to show the 10 most influential microbial genera of bacteria according to Spearman analysis. Consistent with the research results, LA content is significantly positively correlated with *Lactobacillus*, while DM, NH_3-N, pH are negatively correlated with *Lactobacillus* [17]. *Erwinia* is significantly positively correlated with pH because an acidic environment (pH < 5.40) may inhibit *Erwinia* abundance under anaerobic conditions. In this study, *Ruminococcus*, *Prevotella* and *Rikenellaceae*_RC9_gut_treatment showed significant negative correlation with pH and NDF, and significant positive correlation with CP. *Ruminococcus*, *Prevotella* and *Rikenellaceae*_RC9_gut_treatment are considered to be major cellulose degraders in rumen and play a key role in cellulose degradation of rumen microbial community [49]. *Ruminococcus* was originally isolated from ruminant rumen and also exists in other ruminants and non-ruminants. It mainly decomposes cellulose, produces methane, and accumulates glucose in cytoplasm as an iodophilic polymer [5]. *Prevotella* can produce organic acids such as lactic acid and acetic acid through glycolysis to reduce the pH value of silage. Therefore, it is speculated that *Ruminococcus* also has this function [9]. Tang and Yu demonstrated that *Rikenellaceae*_RC9_gut_treatment plays an important role in the degradation of coarse fibers and rumen epithelial morphological structure. It can produce acetic acid and propionic acid and reduce pH and fiber content in the environment, which is consistent with the results of this study [49, 59].

It is important to determine the predictive functional properties and metabolic pathways of the microbiota during silage. Based on the KEGG database, PICRUST evaluated the predictive functional spectrum of grade 1 to 2 bacterial communities. At the initial level of the pathway, "metabolism" appears as the primary metabolic pathway (Fig. 1F). This suggests that bacteria use fermentable substances to convert into multiple metabolites during silage fermentation, leading to an increase in the generality of metabolic pathways. The fermentation process in silage is dominated by the activity of microorganisms that break down substrates or alter metabolites through a series of complex metabolic pathways. This study revealed that adding *L. plantarum* and cellulase could enhance the metabolic capacity of water-soluble carbohydrates (WSC) in silage, which was consistent with the results of Si study [45]. Amino acids are essential substances for human body and play an important role in promoting primary metabolism and protein synthesis in plants. In this study, bacterial enzymes combined with Amino acid metabolism were inhibited, which was consistent with the results of Wang et al. [54]. The relative abundance of "membrane transport" of the bacterial enzyme combination is higher than that of other treatments, followed by CK treatment, which is inconsistent with the previous findings who found that untreated silage has a higher abundance of transporters, and the different results may be caused by different raw materials [45]. Previous reports show that nucleotides can be used to produce and replicate RNA and DNA and serve as the primary source of energy for biological functions [22]. In this experiment, nucleotide metabolism had no significant effect before and after ensiling. The relative abundance of CEL processing was the highest in "replication and repair".

Conclusion

The results showed that the use of cellulase and *L. plantarum* not only improved the nutritional value and silage quality of sorghum straw, but also reduced the harmful bacteria and increased the beneficial bacteria by regulating the microbial community structure, thus affecting the stability of silage. When the two additives were combined, the lactic acid content and pH value were significantly increased. The results showed that combined use had synergistic effect on promoting LA fermentation and improving silage quality. In the future, adding *L. plantarum* and cellulase mixture into silage would be a potential way to improve the quality of silage.

Abbreviations

CP	Crude protein
DM	Dry matter
WSC	Water soluble carbohydrate
NDF	Neutral detergent fibre
ADF	Acid detergent fibre
LA	Lactic acid
AA	Acetic acid
PA	Propionic acid
BA	Butyric acid
CFU	Colony forming units
LAB	Lactic acid bacteria
OTU	Operational taxonomic unit
sPLS-DA	Sparse partial least squares discriminant analysis
rDNA	Ribosomal DNA

Acknowledgements

The author thanks all the authors in the article for their help during the experiment and the writing of the paper. Special thanks go to Associate

Professor Jiang Chao for providing the sorghum seed resources, and Professor Xiao Yanzi, Du Shuai and You Sihan for their guidance and review during the paper—writing process.

Authors' contributions
He Lichao and Jiang Chao: Conceptualization, Data curation, Investigation, Writing – original draft, Software, Validation. Dong He, Wang Yinuo, Tang Jiaxin, Hu Mengjie and Luo Junjie: Investigation. Du Shuai: Writing – review and editing. Jia Yushan: supervision. You Sihan: Writing – review and editing and Funding acquisition. Xiao Yanzi: Funding acquisition.

Funding
This work was supported by Inner Mongolia Nature Foundation Project (2024LHMS03022), Inner Mongolia Autonomous Region Science and Technology Innovation Guidance Award Fund Project (2022CXYD006), Hulunbuir Science and Technology Bureau (NC2023022) and Hulunbuir University Project (2023XJWT03, 2022XKJSZD06).

Data availability
Sequence data that support the findings of this study have been deposited in the NCBI's Sequence Read Archive under Bio-Project accession number PRJNA 1248379.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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Received: 10 February 2025 Accepted: 21 April 2025
Published online: 16 May 2025

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