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Lactobacillus cocktail and cellulase synergistically improve the fiber transformation rate in *Sesbania cannabina* and sweet sorghum mixed silage

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Abstract

Background Elucidating the mechanism of fiber transformation underlying microbial metabolism is critical for improving fiber-rich silage digestibility and preserving silage energy for ruminant nutrient absorption. However, few studies have combined quantitative microbial function and transformation products in silage to explain this mechanism. Here, we constructed a workflow to detect the substrates and products of fiber transformation in mixed silage of *Sesbania cannabina* and sweet sorghum (SS) and combined the absolute quantification 16S rRNA sequencing to reveal this mechanism.

Results The synergistic effect of *Lactobacillus* cocktail and cellulase (LC) simplified the microbial diversity and minimized the microbial quantity, making *Lentilactobacillus buchneri* the dominant species in SS silage. As a result, the LC-treated silage had greater lactic acid content, lower pH value, and less NH₃-N content. The indigestible fibers were significantly decreased due to the synergistic effect of the *Lactobacillus* cocktail and cellulase. Changes in microbial structure during ensiling also resulted in metabolic alterations. The increased levels of microbial enzymes, including β -glucosidase and sucrose phosphorylase, involved in starch and sucrose metabolism led to the enrichment of monosaccharides (including glucose, xylose, mannose, galactose, ribose, rhamnose, and arabinose) in the LC-treated silage. We found that *L. buchneri* was positively associated with β -glucosidase and sucrose phosphorylase, reflecting the crucial contribution of *L. buchneri* to fiber decomposition in SS silage.

Conclusion Using an absolute quantitative microbiome, we found that LC treatment decreased the microbial biomass in SS silage, which in turn promoted the energy preservation in the SS silage. The cooperative interaction of the *Lactobacillus* cocktail and cellulase improved the fiber decomposition and in vitro dry matter digestibility rate by changing the microbiome structure and function in the SS silage, providing guidance and support for future fiber-rich silage production in the saline-alkaline region.

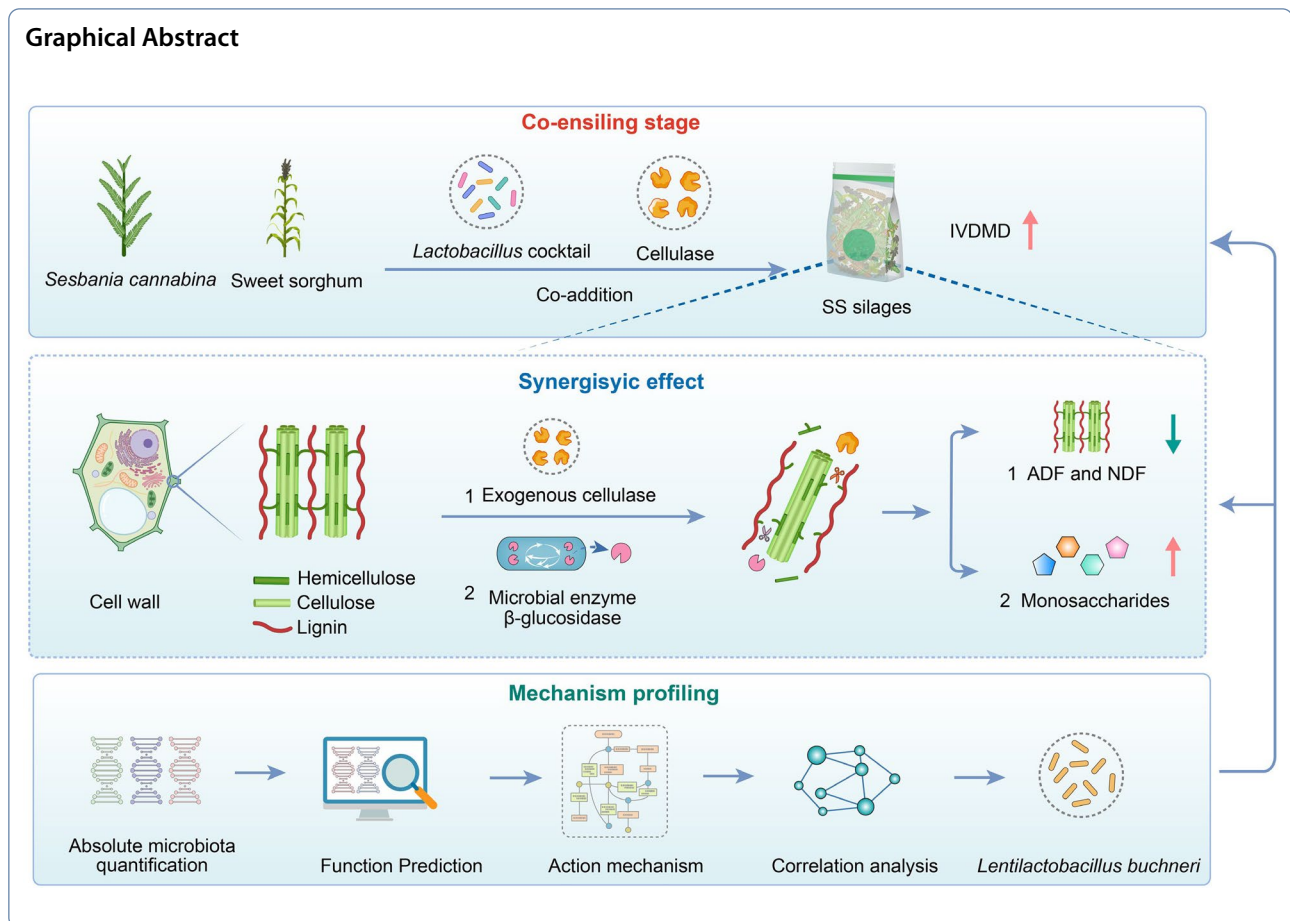
Keywords *Lactobacillus*, Cellulase, Soluble sugar, Fiber transformation

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Graphical Abstract



Introduction

The rising global price of soybean meal has sparked renewed interest in alternative protein feeding strategies for dairy cows. Increasing the amount of protein-rich forage in the diet is an efficient strategy for improving the energy and protein status of dairy cows. *Sesbania cannabina* plays an indispensable role in providing protein for ruminants, especially in saline-alkaline regions, owing to its rich protein content (25% dry matter [DM]), high productivity, and adaptation to nutrient-poor habitats [1]. However, *Sesbania cannabina* silage contains 50–60% DM indigestible fiber, limiting its efficient utilization of protein [2]. Therefore, understanding the mechanism of fiber transformation to improve silage digestibility is crucial for enhancing ruminant performance.

Sesbania cannabina is a pioneer forage that is widely cultivated in coastal saline-alkali soil due to its high biomass accumulation ($45 \text{ t ha}^{-1} \text{ year}^{-1}$) and ability to thrive under conditions of high salinity, waterlogging, and drought [1]. Nevertheless, the utilization of *Sesbania cannabina* as a silage is limited due to its low

water-soluble carbohydrate (WSC) content ($<5\% \text{ DM}$) and high buffer capacity, which often leads to silage fermentation failure. In our previous study, the co-ensiling of *Sesbania cannabina* with sweet sorghum, which contains abundant WSC, achieved good fermentation quality [2]. However, the presence of highly indigestible fiber creates obstacles for further development of mixed silage.

The indigestible fibers in forages typically crosslink with the plant cell wall, which are highly resistant to degradation. This results in reduced feed intake by filling the rumen and prolonging chewing time, which can also influence microbial efficiency in the rumen [3, 4]. Cellulase is a common additive used to improve fiber digestibility and can accelerate fermentation by releasing soluble carbohydrates and potentially enhancing animal performance [5]. Furthermore, silage inoculants, such as ferulic acid esterase (FAE)-producing bacteria, have the potential to break down the bonds between lignin and structural polysaccharides of the forage cell wall, thereby improving silage nutrition and feed conversion efficiency [6, 7]. Numerous studies have demonstrated that the

application of lactic acid bacteria (LAB) inoculants and cellulase can enhance the fermentation quality of silage [8–10], but few studies have focused on soluble sugar accumulation in mixed silage of *Sesbania cannabina* and sweet sorghum (SS). Moreover, previous studies revealed that the combination of LAB and cellulase improved the in vitro dry matter digestibility (IVDMD) in King grass and alfalfa silage [11, 12]. However, the impact of the cellulase and *Lactobacillus* combination on fiber digestibility in SS silage and the mechanism behind fiber transformation by microbial-cellulase synergy action remain to be determined.

Ensiling is driven by microorganisms, which are capable of improving silage fermentation quality and feed nutrition [13]. To optimize silage utilization, it is important to characterize the metabolic functions of microorganisms engaged in silage fermentation, as the ensiling process is influenced by microbial metabolism to transform substrates [14]. However, the quantifiable role of microorganisms and genetic function in the fiber transformation of SS silage remain unknown. Based on relative abundance, high-throughput 16S rRNA sequencing was used to determine the proportions of different taxa in the microbial community. However, it is difficult to determine whether an increase in the relative abundance of a taxon is due to blooming or a decline in other taxa [15]. To address this challenge, it is necessary to use absolute quantification 16S rRNA sequencing (AQ-16S-seq). Herein, AQ-16S-seq was performed to capture absolute microbial changes and quantify microbial metabolism variation involved in fiber transformation in SS silage.

In this study, a combination of a *Lactobacillus* cocktail (containing a FAE-producing strain) and cellulase was applied to ensile SS silage. We hypothesize that the combined additive may enhance fiber degradation to release more soluble sugars to preserve more silage energy for ruminants. This study aimed to elucidate the mechanism of *Lactobacillus* on fiber transformation through the use of a quantitative microbiome, which is crucial for improving the utilization of high-fiber and protein-enriched silage forages by ruminants.

Materials and methods

Materials and silage preparation

Sesbania cannabina (Lujing 5, at the pod-bearing stage) and sweet sorghum (Ketian 14, at the milk-ripe stage) were obtained from the experimental field of the Yellow River Delta Modern Agricultural Technology Innovation Center, Dongying, Shandong Province, China (37°67' N; 118°90' E) on September 9, 2021. The harvested forages were cut into a particle size of 2.0 cm by a crop chopper. The *Sesbania cannabina* and sweet sorghum (SS) materials were mixed at a ratio of 7:3 under 45% DM. The

strains of *Lactiplantibacillus plantarum* B90 (CGMCC No. 13318), *Companilactobacillus farciminis* GMX4 (CGMCC No.19434), *L. buchneri* NX205 (CGMCC No. 16534), and *Lentilactobacillus hilgardii* 60TS-2 (CGMCC No. 19435) were used as compound LAB inoculants. Three treatments were used: CK, sterilized water; LAB; and LC, *Lactobacillus* Cocktail + 0.1% w/w cellulase. Each microbe in the *Lactobacillus* cocktail was sprayed onto the SS silage at a concentration of 10^6 cfu/g fresh weight, while an equal amount of sterile water was sprayed on the CK group. The 500 g of treated forages were packed into vacuum-sealed polyethylene plastic bags (dimensions 225×350 mm) and vacuum-sealed with a vacuum machine (DZ-AS, 2500KW, ANSEN, Fujian, China), followed by 60 days of ensiling at room temperature.

Chemical characteristics analysis

The fermentation quality analysis was performed according to previously reported method [2]. Briefly, 10 g of silage sample was homogenized with 90 ml of sterilized water and shaken for 30 min. The pH of the resulting silage extract was immediately measured using a glass electrode pH meter (pH 213; HANNA; Italy). The quantitative analysis of organic acids, including lactic acid (LA), acetic acid (AA), and butyric acid (BA), was performed by high-performance liquid chromatography (HPLC-treated, 1200, Agilent, California, America) [16]. The mobile stage proceeded at 55 °C: 0.005 M H₂SO₄ was added at a flow rate of 0.6 mL/min. The ammonia-N (NH₃-N) content was analyzed using ninhydrin colorimetric and phenol-hypochlorite methods according to previously reported literature [17]. The ensiled samples were dried in an air-forced oven at 65 °C until a constant weight was reached to determine the DM content. The crude protein (CP), neutral detergent fiber (NDF), and acid detergent fiber (ADF) contents were measured with reference to the Association of Official Analytical Chemists [3], while the WSC content was determined according to a previously reported method [18].

In vitro dry matter digestibility analysis

The in vitro ruminal incubation and sampling procedures were performed according to previously described methods [19]. Briefly, rumen fluid was collected from healthy goats and filtered through four layers of sterile cheesecloth into a pre-warmed insulated bottle. The ground samples of dried silage were incubated in rumen fluid for 72 h at 39 °C. Then the reaction solution was filtered into crucibles and dried at 105 °C for 24 h to measure the IVDMD [12].

Carbohydrate composition analysis

The carbohydrate composition was analyzed by pre-column derivatization high-performance liquid chromatography with slight modifications [20]. Briefly, the monosaccharides (glucose, fructose, mannose, glucosamine, galactosamine, ribose, rhamnose, galactose, arabinose, and xylose) and disaccharides (sucrose and maltose) of silage were extracted by ultrasonication. Then approximately 5 g/L samples were extracted three times with chloroform and filtered. Mixed monosaccharide and disaccharide standard solutions were prepared. Finally, the samples and standard solutions were injected into a Sugar-Pak I column (6.5×300 mm) for analysis by HPLC (Waters 1525) using a differential refractive index detector.

Absolute quantification of 16S sequencing

The microbial DNA of the samples was extracted with a soil DNA Kit (Yeasen, Shanghai, China) in compliance with the manufacturer's guidelines. The V3–V4 regions of the bacterial 16S rRNA gene were amplified with the primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'). Absolute quantification of 16S-seq was performed by Genesky Biotechnologies Inc. (Shanghai, China). Briefly, appropriate proportions of spike-in sequences with known gradient copy numbers were added to sample DNA and then sequenced by Illumina NovaSeq 6000 instrument. The detected spike-in read counts were used for normalization of different DNA samples. Thus, the microbial taxa and functional genes were quantified.

Illumina MiSeq sequence processing and functional analysis

Sequence processing and functional analysis were performed in compliance with previously described methods [21]. Briefly, the raw sequences were processed using QIIME1. The high-quality sequences were clustered into operational taxonomic units (OTUs), and then the OTUs were distributed to different taxonomic categories based on the SILVA database. Bacterial community richness and diversity were assessed with the Shannon, Simpson, Chao 1, ACE, and Goods coverage indices using the UPARSE pipeline [22]. The similarity between bacterial communities was estimated by principal coordinates analysis (PCoA) based on the Bray–Curtis measure. The functional profiles of the microbial communities were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) according to previous reports [23].

Statistical analysis

The statistically analyzed data are shown as the means±standard deviations (SD). The data were analyzed using GraphPad Prism (version 8.0.2, GraphPad Software, San Diego, California, USA). One-way analysis of variance (ANOVA) and Pearson correlation analysis were used to evaluate the differences between operational phases in the CK, LAB, and LC groups. The correlations between the bacterial taxonomic profile and carbohydrates, functional genes, and fermentation indices were analyzed and plotted with R 4.0.2 software packages. Bioinformatic analysis was performed with the OmicStudio tool at <https://www.omicstudio.cn/tool> [24]. Circos plots were generated with Cytoscape 3.7.2. The LEfSe profile was generated by Huttenhower Lab Galaxy Server 2.0 at <http://galaxy.biobakery.org/> [25].

Results

Chemical characteristics and carbohydrates composition of fresh materials

The chemical characteristics and carbohydrates compositions of fresh SS are shown in Table 1. The CP, WSC, NDF and ADF contents were 10.05% DM, 4.40% DM, 52.80% DM, and 65.90% DM, respectively, whereas the IVDMD

Table 1 Chemical characteristics and carbohydrates composition of fresh *Sesbania cannabina* and sweet sorghum (SS) before ensiling

Items	Fresh SS
Chemical composition	
CP (%DM)	10.05
WSC (%DM)	4.40
ADF (%DM)	52.80
NDF (%DM)	65.90
IVDMD (%)	42.13
Carbohydrates composition	
Glucose (mg/g)	7.60
Fructose (mg/g)	7.56
Mannose (mg/g)	0.33
Glucosamine (mg/g)	2.42
Ribose (mg/g)	0.06
Rhamnose (mg/g)	0.06
Aminogalactose (mg/g)	0.00
Galactose (mg/g)	0.11
Xylose (mg/g)	0.00
Arabinose (mg/g)	0.09
Sucrose (mg/g)	0.76
Maltose (mg/g)	0.69

Data are represented as the mean of three replicates

SS *Sesbania cannabina* and sweet sorghum, CP crude protein, WSC water-soluble carbohydrates, ADF acid detergent fiber, NDF neutral detergent fiber, IVDMD in vitro dry matter digestibility

was 42.13%. The contents of glucose, fructose, mannose, glucosamine, ribose, rhamnose, galactose, and arabinose were 7.60 mg/g, 7.56 mg/g, 0.33 mg/g, 2.42 mg/g, 0.06 mg/g, 0.06 mg/g, 0.11 mg/g, and 0.09 mg/g, whereas the contents of sucrose and maltose were 0.76 mg/g and 0.69 mg/g, respectively.

Additives improved the fermentation and nutritional quality of SS silage

The pH of silage is a critical factor during ensiling, and a pH value below 4.2 is desirable for producing high-quality silage [26]. In this study, all the silages had pH values below 4.06. However, the LAB- and LC-treated silages had significantly lower pH values (3.93 and 3.89, respectively) than the CK group (Fig. 1A). The LAB- and LC-treated silages had the higher content of LA (6.41 and 6.51% DM, respectively), compared with the CK silage (5.45% DM) (Fig. 1B), while the AA content was substantially different among silages (Fig. 1C). The $\text{NH}_3\text{-N}$

is an important index for evaluating fermentation quality. The LAB- and LC-treated silages resulted in lower $\text{NH}_3\text{-N}$ contents than that of the CK silage. More specifically, the LC-treated silage showed substantial lower ($p < 0.001$) $\text{NH}_3\text{-N}$ content of 0.04% DM compared to others (Fig. 1D).

The DM content is a crucial index for the nutritional preservation of forages. In this study, the DM content was not influenced by the additives (Fig. 1E). The CP is an essential nutrient for the ruminants. The LC-treated silage showed significantly higher content ($p < 0.05$) of CP (10.22% DM) compared to other silages (Fig. 1F). The WSC is an important substrate for the LAB fermentation. The WSC content was significantly greater in the LC-treated silage compared to the LAB and CK silages (Fig. 1G). The LAB- and LC-treated silages resulted in substantially lower ($p < 0.05$) NDF and ADF contents compared to the CK silage (Fig. 1H, I). Importantly, the NDF content in LC-treated silage (60.94% DM) was substantially lower compared to the LAB-treated silage

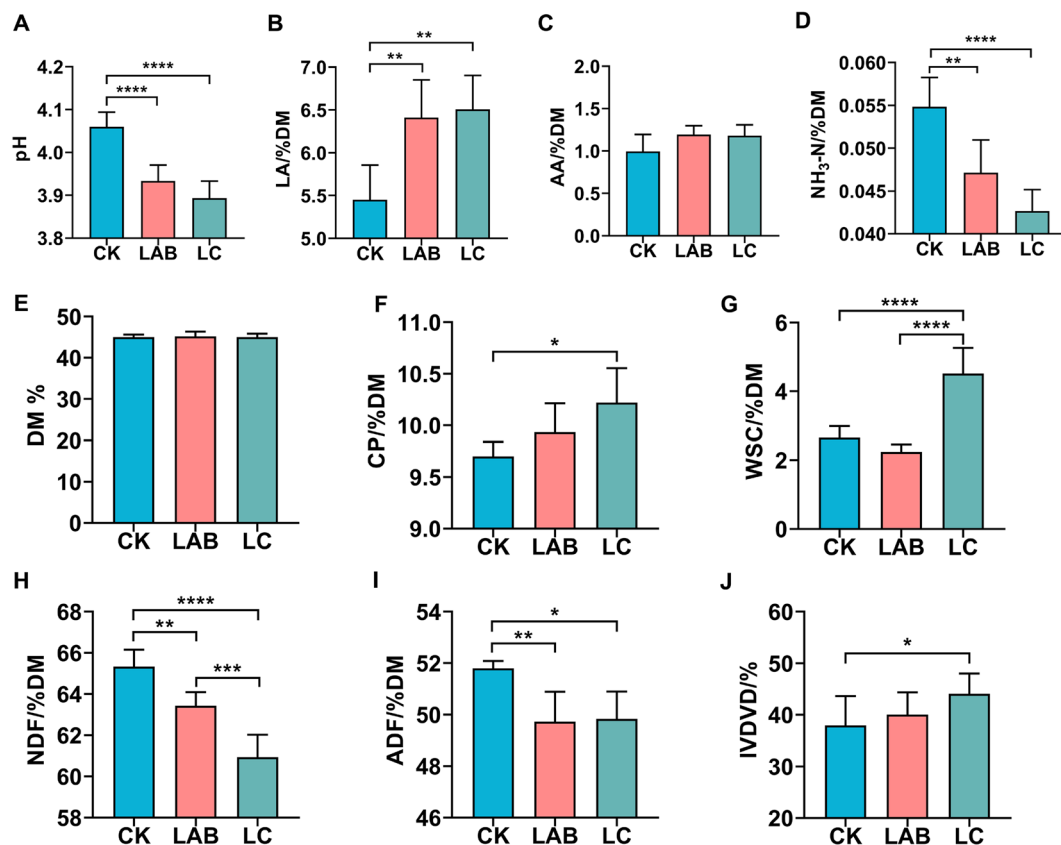


Fig. 1 Fermentative and chemical characteristics of SS silage in response to additives. **A** pH; **B** lactic acid content; **C** acetic acid content; **D** $\text{NH}_3\text{-N}$ content; **E** dry matter; **F** crude protein content; **G** water-soluble carbohydrate content; **H** neutral detergent fiber content; **I** acid detergent fiber content; **J** in vitro dry matter digestibility. CK: sterilized water; LAB: *Lactiplantibacillus plantarum* B90 + *Companilactobacillus farciminis* GMX4 + *Lentilactobacillus buchneri* NX205 + *Lentilactobacillus hilgardii* 60TS-2; LC: LAB + 0.1% w/w cellulase. Bars represent the standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ versus the CK group

(63.70% DM). The IVDMD is a key indicator of nutrient intake by ruminants. The LC-treated silages had substantially ($p < 0.05$) greater IVDMD (44.08%) than the CK silage (Fig. 1J). Taken together, both LAB and LC additives improved the fermentation quality and nutritional quality, but LC additive substantially decreased the indigestible fiber content in SS silage.

Additives improved the soluble sugar content of SS silage

During ensiling, carbohydrates serve as the primary source of energy, and the extent of silage fermentation is directly related to the concentration of soluble carbohydrates in the material. Hence, the contents of monosaccharides (glucose, fructose, mannose, glucosamine, galactose, ribose, rhamnose, xylose, aminogalactose, and arabinose), and disaccharides (sucrose and maltose) were determined (Fig. 2). Compared with the other silages, the LC-treated silage had the highest sugar content (Fig. 2A). In addition, the LC-treated silage exhibited significantly ($p < 0.05$) higher levels of glucose (13.45 mg g⁻¹), mannose (0.61 mg g⁻¹), galactose (0.50 mg g⁻¹), ribose (0.21 mg g⁻¹), rhamnose (0.34 mg g⁻¹), xylose (0.72 mg g⁻¹), and arabinose (0.11 mg g⁻¹) than other silages (Fig. 2B–H). However, the sucrose content in

the LC-treated silage was significantly ($p < 0.05$) lower (1.31 mg g⁻¹) than that in the other silages (Fig. 2L). There were no significant differences in the contents of glucosamine, fructose, aminogalactose, or maltose between the LC-treated silage and the other silages. Taken together, the LC additive substantially increased most of the monosaccharide contents in the SS silage.

Additives altered the microbial community and metabolism in SS silage

To investigate whether the inoculation of a *Lactobacillus* cocktail and cellulase mediates carbohydrate alterations during ensiling, AQ-16S-seq was conducted to assess the dynamic changes in the microbial community. The PCoA analysis of the microbial community structure revealed significant differences among the different groups (Fig. S1A). The LAB- and LC-treated silages showed the lower Shannon indices compared to CK silage and fresh materials (Fig. S1B). After ensiling, the absolute abundance of the total bacteria decreased in all silages compared to that in the fresh forages (Fig. S1C). The quantity of total bacteria in the LC group decreased by 83.82% and 90.93% compared to that in the fresh SS groups, respectively. After ensiling, *Lactobacillus* replaced the *Weissella*

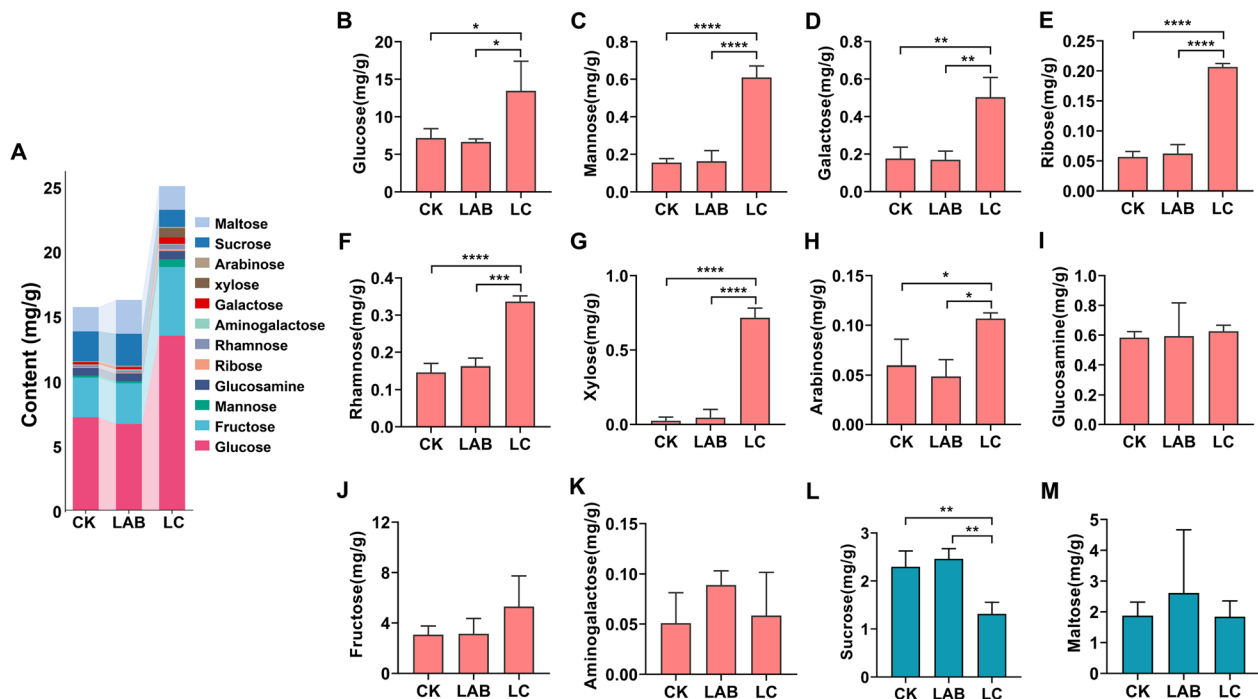


Fig. 2 Carbohydrate composition of SS silage in response to additives. **A** Overview of carbohydrate content in SS silage; **B** glucose content; **C** mannose content; **D** galactose content; **E** ribose content; **F** rhamnose content; **G** xylose content; **H** arabinose content; **I** glucosamine content; **J** fructose content; **K** aminogalactose content; **L** sucrose content; **M** maltose content. CK: SS silage inoculated with sterilized water; LAB: SS silage inoculated with *Lactiplantibacillus plantarum* B90 + *Companilactobacillus farciminis* GMX4 + *Lentilactobacillus buchneri* NX205 + *Lentilactobacillus hilgardii* 60TS-2; LC: SS silage inoculated with LAB + 0.1% w/w cellulase. Bars represent the standard error of the mean. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ versus the CK group

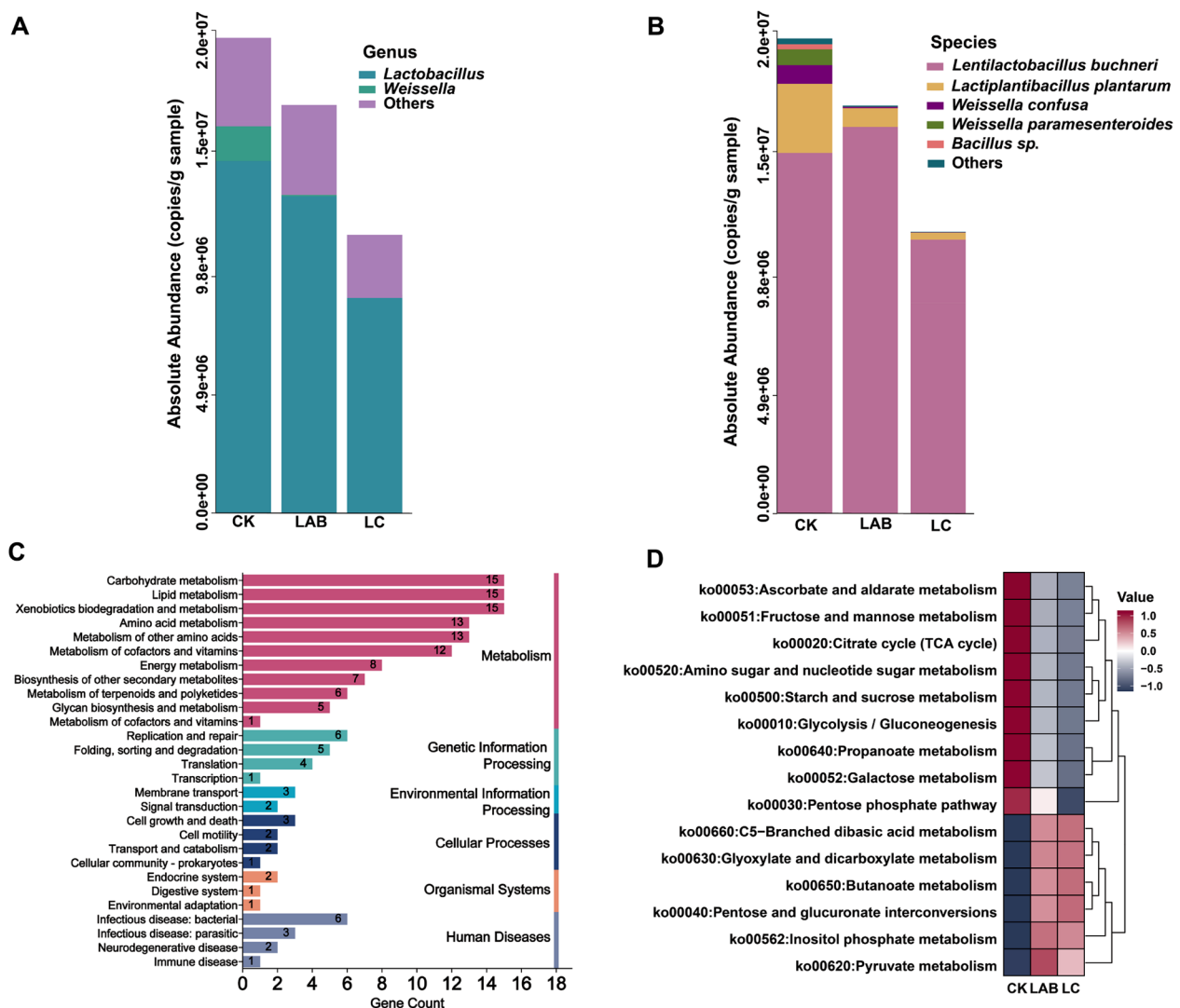


Fig. 3 Microbiome structure and functional profile of SS silage in response to additives. **A** Absolute abundance of the identified microbiota at the genus level in the different groups; **B** absolute abundance of the identified microbiota at the species level in the different groups; **C** metabolic pathways at KEGG levels 1 and 2 predicted via PICRUSt; **D** predicted metabolic pathway of carbohydrate metabolism at KEGG level 3. CK: SS silage inoculated with sterilized water; LAB: SS silage inoculated with *Lactiplantibacillus plantarum* B90 + *Companilactobacillus farciminis* GMX4 + *Lentilactobacillus buchneri* NX205 + *Lentilactobacillus hilgardii* 60TS-2; LC: SS silage inoculated with LAB + 0.1% w/w cellulase

in fresh *Sesbania cannabina* and sweet sorghum and became the dominant bacteria (Fig. S1C). Compared with those of the CK silage, the LAB and LC treatments further led to a decrease in the amounts of *Weissella* (Fig. 3A). At the species level, *L. buchneri* became the most abundant species in all the silages after fermentation (Fig. 3B), followed by *L. plantarum*. Specifically, the absolute quantities of *L. buchneri* in the LAB- and LC-treated silages were 1.60×10^7 copies/g sample (accounting for 94.80%) and 1.14×10^7 copies/g sample (accounting for 97.19%), respectively (Fig. 3B). This result highlights that both LAB and LC additives had a

substantial effect on the microbial composition, and *L. buchneri* may be the key beneficial microbe that competes with undesirable microorganisms.

To reveal the mechanism of the transformation of indigestible fiber into soluble carbohydrates under *Lactobacillus* action, functional pathways were predicted based on KEGG databases. Our study revealed that the main predicted microbiome functions were strongly associated with metabolism, especially carbohydrate metabolism, xenobiotics biodegradation and metabolism, and lipid metabolism (Fig. 3D). A heatmap for carbohydrate metabolism at the KEGG

3rd level was constructed (Fig. 3E). The pathways Ko00040, Ko00630, Ko00650, and Ko00660 were more enriched in LAB- and LC-treated silages, especially in LC-treated silage. These results indicate that both LAB and LC treatment altered microbial metabolism, especially that of LC.

Microbial metabolism contributes to carbohydrate alteration

To gain a better understanding of the role of microbial enzymes in soluble carbohydrate formation, a metabolic scheme for carbohydrate metabolism was reconstructed to analyze the genes involved in carbohydrate transformation (Fig. 4A). The relative levels of all identified enzymes are shown in a heatmap plot (Fig. 4B). The microbial enzymes, such as β -glucosidase (EC: 3.2.1.21), sucrose phosphorylase (EC: 2.4.1.7), xylose isomerase (EC: 5.3.1.5), and xylulokinase (EC: 2.7.1.17), involved in the starch and sucrose metabolism pathway and pentose and glucuronate interconversions, were more enriched in LAB- and LC-treated silages than in CK silage (Fig. 4B). These results

indicate that microbial enzymes may participate in carbohydrate alteration, particularly in the formation of monosaccharides in SS silage.

Identification of the relationships between microbes and metabolites in key bacteria

To further investigate the impact of functional species on the fermentation profiles, carbohydrate fractions, and predicted metabolic functions, Spearman's rank correlation analysis was conducted between distinct species and the aforementioned parameters (Fig. 5). The *L. buchneri* showed positive correlations with LA, mannose, ribose, and rhamnose but exhibited negative relationships with pH and the levels of $\text{NH}_3\text{-N}$, ADF, NDF (Fig. 5A). In addition, the *L. buchneri* was positively associated with enzymes involved in pentose and glucuronate interconversions (K01805, K00854, and K01783), as well as enzymes related to pathways K00690, K00615, and K05349. Conversely, it exhibited a negative correlation with all other enzymes involved in carbohydrate metabolism (Fig. 5B). The *L. plantarum*, *W. confusa*, *W. paramesenteroides*, and *C. farciminis* were positively associated with pH, ADF, NDF, and $\text{NH}_3\text{-N}$ contents but negatively

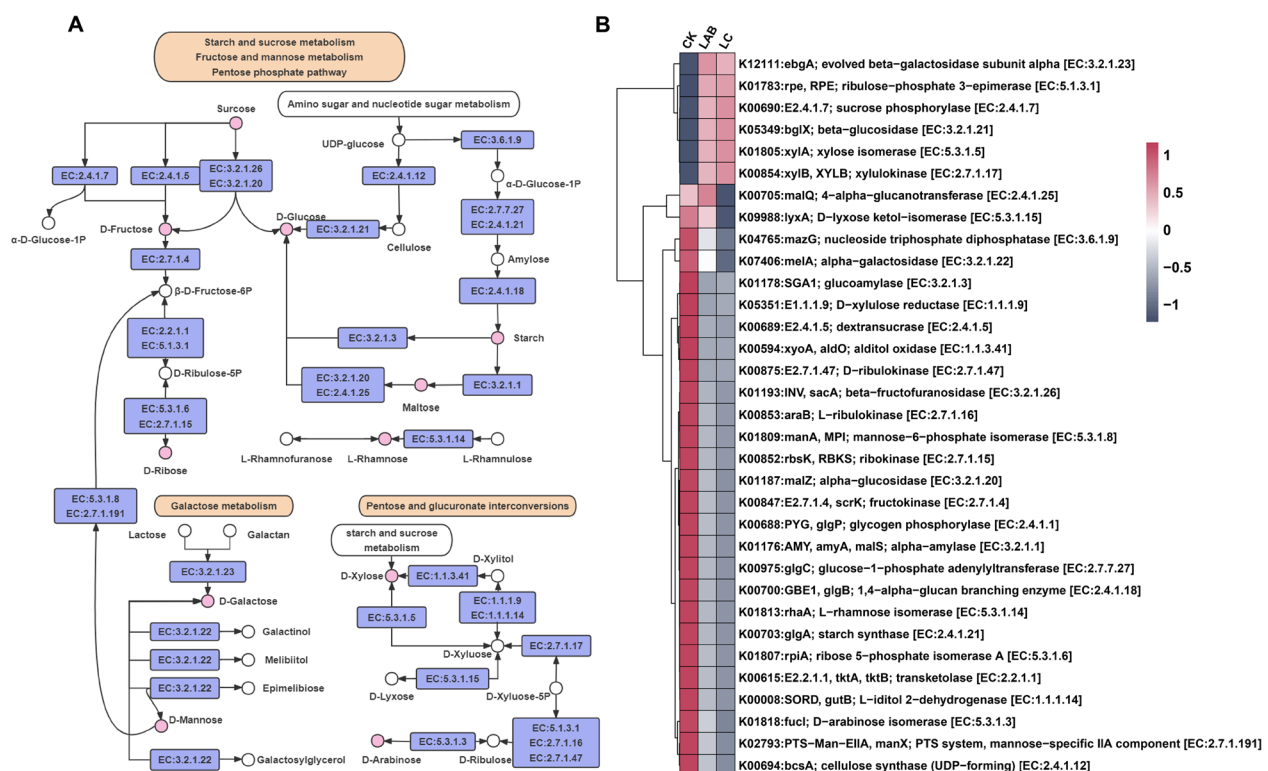


Fig. 4 Microbial metabolism contributes to carbohydrate alteration. **A** Reconstructed metabolic scheme for carbohydrate metabolism. **B** Heatmap showing the abundances of microbial enzymes involved in carbohydrate metabolism. CK: SS silage inoculated with sterilized water; LAB: SS silage inoculated with *Lactiplantibacillus plantarum* B90 + *Companilactobacillus farciminis* GMX4 + *Lentilactobacillus buchneri* NX205 + *Lentilactobacillus hilgardii* 60TS-2; LC: SS silage inoculated with LAB + 0.1% w/w cellulase

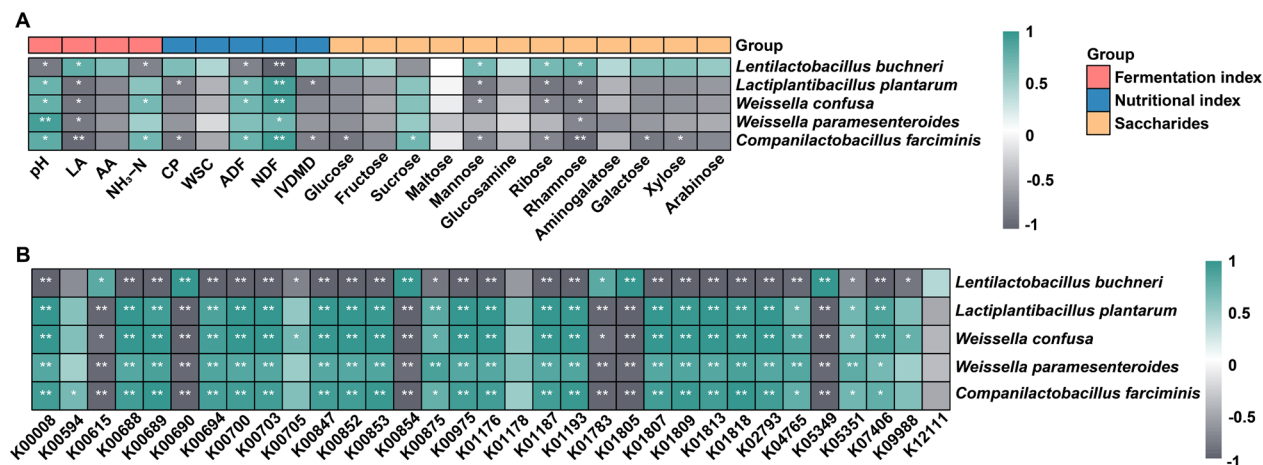


Fig. 5 The relationships among dominant microbes, metabolites and microbial enzymes. **A** The correlation between differential bacterial abundance and fermentation quality indices, nutritional quality indices, and sugars; **B** the correlation between differential bacterial abundance and microbial enzyme-encoding genes

correlated with LA and rhamnose content (Fig. 5A). In addition, these bacteria were negatively correlated with the K00690, K00615, K05349, K01805, K00854, and K01783 enzymes but positively correlated with the other enzymes involved in starch and sucrose metabolism (Fig. 5B). In summary, *L. buchneri* contributed to pH decrease, LA formation, and monosaccharides production, while *L. plantarum*, *W. confusa*, *W. paramesenteroides*, and *C. farciminis* were associated with NH₃-N formation and fiber content.

Discussion

The present study investigated the transformation trajectories of indigestible fiber in SS silage by quantitative microbiome profiling, which revealed the synergistic effect of a *Lactobacillus* cocktail and exogenous cellulase. To the best of our knowledge, this is the first study to depict the network of carbohydrate metabolism involved in microbial enzyme activity and to determine the composition of soluble carbohydrates and their alterations in SS silage. This study significantly advances our fundamental understanding of the role of microbes in increasing silage digestibility which is beneficial for energy preservation of silage and even nutrient uptake for ruminants. LAB and LC treatment improved the fermentation quality of SS silage. The increased LA (pKa of 3.86) content in LAB and LC groups contributes to a rapid reduction in pH due to its strong acidifying capacity [27]. A lower pH stabilizes fermentation by inhibiting undesirable bacteria, resulting in decrease in NH₃-N content as previously reported [28]. Most plants consist of three major lignocellulosic biomass components, namely, cellulose (38–50%), hemicellulose (23–32%), and lignin (10–25%) [29]. Hemicellulose and cellulose are

potentially digestible components of silage NDF [6]. A previous study reported that the combination of *L. plantarum* and hemicellulose effectively decreased the NDF content in rice straw silages [30]. In the present study, the combination of a *Lactobacillus* cocktail and cellulase resulted in a substantial reduction in NDF, consistent with previous findings. This may be mainly ascribed to hemicellulose degradation, which is easily hydrolyzed due to its amorphous structure and lower polymerization level than cellulose [31]. The other reason is that hemicellulose is the most abundant and diverse class of plant cell wall polysaccharide [32]. The ADF is primarily composed of cellulose and lignin [33]. Lignin is recognized as the glue that binds the various components together, providing structural support for the plant and resisting microbial or enzymatic degradation [29]. Cellulose is resistant to enzyme attack due to the physical barrier formed by lignin and hemicellulose, as well as its stabilized crystalline structure being stiffened by hydrogen bonds [34, 35]. This explains why the degradability of ADF is lower than that of NDF. However, the *Lactobacillus* cocktail alone resulted in inferior NDF degradability than the combination with cellulase. Our finding is similar to a previous finding that *Enterococcus faecalis* alone resulted in low NDF degradability in *Pennisetum sinense* silage [36], highlighting the synergistic effect of *Lactobacillus* and cellulase [37]. This phenomenon is potentially due to the alteration of the structural matrix in the cell wall increasing the intrusion of bacteria during anaerobic digestion, resulting in greater exposure to enzymes [30, 38]. In addition, the presence of *Lactobacillus* may enhance cellulase activity, leading to more efficient breakdown of lignocellulosic biomass.

The hydrolysis of lignocellulose to fermentable monosaccharides by cellulase is considered the most crucial step in lignocellulose bioconversion. In the LC-treated silage, glucose made up 60.93% of the monosaccharides, while fructose accounted for 24.02%, with traces of xylose, glucosamine, mannose, galactose, rhamnose, ribose, arabinose, and aminogalactose. The increase in the concentration of most of the monosaccharides in the LC-treated silage was probably due to the enzymatic hydrolysis of lignocellulose, which released more fermentable sugars than were consumed by microorganisms during fermentation. Similarly, a previous study reported an increase in the content of residual saccharides in oat silage when cellulase was applied as an additive in combination with lactic acid bacteria [39]. In addition, studies have confirmed that the presence of enzymes and acids during ensiling could substantially increase the degradation of structural carbohydrates, resulting in the release of soluble carbohydrates [38, 40], which is consistent with the lower structural carbohydrate (NDF and ADF) contents of the LC-treated silage in this study. Microbial enzymes also play a vital role in degrading cellulose with different specificities. For instance, β -glucosidase (EC 3.2.1.21) plays a crucial role in lignocellulose degradation by specifically hydrolyzing β -1,4- and β -1,3-glycosidic bonds [41]. These two bonds link monomeric sugars (D-xylose, D-glucose, D-mannose, L-arabinose, D-galactose, D-galacturonic, etc.) and sugars (D-glucuronic acids), which compose the polysaccharide hemicellulose [42]. The increased content of monosaccharides, such as xylose, glucose, mannose, arabinose, and galactose, in the LC-treated silage may be attributed to the increased level of the microbial enzyme β -glucosidase (EC: 3.2.1.21). Sucrose phosphorylase (EC: 2.4.1.7) is another key enzyme that breaks down sucrose to form fructose and glucose-1-phosphate during carbohydrate metabolism associated with *Lactobacillus* species [43]. The increased level of sucrose phosphorylase (EC: 2.4.1.7) in the LC-treated silage may explain the decreased sucrose content in the LC-treated silage. The synergistic action of microbial enzymes and exogenous cellulase is essential for the hydrolysis of lignocellulose. In addition to cellulase, microbial enzymes clearly play an influential role in the degradation process. Moreover, it could be deduced that additives may offset the richness of metabolic enzymes related to starch and sucrose metabolism pathways by altering the microbiome structure.

Determination of the IVDMD is another way to assess the biodegradation ability of lignocellulosic biomass [44]. Improvements in IVDMD occurred with hemicellulose degradation [45]. Previous studies reported that the cellulolytic fungus *Trichoderma reesei* improved IVDMD in

corn distiller grains [46], the co-addition of soluble sugar and cellulase enhanced IVDMD in King grass silage [11], and *L. buchneri* improved IVDMD in whole-crop oat silages [47]. Our results are consistent with previous findings that both *Lactobacillus* alone and the combination of *Lactobacillus* and cellulase enhanced the IVDMD in SS silage, while the latter was better. The higher IVDMD in the LC-treated silage may be ascribed to hemicellulose degradation under the synergistic effect of *Lactobacillus* and cellulase, as this result corresponds to the lower NDF content. A previous study reported a regression equation illustrating the positive correlation between IVDMD and in vivo digestibility in animals [48]. Our results indicated that treating SS silage with *Lactobacillus* and cellulase may enhance the in vivo digestibility in animals.

The ensiling process simplified the microbial community structure of the silages. Undesirable microbes such as *Weissella* are replaced by beneficial *Lactobacillus* due to the accumulation of organic acids and a decrease in the pH of silage [49]. Similarly, *L. buchneri* became the dominant species in all silages after ensiling in this study. However, the absolute abundance of total bacteria was reduced in the inoculated silage, especially in the LC-treated silage. A lower quantity of bacteria prevented the consumption of released sugars, which corresponded to the richest sugar accumulation in the LC-treated silage.

Conclusion

In summary, we analyzed the substrates and products of fiber transformation under a co-addition *Lactobacillus* cocktail and cellulase and revealed the potential connections between the microbial enzymes and silage metabolites. We showed a decrease in indigestible fiber content and an increase in monosaccharide levels, particularly xylose, as well as improved IVDMD under the LC treatment. We also highlighted the importance of *Lactobacillus* in carbohydrate metabolism. We further presented a comprehensive view of fiber transformation during ensiling and provided insight into the influence of microbes on silage metabolism. Taken together, *Lactobacillus* cocktail and cellulase are recommended to achieve better fermentation quality and nutrient preservation of protein-rich SS silage in saline-alkaline regions.

Abbreviations

SS	<i>Sesbania cannabina</i> and sweet sorghum
LAB	Lactic acid bacteria
LC	<i>Lactobacillus</i> Cocktail and cellulase
CK	Control
FR	Fresh forages
LA	Lactic acid
AA	Acetic acid
NH ₃ -N	Ammonia-N
WSC	Water-soluble carbohydrate
CP	Crude protein
NDF	Neutral detergent fiber

ADF	Acid detergent fiber
DM	Dry matter
IVDMD	In vitro Dry matter digestibility
AQ-16S-seq	Absolute quantification 16S rRNA sequencing
FAE	Ferulic acid esterase

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40538-024-00605-w>.

Supplementary Material 1: **Figure S1.** Alpha diversity and bacterial composition at the species level of SS silage. A, principal component analysis of the bacterial community in SS silage; B, alpha diversity indices of bacteria; C, bacterial community at the genus level in different groups. FC: fresh forage of *Sesbania cannabina*; FS: fresh forage of sweet sorghum; CK: SS silage inoculated with sterilized water; LAB: SS silage inoculated with *Lactiplantibacillus plantarum* B90 + *Companilactobacillus farciminis* GMX4 + *Lentilactobacillus buchneri* NX205 + *Lentilactobacillus hilgardii* 60TS-2; LC: SS silage inoculated with LAB + 0.1% w/w cellulase.

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Author contributions

TQX conducted the experiments design and the original draft. MT and TWW revised the manuscript. YDW performed the experiments. XMZ, SJL, KLT, and ZHF performed the data analysis. FFY, SYW, SJJ, and JCH contributed to visualization. JZ and TWW contributed to project administration and funding acquisition. All authors have read and approved the final manuscript.

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Availability of data and materials

The raw sequencing files and associated metadata have been deposited in the NCBI Sequence Read Archive (PRJNA1083345).

Declarations

Competing interests

The authors declare that the research was conducted without any commercial or financial relationships construed as potential competing interests.

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