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Metagenomics analysis reveals the performance of homo- and heterofermentative lactic acid bacteria in alfalfa silage fermentation, bacterial community, and functional profiles

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Abstract

Alfalfa (*Medicago sativa* L.) is a kind of roughage frequently utilized as an animal feed but challenging to be ensiled due to its low water-soluble carbohydrate (**WSC**), high water content, and elevated buffering capacity, thus requiring the application of lactic acid bacteria (**LAB**) to improve its fermentation. This study employed high-throughput metagenomic sequence technology to reveal the effects of homofermentative LAB, *Lactobacillus plantarum* (**Lp**), or *Pediococcus pentosaceus* (**Pp**), and heterofermentative LAB, *L. buchneri* (**Lb**), or their combinations (LbLp or LbPp) (applied at 1.0×10^9 colony forming units (**cfu**) per kilogram of alfalfa biomass fresh material) on the fermentation, microbial community, and functional profiles of alfalfa silage after 7, 14, 30, and 60 ensiling days. The results indicated a reduction ($P < 0.05$) in glucose and pH and higher ($P < 0.05$) beneficial organic acid contents, xylose, crude protein, ammonia nitrogen, and aerobic stability in Lb-, LbPp-, and LbLp-inoculated alfalfa silages after 30 and 60 d. Also, higher ($P < 0.05$) WSC contents were recorded in LbLp-inoculated alfalfa silages after 30 d (10.84 g/kg dry matter [**DM**]) and 60 d (10.92 g/kg DM). Besides, LbLp-inoculated alfalfa silages recorded higher ($P < 0.05$) LAB count (9.92 $\log_{10}$ cfu/g) after 60 d. Furthermore, a positive correlation was found between the combined LAB inoculants in LbLp-inoculated alfalfa silages and dominant LAB genera, *Lactobacillus* and *Pediococcus*, with fermentation properties after 30 and 60 d. In addition, the 16S rRNA gene-predicted functional analyses further showed that the *L. buchneri* PC-C1 and *L. plantarum* YC1-1-4B combination improved carbohydrate metabolism and facilitated further degradation of polysaccharides in alfalfa after 60 d of ensiling. These findings reveal the significant performance of *L. buchneri* and *L. plantarum* in combination with dominant LAB species in suppressing the growth of *Clostridia,* molds, and yeasts and improving the fermentation characteristics and functional carbohydrate metabolism of alfalfa after 60 d ensiling, thus suggesting the need for further studies to uncover the diverse performance of the LAB combination and their consortium with other natural and artificial inoculants in various kinds of silages.

Lay Summary

Current studies are aimed towards utilizing certain lactic acid bacteria (**LAB**) strains with high-yielding beneficial organic acid production for enhancing the quality of forages during preservation (otherwise known as ensiling) and to improve animal feed production. This study addresses the challenges of ensiling alfalfa (a kind of roughage frequently utilized as an animal feed in the livestock industries), such as low water-soluble carbohydrate, high water and fiber content, and high buffering capacity, by unraveling the positive interaction mechanisms of mixed LAB inoculants (homo-and heterofermentative LAB strains) and dominant epiphytic LAB in altering the development of pathogenic microorganisms and consequently improving the fermentation characteristics, chemical compositions, bacterial community, and functional profile of alfalfa after 60 d of ensiling.

Key words: carbohydrate metabolism, ensiling, inoculants, organic acids, polysaccharides

Abbreviations: ADF, acid detergent fiber; ADL, acid detergent lignin; aNDF, amylase-treated neutral detergent fiber; BA, butyric acid; CAZymes, carbohydrate-active enzymes; *cfu*, colony-forming units; CP, crude protein; DM, dry matter; FM, alfalfa biomass fresh material; IA, isobutyric acid; LA, lactic acid; LAB, lactic acid bacteria; OD, optical density; PA, propionic acid; WSC, water-soluble carbohydrate

Introduction

Alfalfa (*Medicago sativa* L.) is considered an exceptional animal feed item maintained as silage due to its high feeding value among leguminous forages (Hu et al., 2020). It is more abundant in minerals, organic acids, and crude protein (CP) than grasses, although it is more challenging to ensile based on its low water-soluble carbohydrate (WSC) concentrations, high fiber content, and buffering capacity in acidic environments (Ogunade et al., 2016). Besides, it is crucial to preserve alfalfa due to its seasonal harvest to deal with the shortage of winter animal feed (Fan et al., 2021). Ensiling is a fermentative activity by epiphytic microorganisms in conserving green forage crops or plant biomasses using low-tech improvements and storage time (Wang et al., 2020; Nazar et al., 2022). Several studies have recommended silage additives

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and inoculants to enhance ensiling since alfalfa fermentation is often dominated by pathogenic microorganisms, such as Clostridia, yeasts, and molds, which sometimes prevail over beneficial bacteria, thus providing optimal conditions for mycotoxins and spoilage (Jiang et al., 2020).

Lactic acid bacteria (**LAB**) are typically employed as inoculants due to their ideal performance in inhibiting pathogenic microorganisms' growth and improving the silage fermentation process (Blajman et al., 2020; Okoye et al., 2023d). The activities of different LAB strains participating in ensiling significantly impact silage fermentation quality. For instance, homofermentative LAB, *Lactobacillus plantarum,* and *Pediococcus* sp. are the most frequently utilized LAB in ensiling due to their ability to produce substantial concentrations of lactic acid (**LA**), which facilitates the reduction of pH during the silage fermentation process (Okoye et al., 2023b, 2023c). In contrast, heterofermentative LAB, *Lactobacillus buchneri,* is frequently used based on its ability to synthesize acetic acid (**AA**) from LA, which facilitates the prevention of aerobic deterioration caused by pathogenic microorganisms (Okoye et al., 2023c). The pace at which WSC is utilized and the production of organic acids during ensiling are considerably accelerated by both LAB inoculants and native LAB, although the presence of pathogenic microorganisms could alter this process (Peng et al., 2021). Moreover, due to variations in the constituents of different forages and the prevalence of pathogenic bacteria over epiphytic LAB, inconsistency in silage quality in recent times could be interpreted by the lack of information on microbial community dynamics and functional metabolite changes of LAB involved in silage production. Although, most LAB strains are applicable for fermenting crops or forage biomass such as corn straw with relatively low moisture, high WSC, and low buffering capacity. Still, some forages like alfalfa, whose biomass is characterized by low WSC, high moisture, high buffering capacity, and yeast growth during aerobic exposure, require LAB inoculants that can ferment biomass rapidly to reduce the pH of silage in a short time and suppress the growth of pathogens during ensiling and aerobic exposure. This makes it necessary to continue the search for novel LAB strains with unique fermentation characteristics. Despite the advances in LAB improvement for silage production, they are still faced with some challenges, such as the efficacy of LAB strains, the viable LAB concentration to be applied on fresh forage, and the fermentation type of LAB, which are determinants of adequate silage fermentation. Since both LAB types are promising inoculants for silage production, their combination has been considered valuable for diverse crop or forage biomasses. The mixture typically contains *L. buchneri* and homofermentative LAB, such as *L. plantarum* or *Pediococcus pentosaceus*, which dominate initial fermentation to realize an effective fermentation with rapid pH reduction and aerobic stability to enhance the silage quality (Okoye et al., 2023b, 2023c).

Previous studies have demonstrated the ability of novel LAB strains isolated from fermented vegetables that produce beneficial organic acids, including propionic acids (**PA**), LA, AA, etc., to enhance silage fermentation (Okoye et al., 2022, 2023c). Also, when comparing changes in the fermentation characteristics of silage at various phases of fermentation with the succession of the complex interaction of epiphytic microbial communities, the performance of these LAB inoculants or their combinations could be assessed. Therefore, it is crucial to fully comprehend the impact of inoculating homofermentative or heterofermentative LAB on the fermentation profile, microbial community, and metabolic functions of silage. Several studies have mostly employed denaturing gradient gel electrophoresis or culture-dependent methods in analyzing the complex microbial communities in silage (Muck et al., 2018), with a few studies using high-throughput next-generation sequencing technologies, which provide detailed information with reduced sequencing time and cost (Wang et al., 2022; Okoye et al., 2023e). Until now, very few studies have extensively used metagenomic sequencing in conjunction with functional analyses predicted by the 16S rRNA gene to characterize changes in the microbial community and functional metabolic profile of crops or forage biomasses during ensiling.

Our previous study identified distinct strains from several LAB species isolated from edible Chinese traditional fermented vegetables that were propagated for over 10 yr in the liquid fermentation media. A preliminary assessment showed that the LAB strains are salt-tolerant and with longevity and showed the potential to ferment biomass with high water content due to their adaptation in high-moisture conditions (Okoye et al., 2023a). Based on the preceding, we hypothesized that novel LAB strains synthesizing beneficial organic acids could enhance the alfalfa fermentation process by improving the silage microbiota and functional profile during ensiling. Also, combining different LAB types could facilitate alfalfa improvement, particularly during short or long ensiling periods. Even though previous studies have investigated the application of distinct LAB types in the alfalfa ensilage process, very few studies have utilized shotgun metagenomics approaches to evaluate the fermentation, bacterial community, and functional profiles of alfalfa inoculated with homo- and heterofermentative LAB strains or their combinations at various ensiling days. Therefore, we aimed to assess the effects of *L. buchneri* PC-C1, *L. plantarum* PC1-1, *P. pentosaceus* PC2-1(F2), or their combinations on the fermentation, bacterial community dynamics, and functional profiles of alfalfa after 7, 14, 30, and 60 ensiling days.

Material and Methods

LAB strains

Distinct LAB strains were isolated from an exceptional traditional fermented vegetable with high water content made in Hunan, China, and preserved in our laboratory. Using 16S rDNA fragment sequencing, their taxa were identified. Preliminary investigation showed that three unique LAB strains, including *L. buchneri* PC-C1 (**Lb**), *L. plantarum* PC1-1 (**Lp**), and *P. pentosaceus* PC2-1(F2) (**Pp**), have high-yielding organic acid abilities, potent antimicrobial inhibition, and better growth performance with exceptional biopreservative potential (Supplementary Tables S1 and S2). These three strains were chosen for this study, and the LAB inoculums were produced using the deMan Rogosa Sharpe (**MRS**) medium prepared in our laboratory.

Silage making

Alfalfa cultivar "Huaiyang-4" was grown at an experimental farm of Yangzhou University, Yangzhou, Jiangsu, China. Fresh alfalfa biomasses were harvested at their maturity stages, chopped to about 2 cm sizes, and promptly taken to the laboratory for processing. LAB inoculants, Lb, Lp, Pp, or their combinations LbLp and LbPp, were diluted in distilled

water and applied at 1.0×10^9 colony forming units (**cfu**) per kilogram of alfalfa biomass fresh material (**FM**). The control (**CK**) was inoculated using an equivalent measure of distilled water. The FM was loaded into plastic vacuum bags of specific size (30 × 40 cm) (N-14915, Deli Group Co., Ltd, Zhejiang, China) after that by compaction to remove air with a vacuum sealer (N-14886, Deli Group Co., Ltd,). A total of 12 vacuum bags (four ensiling days × three replicates) of FM for each inoculant or their combination were fermented anaerobically under room temperature (25 ± 2 °C) for 7, 14, 30, and 60 d, respectively.

Fermentation characteristics and microbial population count

The fermentation characteristics and microbial counts were determined in FM and alfalfa silage samples by infusing 30 g of samples in 70 mL sterilized water. The mixture is kept for 24 h at 4 °C before sieving using four layers of gauze to determine organic acids, ammoniacal nitrogen (NH_3-N), and pH. The pH values were determined promptly with the LAQUAtwin Compact pH meter (ASpH22, Horiba Advanced Techno Co., Ltd, Japan). A filtrate fraction was centrifuged for 10 min at 10,000 × *g* and the supernatant was filtered with a 0.22 µm membrane filter before determining organic acids using HPLC (Prominence LC, Shimadzu Co., Tokyo, Japan) equipped with a UV–Vis detector (SPD-20A), liquid chromatography (LC-20AD), and Supersil column (ODS-B 5 m, 250 × 4.6 mm). The HPLC condition includes mobile phase: methanol (86:14) and 0.02 mol/L NaH_2PO_4 (pH 2.7); column temperature: 30 °C; wavelength: 210 nm; flow rate: 1.0 mL/min; injection volume: 20 µL; and single sample run time: 25 min. The remaining filtrate was used for determining NH_3-N following the modified phenol-hypochlorite technique (Broderick and Kang, 1980). For the microbial population count, 25 g of alfalfa silage samples were mixed with 75 mL of sterile saline solution (8.5 g/L NaCl). This mixture was then agitated for 2 h at 150 rpm. After filtration with medical gauze, a sterile saline solution was used to dilute 1 mL of the filtrate serially in ten folds. At the same time, the remaining samples were kept for DNA extraction at –20 °C. The LAB populations were evaluated after growing MRS media incubated anaerobically at 37 °C for 48 h. Yeasts and molds were counted on potato dextrose agar following incubation for 24 h at 37 °C. Each experiment was carried out in triplicate, and the cfu of microorganisms was log-transformed.

Chemical analyses

The chemical compositions of dry matter (**DM**), neutral detergent fiber (**aNDF**), WSC, acid detergent fiber (**ADF**), CP, and acid detergent lignin (**ADL**) were analyzed before and after ensiling. Alfalfa biomass FM and silage samples DM were analyzed following a consistent weight after oven-drying at 65 °C. An FW100 Wiley mill (Taisite Instrument Co., Ltd, Tianjin, China) was used to pulverize the samples to a 1 mm mesh. Nitrogen contents were evaluated following the Kjeldahl method (DK series - UDK 149, VELP Scientifica Co., Shanghai, China), and then CP was estimated by multiplying N content by 6.25 (AOAC, 1990). Lignocellulose contents, including ADL, ADF, and aNDF, were determined with a Fiber Analyzer (FIWE 6, VELP Scientifica Co., Ltd, Usmate, Italy) based on standard procedures (Van Soest et al., 1991; Mertens, 2002). Crude ash was evaluated after 3 h incineration at 525 °C. The Anthrone procedure was used to evaluate the WSC (Tian et al., 2018).

To determine mono- and disaccharide contents (arabinose, cellobiose, galactose, glucose, and xylose), about 0.1 mg of FM and ensiled alfalfa were hydrolyzed in a 100 mL Erlenmeyer flask containing 0.1 mol/L sodium acetate buffer (pH 7.0) and resuspended by vortexing. A commercial cellulose, Cellic Ctec3, purchased from Novozymes, was put into the blend at a final concentration of 20 FPU/g biomass, consisting of cellulase (10 FPU/g) and β-glucosidase (10 CBU/g) after pretreatment with 1% HCl (w/v) for 24 h at 40 °C. To prevent microbial contamination, 0.1% w/v sodium azide was added to the hydrolysis reaction mixture. Hydrolysis was carried out at 40 °C for 48 h with shaking (150 rpm). After extraction, the hydrolysates were centrifuged for 10 min at 10,000 × *g*, and the supernatants were measured using HPLC (Prominence LC, Shimadzu Co.) equipped with an HPX-87H ion exclusion column (Bio-Rad, USA) and a refractive index detector. The supernatants were eluted with a mobile phase (4 mmol/L H_2SO_4) at a flow rate of 0.4 mL/min. An Agilent C18 analytical column (4.6 × 150 mm, 5 µm particle size) and a UV detector were used to run each sample for 35 min at an injection volume of 20 µL.

Aerobic stability

The aerobic stability of the alfalfa silages was analyzed following the opening of the silos on different ensiling days. Briefly, silage samples were loaded into 0.5 L sterile plastic bottles, enclosed with two cheesecloth layers to minimize contamination and desiccation, and kept in an incubator at an ambient temperature of 25 °C. A data logger (YSUP-15P, Shenzhen Yuwen Sensor System Co., Ltd, China) was geometrically placed in the middle of the silage mass to measure the silage temperature. The aerobic stability was determined after a 2 °C and above elevation in silage temperature.

DNA extraction, amplification, and high-throughput sequence-based metagenomics

For the microbial DNA extraction, 30 mL of every stored sample filtrate was centrifuged for 3 min at 10,000 × *g*. According to the manufacturer's instructions, the pellets were collected for genomic DNA extraction using EZNA Mag-Bind Soil DNA Kit (Omega Biotech, Norcross, GA, USA). After accurate quantification of genomic DNA using a Qubit3.0 Fluorometer (Life Technologies, Paisley, UK), amplification of bacterial 16S rDNA gene was done with the primer pairs of 341F (5′- CCT ACG GGN GGC WGC AG- 3′) and 805R (5′- GAC TAC HVG GGT ATC TAA TCC- 3′). The PCR reactions were as follows: predenaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and a final extension at 72 °C for 5 min. The DNA quality was checked with a Nanodrop 2000 UV–Vis Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA). High-throughput sequence analysis was performed by Sangon Biotech (Shanghai) Co., Ltd. UPARSE 7.1 (https://drive5.com/uparse/) was used to cluster the operational taxonomic units (**OTUs**) at a 97% sequence similarity level. The Ribosomal Database Project (http://rdp.cme.msu.edu/) was used to evaluate the taxonomy of the 16S rDNA gene sequences. Mouther 1.30.1 (http://www.mothur.org/wiki/Classify.seqs) was used to evaluate the alpha diversity, particularly the Simpson index, Shannon index, Good's coverage ACE, and Chao richness.

Functional profile analysis

By using the PICRUSt bioinformatics tool to assign 16S rRNA marker gene sequences to functional annotations of metagenomic sequences, the metabolic profile of the bacterial community and the functional genes were predicted based on the Kyoto Encyclopedia of Genes and Genomes (**KEGG**) on the first-level, second-level, and third-level pathways (You et al., 2022). The sequence data presented in this study were deposited at the National Center for Biotechnology Information database with the BioProject accession number "PRJNA760836".

Statistical analysis

The obtained data were analyzed with the Statistical Package for the Social Sciences software version 25.0 (IBM Corporation, Armonk, USA). The study employed a completely randomized factorial design in a 6 (treatments [one control and five LAB inoculants]) × 4 (ensiling days [7, 14, 30, and 60 d]) factorial arrangement of treatments. Treatment level means were matched at $P < 0.05$ with Tukey's Honest Significant Difference after the homogeneity assumption among variables was met. Alpha diversity indices were determined using the analysis of variance. A comparison of bacterial species at different ensiling days was performed using Welch's *t*-test at a 95% confidence interval. A significant correlation between bacterial taxa and fermentation profile was made using Spearman's correlation heatmap, and RStudio Package was used to infer the correlation matrix after dislodging highly insignificant variables. Plots and graphs were performed using OriginPro software version 2021 (OriginLab Corporation, Northampton, USA).

Results

Chemical composition and microbial population of fresh alfalfa

Table 1 presents the chemical composition and microbial population of alfalfa biomass FM before ensiling. The pH, DM, WSC, aNDF, ADF, ADL, CP, arabinose, cellobiose, galactose, glucose, and xylose were 5.72, 294.40 g/kg FM, 44.53 g/kg DM, 58.21%, 46.54%, 31.03%, 38.89%, 1.64%, 4.95%, 2.46%, 2.95%, and 2.14%, respectively. Microbial counts in the FM for LAB, yeasts, and molds were 7.25, 5.23, and 6.48 log10 cfu/g FM, respectively.

Fermentation profile, microbial population, and aerobic stability of alfalfa after 7, 14, 30, and 60 ensiling days

Table 2 shows the fermentation profile, microbial counts, and aerobic stability of alfalfa after 7, 14, 30, and 60 d of ensiling. Compared to the CK, the pH was reduced ($P < 0.05$) from day 7 to day 60 in LAB-inoculated silages. Exceptionally, there was a reduction ($P < 0.05$) in the Lb- and LbPp-inoculated silages after 30 and 60 d ensiling. The LA and AA contents increased ($P < 0.05$) in Lb-, LbLp-, and LbPp-inoculated silages after 60 d compared with other LAB-inoculated silages and CK. The ratio of LA/AA was reduced ($P < 0.05$) in LAB-inoculated silages and CK after 60 d. Also, the PA contents increased ($P < 0.05$) in LbLp-inoculated silage after 60 d (1.95 g/kg DM). Besides, isobutyric acid (**IA**) and butyric acid (**BA**) decreased ($P < 0.05$) in LAB-inoculated silages and CK after 60 d. Compared with CK, the LAB counts increased ($P < 0.05$) in LAB-inoculated silages after 14, 30, and 60 d. However, LbLp-inoculated silages detected higher LAB counts (9.92 $\log_{10}$ cfu/g) after 60 d. Moreover, mold and yeast counts were only detected in CK silages after different ensiling days. Also, the aerobic stabilities of LAB-inoculated silages were higher (>578 h) than CK after 60 d of ensiling.

Chemical and carbohydrate compositions of alfalfa after 7, 14, 30, and 60 ensiling days

Table 3 presents the chemical compositions of alfalfa after 7, 14, 30, and 60 d of ensiling. The DM content of all the inoculated silages and CK decreased after ensiling. Compared to CK with a declining trend ($P < 0.05$) in DM contents, LbPp-inoculated silages increased after 60 d of ensiling (276.10 g/kg DM). After ensiling, a declining trend in CP was detected in LAB-inoculated alfalfa silages and CK, with LbLp- and LbPp-inoculated silage having higher CP content (25.48% and 26.70% DM, respectively) after 60 d. After 60 d, NH_3-N contents decreased ($P < 0.05$) in LbLp- and Lp-inoculated silage 27.73 g/kg TN and 28.37 g/kg TN, respectively. An increase ($P < 0.05$) in WSC was detected in Lb-, LbPp-, and LbLp-inoculated silages after 30 d (10.46 g/kg DM), 60 d (10.33 g/kg DM), 30 d (10.84 g/kg DM), and 60 d (10.92 g/kg DM), respectively. A decrease ($P < 0.05$) was detected in ADF and ADL contents of LAB-inoculated silages after 60 d. In contrast, the aNDF contents increased ($P < 0.05$) in Lb- (48.42% DM) and LbLp- (48.64% DM) inoculated silages after 60 d. The variations in the compositions of mono- and disaccharides in LAB-inoculated alfalfa samples and CK after different ensiling days are shown in Fig. 1. Xylose concentrations increased ($P < 0.05$), while cellobiose, galactose, and glucose contents significantly reduced in LAB-inoculated alfalfa silages and the CK. Moreover, arabinose contents increased ($P < 0.05$) in LbLp- (2.10%–2.90% DM) and LbPp- (2.14%–2.94% DM) inoculated silages

Table 1. Chemical composition, carbohydrate content, and microbial populations of alfalfa biomass fresh material before ensiling (mean ± SD)

Item	Fresh alfalfa biomass
pH	5.72 ± 0.05
DM, g/kg FM	294.40 ± 0.50
WSC, g/kg DM	44.53 ± 0.07
Arabinose, % DM	1.64± 0.22
Cellobiose, % DM	4.95± 0.04
Galactose, % DM	2.46± 0.12
Glucose, % DM	2.95± 0.02
Xylose, % DM	2.14± 0.03
aNDF, % DM	58.21 ± 0.45
ADF, % DM	46.54 ± 0.34
ADL, % DM	31.03 ± 0.07
CP, % DM	38.89 ± 0.02
LAB, $\log_{10}$ cfu/g FM	7.25 ± 0.20
Yeast, $\log_{10}$ cfu/g FM	5.23 ± 0.34
Mold, $\log_{10}$ cfu/g FM	6.48 ± 0.12

aNDF, amylase-inoculated neutral detergent fiber; ADL, acid detergent lignin; ADF, acid detergent fiber; cfu, colony-forming unit; CP, crude protein; FM, alfalfa biomass fresh material; DM, dry matter; WSC, water-soluble carbohydrate.

Table 2. Fermentation profile, microbial populations, and aerobic stability of alfalfa silage inoculated at different ensiling days[1]

Item	Treatment	Ensiling days				SEM (n = 3)	P-value
		7 d	14 d	30 d	60 d		
pH	CK	5.23^{o}	4.95^{n}	4.77^{k}	4.68^{h}	0.019	<0.0001
	Lp	4.74^{j}	4.71^{i}	4.65^{fg}	4.56^{c}		
	Lb	4.77^{jk}	4.65^{g}	4.55^{bc}	4.53^{b}		
	Pp	4.86^{m}	4.84^{m}	4.78^{kl}	4.60^{d}		
	LbLp	4.68^{h}	4.63^{ef}	4.61^{de}	4.55^{bc}		
	LbPp	4.80^{l}	4.77^{k}	4.53^{b}	4.48^{a}		
Lactic acid, g/kg DM	CK	1.50^{b}	1.66^{b}	1.68^{b}	0.74^{a}	0.260	<0.0001
	Lp	4.10^{def}	4.37^{efg}	7.27^{m}	8.03^{n}		
	Lb	2.87^{c}	4.29^{defg}	5.59^{k}	4.60^{fgh}		
	Pp	4.91^{hij}	5.16^{ijk}	7.91^{n}	9.92^{o}		
	LbLp	3.98^{de}	4.10^{def}	6.84^{lm}	5.35^{jk}		
	LbPp	4.10^{def}	4.75^{ghi}	6.45^{l}	3.80^{d}		
Acetic acid, g/kg DM	CK	3.29^{ef}	3.66^{fg}	3.65^{fg}	2.74^{cde}	0.225	<0.0001
	Lp	1.16^{a}	1.37^{a}	2.27^{bc}	2.74^{cde}		
	Lb	3.63^{f}	5.10^{i}	8.04^{m}	6.92^{kl}		
	Pp	1.31^{a}	1.71^{ab}	2.55^{cd}	3.08^{def}		
	LbLp	4.26^{gh}	5.17^{ij}	7.17^{l}	6.56^{k}		
	LbPp	4.26^{gh}	4.29^{i}	5.74^{j}	4.70^{hi}		
Propionic acid, g/kg DM	CK	0.86^{bcd}	0.95^{cd}	0.94^{cd}	0.17^{a}	0.070	<0.0001
	Lp	0.78^{b}	0.85^{bc}	1.22^{gh}	0.79^{b}		
	Lb	1.13^{g}	3.12^{k}	2.27^{j}	1.23^{gh}		
	Pp	0.84^{bc}	0.85^{bc}	1.38^{h}	0.95^{cd}		
	LbLp	1.08^{ef}	1.11^{fg}	1.86^{i}	1.95^{i}		
	LbPp	1.08^{ef}	1.08^{ef}	1.24^{gh}	0.75^{b}		
Isobutyric acid (g/kg DM)	CK	0.95^{l}	0.81^{h}	0.84^{i}	0.46^{a}	0.016	<0.0001
	Lp	0.88^{j}	0.73^{f}	0.67^{e}	0.64^{d}		
	Lb	0.88^{j}	0.79^{g}	0.65^{de}	0.59^{c}		
	Pp	0.93^{kl}	0.87^{ij}	0.75^{f}	0.67^{e}		
	LbLp	0.92^{k}	0.87^{ij}	0.62^{d}	0.57^{bc}		
	LbPp	0.78^{g}	0.67^{e}	0.59^{c}	0.56^{b}		
Butyric acid, g/kg DM	CK	0.65^{k}	0.45^{i}	0.30^{c}	0.10^{a}	0.023	<0.0001
	Lp	0.50^{j}	0.35^{g}	0.20^{b}	0.10^{a}		
	Lb	0.32^{f}	0.25^{e}	0.20^{b}	0.10^{a}		
	Pp	0.40^{h}	0.20^{d}	0.20^{b}	0.10^{a}		
	LbLp	0.40^{h}	0.25^{e}	0.10^{a}	0.10^{a}		
	LbPp	0.35^{g}	0.25^{e}	0.10^{a}	0.10^{a}		
LA/AA	CK	0.46^{b}	0.45^{b}	0.46^{b}	0.28^{a}	0.142	<0.0001
	Lp	3.53^{l}	3.18^{j}	3.21^{ik}	3.03^{h}		
	Lb	0.79^{d}	0.84^{e}	0.70^{c}	0.67^{c}		
	Pp	3.76^{m}	3.03^{h}	3.10^{i}	3.23^{k}		
	LbLp	0.94^{f}	0.79^{de}	0.95^{f}	0.83^{de}		
	LbPp	0.96^{f}	1.11^{g}	1.12^{g}	0.83^{de}		
LAB, $\log_{10}$ cfu/g FM	CK	7.23^{c}	6.20^{b}	5.65^{a}	5.45^{a}	0.264	<0.0001
	Lp	7.74^{d}	8.25^{e}	8.88^{f}	9.15^{g}		
	Lb	7.26^{c}	8.19^{e}	8.67^{f}	9.11^{g}		
	Pp	7.19^{c}	8.14^{e}	8.95^{f}	9.08^{g}		
	LbLp	7.16^{c}	8.27^{e}	9.70^{h}	9.92^{i}		
	LbPp	7.74^{d}	8.35^{e}	9.45^{gh}	9.63^{h}		
Yeast, $\log_{10}$ cfu/g FM	CK	3.41^{d}	2.45^{c}	0.21^{b}	0.14^{a}	0.242	<0.0001
	Lp	ND	ND	ND	ND		
	Lb	ND	ND	ND	ND		

Table 2. Continued

Item	Treatment	Ensiling days				SEM (n = 3)	P-value
		7 d	14 d	30 d	60 d		
	Pp	ND	ND	ND	ND		
	LbLp	ND	ND	ND	ND		
	LbPp	ND	ND	ND	ND		
Mold, log_{10} cfu/g FM	CK	4.22[c]	2.19[b]	0.43[a]	0.41[a]	0.156	<0.0001
	Lp	ND	ND	ND	ND		
	Lb	ND	ND	ND	ND		
	Pp	ND	ND	ND	ND		
	LbLp	ND	ND	ND	ND		
	LbPp	ND	ND	ND	ND		
Aerobic stability, h	CK	96	128	>508	>508		
	Lp	124	154	>578	>578		
	Lb	128	196	>578	>578		
	Pp	140	196	>578	>578		
	LbLp	136	240	>578	>578		
	LbPp	138	240	>578	>578		

[1]Mean values with uncommon superscript letters vary significantly (P < 0.05) among different items; ND, not detected; DM, dry matter; SEM, error of the means; LA/AA, lactic to acetic acid ratio; cfu, colony-forming unit; CK, alfalfa with no inoculant; Lp, alfalfa inoculated with *Lactobacillus plantarum*; Lb, alfalfa inoculated with *Lactobacillus buchneri*; Pp, alfalfa inoculated with *Pediococcus pentosaceus;* LbLp, alfalfa inoculated with combined *L. buchneri* and *L. plantarum*; LbPp, alfalfa inoculated with combined *L. buchneri* and *P. pentosaceus*, after 7, 14, 30, and 60 ensiling days.

but experienced a slight reduction in Lp- (2.52% DM), Lb- (2.48% DM), and Pp- (2.50% DM) inoculated alfalfa silages after 60 d.

Bacterial community dynamics of alfalfa after 7, 14, 30, and 60 ensiling days

The alpha diversity of FM and ensiled alfalfa is shown in Table 4. There was an apparent distinction in the bacterial community dynamics of fresh alfalfa compared with the ensiled alfalfa. The bacterial diversity of ensiled alfalfa was reduced compared to fresh alfalfa. The indices of OTUs, Shannon index, ACE, and Chao1 in FM were higher than the LAB-inoculated alfalfa and CK, except for the Simpson index, which had a relatively lower value in FM. LAB-inoculated alfalfa and CK had a very high Good's coverage of 99.9%. The relative abundances of the bacteria community in FM and ensiled alfalfa are shown in Fig. 2. The dominant bacteria phyla of FM were *Firmicutes* and *Proteobacteria*. However, *Proteobacteria* decreased after ensiling, and *Firmicutes* dominated the LAB-inoculated alfalfa silages (Fig. 2a). Besides, LbLp-inoculated alfalfa was highly dominated by *Firmicutes* after 30 and 60 d. The most abundant bacterial genera in FM were *unclassified Rhizobiaceae* (28.81%), *Sphingomonas* (11.06%), and *Streptophyta* (6.99%). In contrast, the most prominent genera among all the treatments after different ensiling days were *Lactobacillus* and *Pediococcus* (Fig. 2b). The highest abundance of *Lactobacillus* was detected in Lp- (93.3%), Lb- (94.5%), and LbLp- (96.02%) inoculated alfalfa silages after 60 d of ensiling. Also, the highest abundance of *Pediococcus* was found in Pp- and LbPp-inoculated alfalfa silages after 14 d (79.9% and 56.6%), 30 d (63.2% and 55.8%), and 60 d (42.8% and 22.4%) in a decreasing trend.

Figure 3 further summarizes the bacterial community dynamics in FM and ensiled alfalfa samples at OTUs, genus, and phylum levels, respectively, using the Bray–Curtis distance plots based on principal coordinates analysis (**PCoA**), nonmetric multidimensional scaling (**NMDS**), and distance heatmap. The ANOSIM value of R^2 = 0.99 revealed that the bacterial community composition in FM and ensiled alfalfa varies more between groups than within groups at the OTUs, genus, or phylum level. Moreover, a clear similarity was found among treatments of the same day, except for CK at 7, 14, and 30 d, which clustered with FM (P < 0.72).

Correlation of the microbial taxa and fermentation profile of alfalfa after 7, 14, 30, and 60 ensiling days

Figure 4 shows the spearman correlation heatmaps between the dominant bacterial genera and fermentation profile of alfalfa silages after 7, 14, 30, and 60 d of ensiling. *Pediococcus* was negatively correlated with pH, molds, and yeasts in LAB-inoculated silages after 7 d but positively correlated with pH, LA, LA/AA after 14 d. *Lactobacillus* was positively correlated with pH, LA, AA, PA, LA/AA, and LAB counts in LAB-inoculated alfalfa silages after 30 d. Although significant negative correlations were found among *Pediococcus* with pH, ADL, and aNDF in LAB-inoculated alfalfa silages after 60 d of ensiling, *Lactobacillus* was positively correlated with pH, CP, WSC, LA, AA, PA, LA/AA, and LAB and negatively correlated with molds and yeasts in Lb-, Pp-, LbLp-, and LbPp-inoculated alfalfa silages after 60 d.

Functional profiles and carbohydrate degradation enzymes of alfalfa after 7, 14, 30, and 60 ensiling days

The dynamic changes in alfalfa silage quality were further evaluated based on the functional profiles to understand the impact of the bacterial community. Figure 5a and b show the differences in the first and second pathway levels of 16S rRNA gene-predicted functional profiles of bacterial communities before and after ensiling. The relative abundance of the metabolism in LAB-treated alfalfa silages was higher (P < 0.05), followed by the genetic information processing in

Table 3. Chemical compositions of alfalfa inoculated at different ensiling days[1]

Item	Treatment	Ensiling days				SEM (n = 3)	P-value
		7 d	14 d	30 d	60 d		
DM, g/kg FM	CK	259.92^{j}	248.73^{de}	243.68^{a}	252.67^{h}	0.814	<0.0001
	Lp	262.43^{k}	246.55^{bc}	246.63^{bc}	248.52^{cde}		
	Lb	245.20^{ab}	251.79^{gh}	250.45^{efg}	249.20^{de}		
	Pp	249.40^{def}	248.57^{cde}	247.91^{de}	251.30^{fgh}		
	LbLp	250.45^{efg}	248.15^{cd}	244.65^{ab}	252.23^{gh}		
	LbPp	245.67^{c}	252.08^{gh}	256.37^{i}	276.10^{l}		
WSC, g/kg DM	CK	11.81^{l}	10.74^{hij}	7.78^{b}	7.12^{a}	0.135	<0.0001
	Lp	11.73^{l}	9.61^{cd}	9.46^{cd}	9.27^{c}		
	Lb	10.76^{hij}	9.61^{cd}	10.46^{fghi}	9.94^{de}		
	Pp	11.53^{kl}	10.30^{efgh}	10.27^{efgh}	9.32^{c}		
	LbLp	11.93^{l}	10.63^{ghij}	10.84^{ij}	10.92^{ij}		
	LbPp	11.10^{jk}	10.23^{efg}	10.12^{ef}	10.33^{efgh}		
NH_3-N, g/kg TN	CK	52.84^{j}	44.67^{g}	38.74^{f}	35.29^{d}	0.147	<0.0001
	Lp	57.31^{l}	51.38^{ij}	44.98^{g}	28.37^{b}		
	Lb	56.67^{k}	50.55^{i}	45.64^{gh}	32.48^{c}		
	Pp	58.67^{lm}	52.34^{j}	47.32^{h}	32.06^{c}		
	LbLp	65.91^{n}	59.43^{m}	48.68^{hi}	27.73^{a}		
	LbPp	65.95^{n}	56.49^{k}	44.56^{g}	37.35^{e}		
CP, % DM	CK	31.26^{l}	28.77^{i}	26.43^{ef}	20.17^{a}	0.287	<0.0001
	Lp	29.70^{j}	28.36^{hi}	26.24^{e}	23.38^{b}		
	Lb	30.40^{k}	29.86^{j}	27.70^{g}	23.63^{bc}		
	Pp	31.21^{l}	29.48^{j}	27.54^{g}	24.05^{c}		
	LbLp	28.27^{h}	27.67^{g}	25.75^{d}	25.48^{d}		
	LbPp	30.45^{k}	29.57^{j}	26.84^{f}	26.70^{f}		
ADF, % DM	CK	32.62^{j}	31.37^{g}	30.93^{f}	30.94^{f}	0.046	<0.0001
	Lp	32.72^{k}	31.62^{i}	30.63^{d}	30.45^{c}		
	Lb	31.43^{h}	31.45^{h}	30.45^{c}	30.22^{a}		
	Pp	31.34^{g}	30.46^{c}	30.64^{d}	30.33^{ab}		
	LbLp	31.39^{g}	30.70^{de}	30.39^{ab}	30.13^{a}		
	LbPp	31.35^{g}	31.36^{g}	30.37^{ab}	30.23^{a}		
ADL, % DM	CK	29.75^{i}	27.75^{h}	24.89^{cd}	25.94^{e}	0.021	<0.0001
	Lp	26.85^{g}	24.37^{c}	26.73^{fg}	24.26^{c}		
	Lb	25.45^{de}	27.88^{h}	23.28^{ab}	23.10^{ab}		
	Pp	26.12^{efg}	23.45^{b}	23.29^{ab}	22.84^{ab}		
	LbLp	25.92^{e}	23.26^{ab}	22.63^{a}	22.83^{ab}		
	LbPp	24.53^{c}	24.60^{c}	26.09^{ef}	23.09^{ab}		
aNDF (% DM)	CK	58.55^{l}	50.07^{de}	53.17^{j}	55.37^{k}	0.306	<0.0001
	Lp	52.40^{ij}	47.52^{a}	50.40^{ef}	49.03^{bc}		
	Lb	49.27^{cd}	51.39^{gh}	48.32^{abc}	48.42^{abc}		
	Pp	51.19^{fgh}	48.64^{bc}	50.64^{efg}	48.14^{ab}		
	LbLp	51.08^{fg}	48.25^{ab}	48.33^{abc}	48.64^{bc}		
	LbPp	48.70^{bc}	48.41^{abc}	52.11^{hi}	48.45^{abc}		

[1]Mean values with uncommon superscript letters vary significantly (P < 0.05) among different items; SEM, error of the means. FM, alfalfa biomass fresh material; DM, dry matter; WSC, water-soluble carbohydrate; CP, crude protein; aNDF, amylase-inoculated neutral detergent fiber; ADL, acid detergent lignin; ADF, acid detergent fiber; NH_3-N, ammoniacal nitrogen; CK, alfalfa with no inoculant; Lp, alfalfa inoculated with *Lactobacillus plantarum*; Lb, alfalfa inoculated with *Lactobacillus buchneri*; Pp, alfalfa inoculated with *Pediococcus pentosaceus;* LbLp, alfalfa inoculated with combined *L. buchneri* and *L. plantarum*; LbPp, alfalfa inoculated with combined *L. buchneri* and *P. pentosaceus*, after 7, 14, 30, and 60 ensiling days.

the first-level pathway (Fig. 5a). Besides, the relative abundance of carbohydrate, membrane transport, and amino acid metabolism was higher (P < 0.05) than other pathways and was significantly enhanced in LbLp-inoculated silage after 60 d of ensiling (Fig. 5b). Other secondary metabolites biosynthesis and polyketides and terpenoids metabolism were predicted to be reduced (P < 0.05) in LAB-inoculated silages after 60 d but increased in control throughout the ensiling.

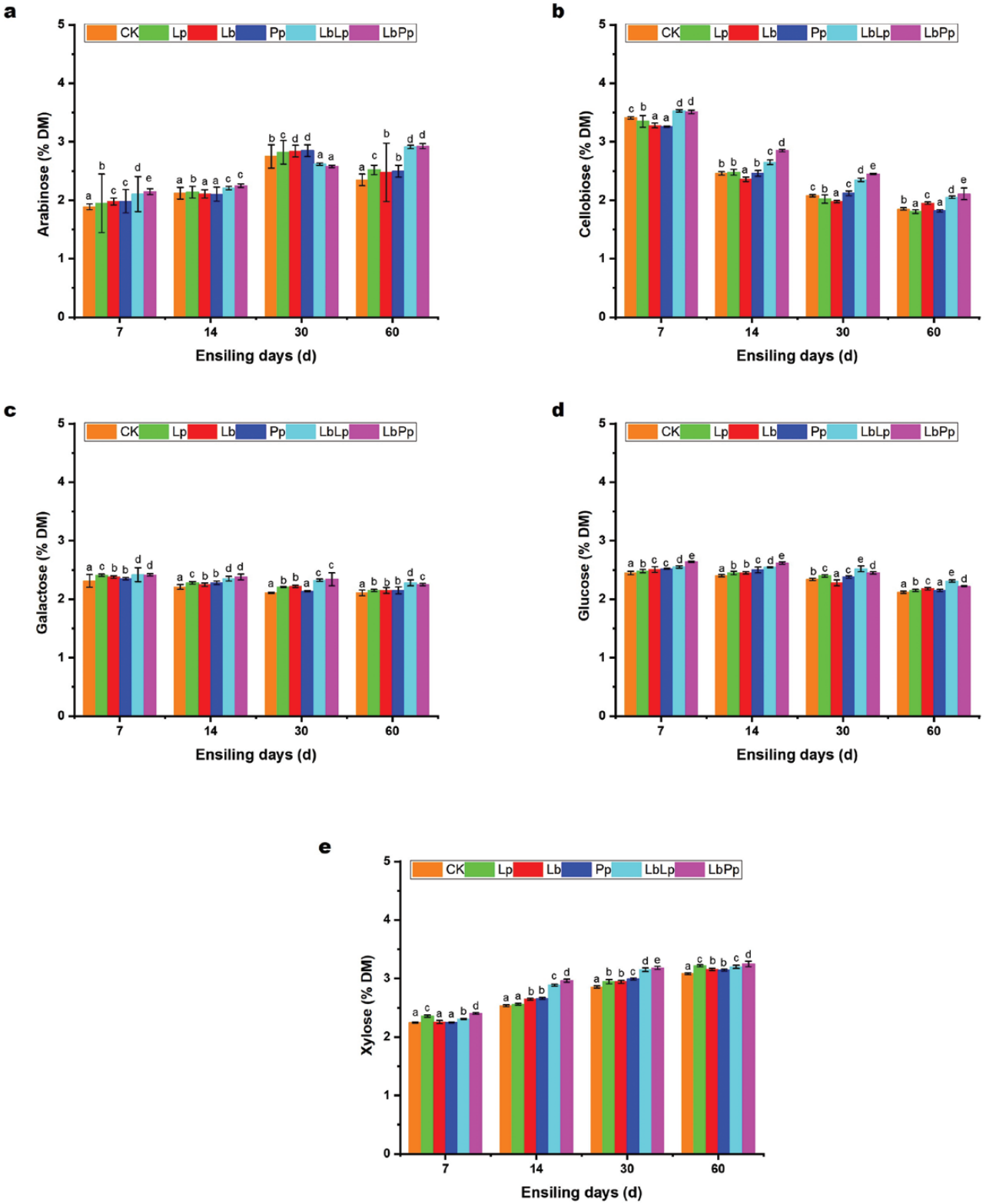

Figure 1. Comparisons of mono- and disaccharide compositions in alfalfa silage inoculated at different ensiling days. (**a**) arabinose, (**b**) cellobiose, (**c**) galactose, (**d**) glucose, and (**e**) xylose. Values with uncommon letters show a significant difference ($P < 0.05$) among treatments. CK, alfalfa with no inoculant; Lp, alfalfa inoculated with *Lactobacillus plantarum*; Lb, alfalfa inoculated with *Lactobacillus buchneri*; Pp, alfalfa inoculated with *Pediococcus pentosaceus;* LbLp, alfalfa inoculated with combined *L. buchneri* and *L. plantarum*; LbPp, alfalfa inoculated with combined *L. buchneri* and *P. pentosaceus*, after 7, 14, 30, and 60 ensiling days.

Table 4. Alpha diversity indices of the bacterial community in alfalfa before and after different ensiling days

Ensiling days	Treatment	Sequence number	OTUs	Shannon index	Simpson index	Chao 1	ACE	Good's coverage
	FM	218707	634	3.352	0.110	639.0	635.7	0.999
7 d	CK	197261	212	2.365	0.155	255.1	299.7	0.999
	Lp	179620	311	1.583	0.419	372.1	388.1	0.999
	Lb	189421	227	1.927	0.287	309.5	391.0	0.999
	Pp	164380	221	0.896	0.661	288.4	303.7	0.999
	LbLp	201157	245	1.669	0.307	312.7	331.9	0.999
	LbPp	179572	235	1.322	0.405	267.3	283.1	0.999
14 d	CK	165670	256	2.233	0.219	291.8	299.3	0.999
	Lp	209219	334	1.714	0.319	384.9	394.3	0.999
	Lb	166354	259	1.586	0.321	296.6	296.6	0.999
	Pp	194418	272	0.847	0.652	336.8	337.9	0.999
	LbLp	170092	242	1.475	0.400	281.4	293.7	0.999
	LbPp	171749	215	1.4227	0.340	287.7	272.1	0.999
30 d	CK	169202	246	2.350	0.141	312.1	291.3	0.999
	Lp	161614	250	1.765	0.236	321.0	316.6	0.999
	Lb	153050	833	2.090	0.354	845.6	843.5	0.999
	Pp	161607	175	1.209	0.442	287.9	338.2	0.999
	LbLp	209592	263	1.677	0.270	311.0	322.1	0.999
	LbPp	122132	101	1.369	0.371	107.1	107.9	0.999
60 d	CK	163320	543	1.840	0.288	558.0	550.9	0.999
	Lp	190379	195	1.611	0.272	216.7	220.7	0.999
	Lb	190015	186	1.589	0.282	204.0	210.2	0.999
	Pp	205221	177	1.468	0.318	194.2	192.1	0.999
	LbLp	208894	231	2.016	0.234	268.4	247.8	0.999
	LbPp	202145	230	1.921	0.197	259.1	261.5	0.999

OTUs, operational taxonomic units; FM, alfalfa biomass fresh material; CK, alfalfa with no inoculant; Lp, alfalfa inoculated with *Lactobacillus plantarum*; Lb, alfalfa inoculated with *Lactobacillus buchneri*; Pp, alfalfa inoculated with *Pediococcus pentosaceus;* LbLp, alfalfa inoculated with combined *L. buchneri* and *L. plantarum*; LbPp, alfalfa inoculated with combined *L. buchneri* and *P. pentosaceus*, after 7, 14, 30, and 60 ensiling days.

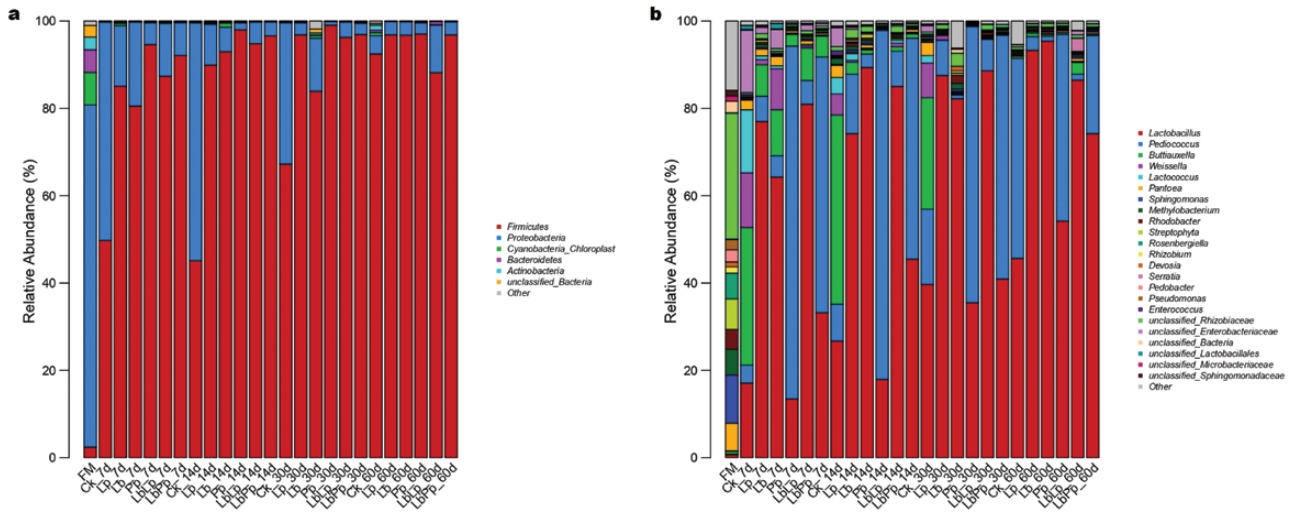

Figure 2. Bacterial community dynamics in alfalfa before and after different ensiling days. Relative abundance of bacterial community at (**a**) phylum and (**b**) genus levels. FM, alfalfa biomass fresh material; CK, alfalfa with no inoculant; Lp, alfalfa inoculated with *Lactobacillus plantarum*; Lb, alfalfa inoculated with *Lactobacillus buchneri*; Pp, alfalfa inoculated with *Pediococcus pentosaceus*; LbLp, alfalfa inoculated with combined *L. buchneri* and *L. plantarum*; LbPp, alfalfa inoculated with combined *L. buchneri* and *P. pentosaceus*, after 7, 14, 30, and 60 ensiling days.

Moreover, the distinctions in 16S rRNA gene-predicted metabolism of carbohydrates and amino acids on the third pathway level are presented in Fig. 5c and d, respectively. The carbohydrate metabolism genes involved in glycolysis and gluconeogenesis, fructose and mannose metabolism, amino sugar and nucleotide sugar metabolism, pyruvate metabolism, starch and sucrose metabolism, and pentose phosphate pathway (**PPP**) in LAB-inoculated alfalfa silages were

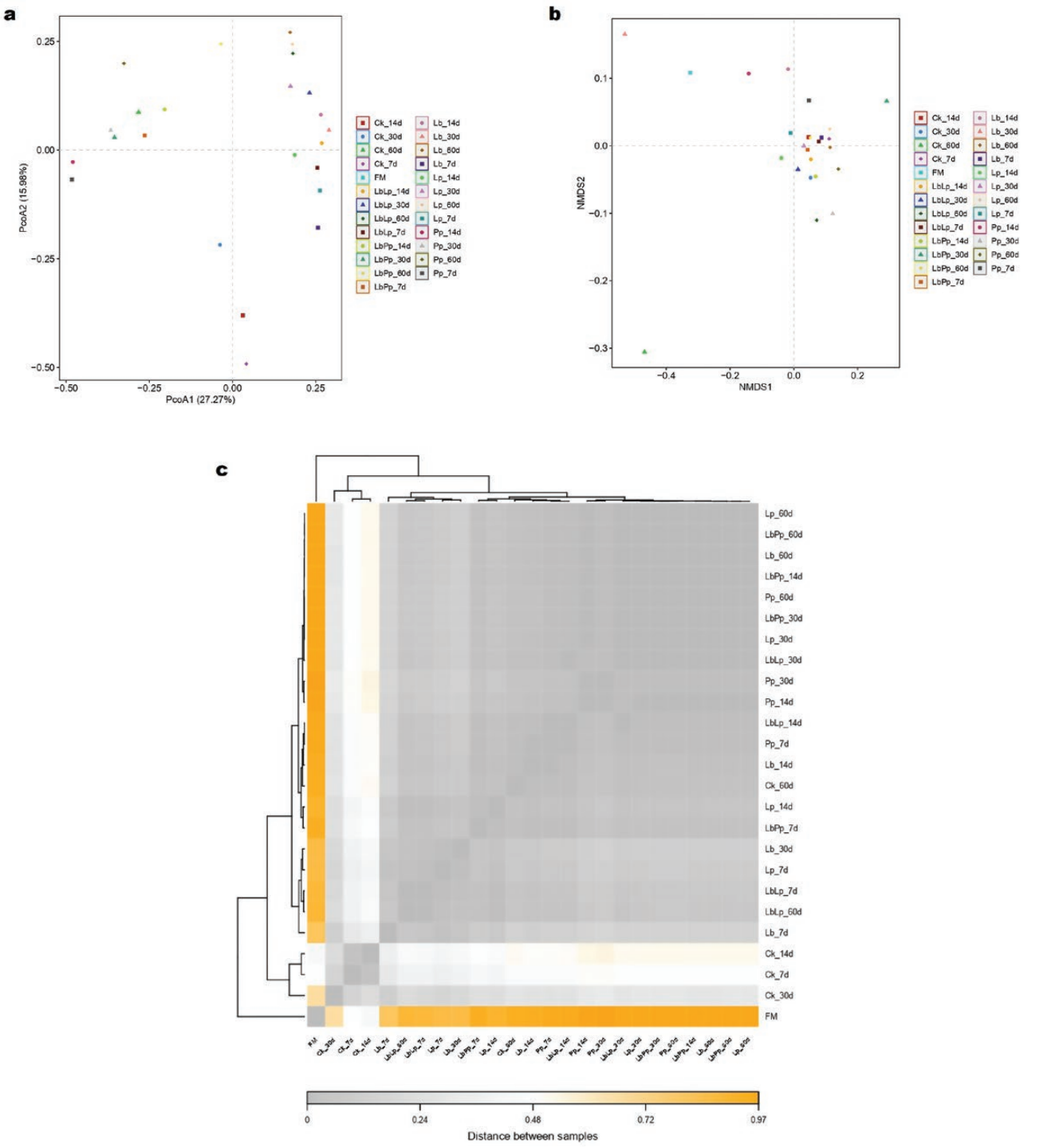

Figure 3. Bray–Curtis distance plots based on (**a**) principal coordinates analysis (PCoA) at OTUs level, (**b**) nonmetric multidimensional scaling (NMDS) with unweighted Unifrac dissimilarity at the genus level, and (**c**) phylum level distance heatmap of the bacterial community in alfalfa before and after different ensiling days. Stress value: 0.07; ANOSIM value, R^2: 0.99, $P = 0.001$. FM, alfalfa biomass fresh material; CK, alfalfa with no inoculant; Lp, alfalfa inoculated with *Lactobacillus plantarum*; Lb, alfalfa inoculated with *Lactobacillus buchneri*; Pp, alfalfa inoculated with *Pediococcus pentosaceus*; LbLp, alfalfa inoculated with combined *L. buchneri* and *L. plantarum*; LbPp, alfalfa inoculated with combined *L. buchneri* and *P. pentosaceus*, after 7, 14, 30, and 60 ensiling days.

enhanced during ensiling. On the other hand, butanoate, inositol phosphate metabolism, galactose, glyxylate and dicarboxylate, propanoate, ascorbate and aldarate, citrate cycle, C5-branched dibasic acid, and pentose and glucuronate interconversions were inhibited in LAB-inoculated alfalfa after prolonged ensiling. However, higher ($P < 0.05$) abundances of these carbohydrate metabolism genes were detected in LbLp-inoculated alfalfa silage after 60 d (Fig. 5c).

The amino acid metabolism genes involved in arginine and proline metabolism, cysteine and methionine metabolism, and alanine, aspartate, and glutamate metabolism were increased ($P < 0.05$) in LAB-treated alfalfa after 30 and 60 d

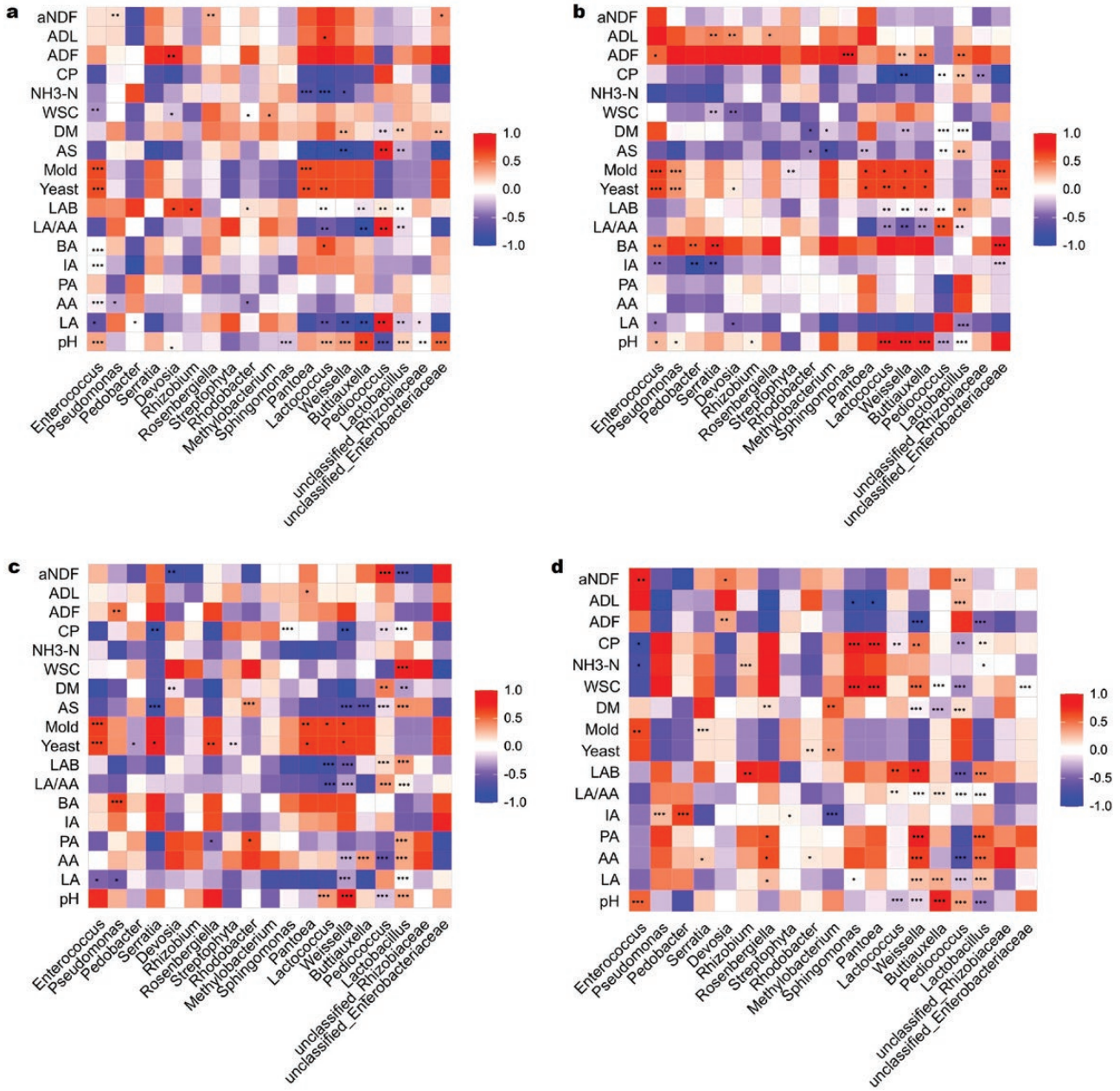

Figure 4. Spearman correlation analysis heatmaps of fermentation profile and bacterial genera in alfalfa silage inoculated at different ensiling days. (**a**) 7, (**b**) 14, (**c**) 30, and (**d**) 60 ensiling days. AA, acetic acid; BA, butyric acid; IA, isobutyric acid; LA, lactic acid; AS, aerobic stability; aNDF, amylase-inoculated neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; CP, crude protein; DM, dry matter; NH_3-N, ammoniacal nitrogen; WSC, water-soluble carbohydrate; significant correlation levels, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

of ensiling. In contrast, the abundance of genes involved in lysine biosynthesis and degradation, phenylalanine, tyrosine, and tryptophan biosynthesis, glycine, serine, and threonine metabolism, tryptophan metabolism, phenylalanine metabolism, tyrosine metabolism, valine, leucine, and isoleucine biosynthesis and degradation, and histidine metabolism were reduced ($P < 0.05$) after 7 and 14 d of ensiling (Fig. 5d).

Figure 6 shows the relative abundances of functional carbohydrate degradation enzymes. The oligo-1,6-glucosidase [EC:3.2.1.10], esterase/lipase [EC:3.1.1.-], glycosyl transferase, family 25“] [EC:2.4.1.50], β-glucosidase [EC:3.2.1.21], endo-1,4-β-xylanase [EC:3.2.1.8], β-galactosidase [EC:3.2.1.23], xylan 1,4-β-xylosidase [EC:3.2.1.37], leucyl aminopeptidase [EC:3.4.11.10], and alpha-mannosidase [EC:3.2.1.24] were increased ($P < 0.05$) in Lb-, LbLp-, and LbPp-inoculated silages after 60 d of ensiling. Also, carboxylesterase [EC:3.1.1.1], alpha-amylase [EC:3.2.1.1], chitooligosaccharide deacetylase [EC:3.5.1.-], xaa-Pro dipeptidase [EC:3.4.13.9], aminopeptidase Y [EC:3.4.11.15], and aminopeptidase [EC:3.4.11.-] were reduced ($P < 0.05$) in CK and LAB-inoculated silages after 7 and 14 d of ensiling.

Discussion

The epiphytic LAB count in FM was higher than the recommended range of 5.0 $\log_{10}$ cfu/g FM. According to Li et al.

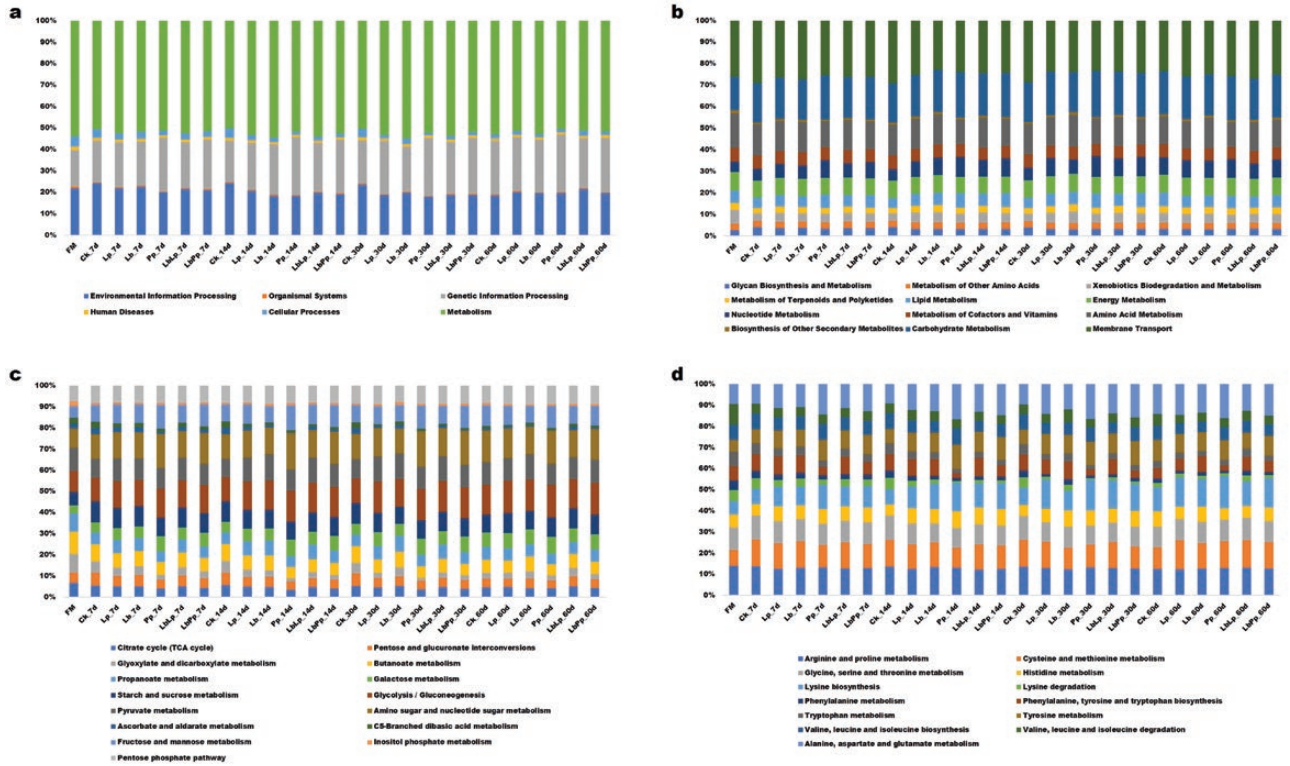

Figure 5. Bar graphs showing the 16S rRNA gene-prediction of the first (**a**) and second (**b**) level metabolic functions, third level KEGG Orthology for carbohydrate metabolism (**c**), and amino acid metabolism (**d**) before and after different ensiling days using PICRUSt. FM, alfalfa biomass fresh material; CK, alfalfa with no inoculant; Lp, alfalfa inoculated with *Lactobacillus plantarum*; Lb, alfalfa inoculated with *Lactobacillus buchneri*; Pp, alfalfa inoculated with *Pediococcus pentosaceus;* LbLp, alfalfa inoculated with combined *L. buchneri* and *L. plantarum*; LbPp, alfalfa inoculated with combined *L. buchneri* and *P. pentosaceus*, after 7, 14, 30, and 60 ensiling days.

(2022), a higher number of epiphytic LAB in FMs is critical for successful silage preservation. Also, the WSC content of FM was lower than the recommended value of 50 g/kg DM required for silage fermentation. WSC in FM is a significant factor during ensiling since it ensures the success of silage fermentation (Ni et al., 2016). The WSC content and LAB number influence the rate of decrease in pH during the early stages of ensiling, which are critical for successful silage fermentation (Li et al., 2022). In the present study, the WSC content of fresh alfalfa was 44.53 g/kg DM (Table 1), which shows the potential ensiling difficulty without inoculants, making it necessary to add LAB inoculants to enhance silage fermentation.

Maintaining a low pH during ensiling is a critical property of LAB inoculants for producing high-quality silages. The significant pH reduction in LbPp-inoculated alfalfa silages after 30 and 60 d of ensiling could be linked to the dominance of *Lactobacillus* and *Pediococcus*, which rapidly ferments WSC to synthesize LA with a rapid pH decrease. As expected, LA concentration was increased in Lp- and Pp-inoculated alfalfa silages but decreased in Lb-, LbLp-, and LbPp-inoculated silages. On the other hand, significantly higher AA contents were detected in Lb-inoculated alfalfa silages after 30 d. Until now, heterofermentative LAB has been the most prevalent LAB synthesizing significant amounts of AA from LA conversion during fermentation (Muck et al., 2018). The LA/AA contents in Lb-, LbLp-, and LbPp-inoculated silages were lower (<3.0) throughout the ensiling days. According to Nazar et al. (2020), fermentation processes with LA/AA ratios below 3.0 are regarded as heterolactic. Low PA concentrations were detected in LAB-inoculated silages after 7 and 14 d ensiling. This supports the previous speculation that most silages contain a very low PA concentration. Also, very low BA and IA concentrations were detected in LAB-inoculated alfalfa silages after different days of ensiling. On the contrary, Sikora et al. (2021) reported high BA concentrations in high-moisture stem alfalfa silages inoculated with *L. plantarum*. This was attributed to the static pH values from 0 to 350 d ensiling, unlike the present study that detected a declining trend in pH after 60 d ensiling. Higher BA concentrations have been found in poorly fermented silages caused by clostridial fermentation, resulting in reduced nutritional value because of the breakdown of soluble nutrients (Okoye et al., 2023c).

Although the LAB count in the FM (7.25 $\log_{10}$ cfu/g FM) was higher than the required minimum LAB count (>5.0 $\log_{10}$ cfu/g FM) for ensiling, the LAB counts increased after 14, 30, and 60 d of ensiling. However, the significantly higher LAB counts detected in LbLp-inoculated alfalfa silage after 60 d could be attributed to *L. buchneri* ability to produce AAs, which incorporate LA produced by *L. plantarum* to increase the growth of desirable epiphytic LAB. Wang et al. (2022) found a reduction in the LAB population after 7 d ensiling, which was attributed to the inhibition of desirable LAB development due to silage acidic environment. Also, the absence of molds and yeasts and the high aerobic stabilities in LAB-inoculated alfalfa silage at different ensiling days could be linked to the ability of LAB to reduce pH and prevent pathogenic microbes development that causes aerobic spoilage during feed out.

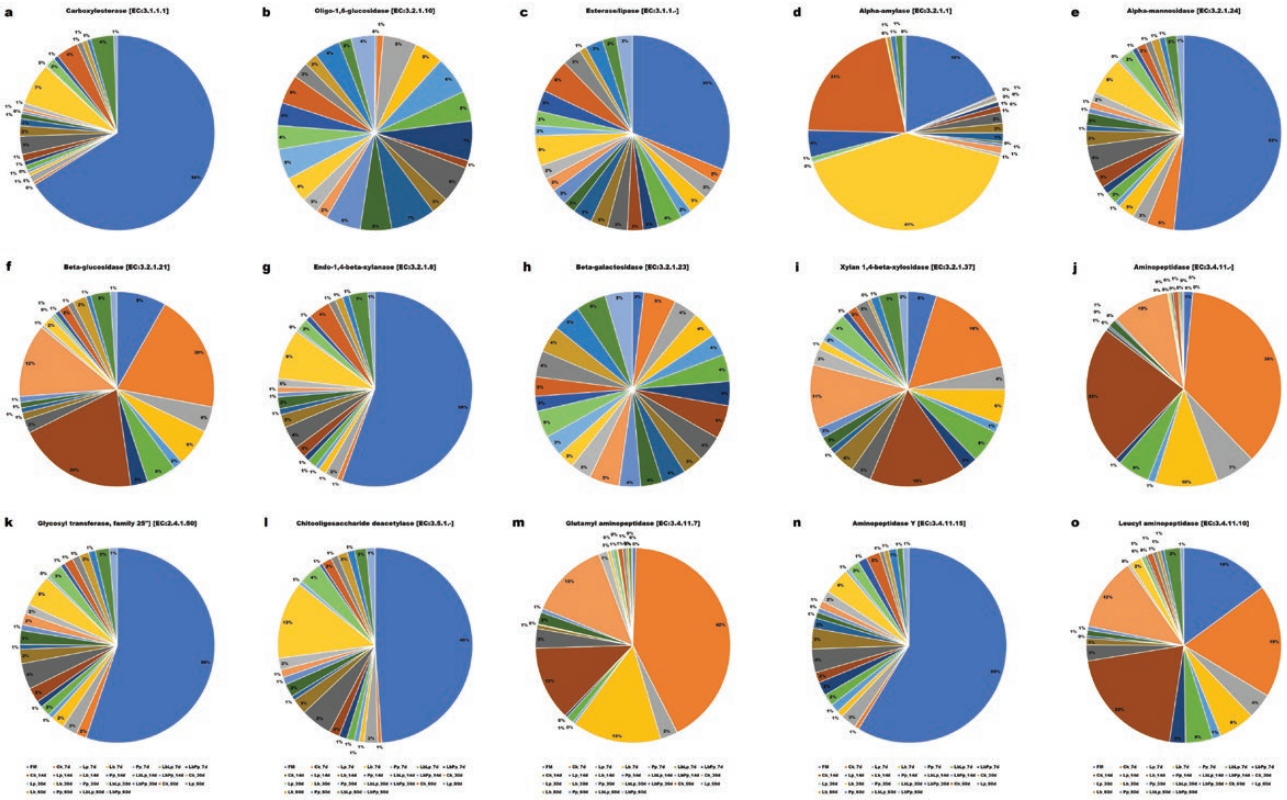

Figure 6. Venn diagrams showing the 16S rRNA gene prediction of functional carbohydrate degradation enzymes before and after different ensiling days using PICRUSt. (**a**) oligo-1,6-glucosidase, (**b**) carboxylesterase, (**c**) esterase/lipase, (**d**) alpha-amylase, (**e**) alpha-mannosidase, (**f**) β-glucosidase, (**g**) endo-1,4-β-xylanase, (**h**) β-galactosidase, (**i**) xylan 1,4-β-xylosidase, (**j**) aminopeptidase, (**k**) glycosyl transferase, family 25", (**l**) chitooligosaccharide deacetylase, (**m**) xaa-Pro dipeptidase, (**n**) aminopeptidase Y, and (**o**) leucyl aminopeptidase. FM, alfalfa biomass fresh material; CK, alfalfa with no inoculant; Lp, alfalfa inoculated with *Lactobacillus plantarum*; Lb, alfalfa inoculated with *Lactobacillus buchneri*; Pp, alfalfa inoculated with *Pediococcus pentosaceus;* LbLp, alfalfa inoculated with combined *L. buchneri* and *L. plantarum*; LbPp, alfalfa inoculated with combined *L. buchneri* and *P. pentosaceus*, after 7, 14, 30, and 60 ensiling days.

Contrary to a similar study that attributed the stable DM content during ensiling to LA fermentation by *L. plantarum* inoculant (You et al., 2022), the present study detected an unstable DM in LAB-inoculated silages, with a decline after 30 d but increase after 60 d of ensiling in Lb- and LbLp-inoculated silages. The unstable DM contents could be linked to the production of several organic acids during heterolactic fermentation. It has been reported that decreased DM resulting from LA fermentation during ensiling should not exceed 6% (Villa et al., 2020). Although the WSC content of alfalfa biomass FM (44.53 ± 0.07 g/kg DM) did not satisfy the recommended value of 50 g/kg DM required for improved silage fermentation, the WSC contents in Lb-, LbLp-, and LbPp-inoculated silages did not experience a significant decline after 30 and 60 d. Li et al. (2019) noted that the WSC content of fresh silage material is essential for LA fermentation. Even though WSC decrease is a known problem with alfalfa, a sufficient supply of substrate for microorganisms is suggested for efficient WSC recovery in alfalfa silages. Moreover, the higher decline in Lp-and Pp-inoculated silages could be linked to homolactic fermentation, which quickens the early phase of ensiling through swift fermentation of WSC to produce LA. The protein requirements of feeding animals range from 10% to 14% DM, which was substantially higher in LbLp- and LbPp-inoculated alfalfa silage after 60 d, showing that the LAB combination prevented proteolysis and enhanced the protein content of alfalfa silage. Besides, protein degradation in silages is frequently detected by measuring the NH_3-N content, which must be lower than 100 g/kg TN for quality silages (Wang et al., 2022). The NH_3-N content in this study was significantly lower in LbLp- (27.73 g/kg TN) and Lp- (28.37 g/kg TN) inoculated alfalfa silages after 60 d. NH_3-N production is often caused by the interaction of plant proteolytic enzymes and *Clostridium*, which are effective at pH 5–6 (Bao et al., 2022). The lower pH facilitated by homofermentative LAB in LbLp- and Lp-inoculated alfalfa silages could have inhibited plant proteases activity and pathogen development in these silages over time.

The present study detected a significant decrease in ADL and ADF contents of LAB-inoculated silages after 60 d. Also, the aNDF contents maintained a steady increase in Lb- and LbLp-inoculated silages after 60 d. According to Cai et al. (2020), LAB-inoculated corn stover silage showed increased aNDF levels during ensiling, making corn stover a probable silage resource that could solve animal feed issues. Nevertheless, the efficient degradation of ADF and aNDF contents by LAB causes a significant decrease in cell wall carbohydrates by releasing lignocellulose enzymes, which facilitate breakdown to WSC, thus making glucose available for subsequent breakdown to lactate (Ebrahimi et al., 2016). In this study, xylose contents increased significantly, while cellobiose, galactose, and glucose contents were reduced in LAB-inoculated alfalfa after 60 d ensiling. Similar studies have reported decreased sucrose (disaccharide) and glucose

(monosaccharide) in alfalfa and sorghum silages (Bai et al., 2011; Wang et al., 2022). The reduction in glucose in the present study showed that monosaccharide was the initial fermentation substrate acted upon by the LAB inoculants during ensiling and was more receptive to breakdown than other carbohydrates. On the other hand, the increased xylose contents could be attributed to acid hydrolysis during ensiling. Besides, arabinose contents increased in LbLp- and LbPp-inoculated silages after 60 d. This could be linked to the synergistic effects of LAB inoculants and dominant bacteria in reducing the pH during fermentation since structural carbohydrates are first digested by enzymes before acid at low pH. Therefore, we speculate that the increased xylose and arabinose contents in LbLp-and LbPp-inoculated alfalfa silages could have resulted from significant hemicellulose degradation after 60 d ensiling.

The decrease in bacterial diversity of ensiled alfalfa compared to fresh alfalfa in this study could be affirmed by the study of Ogunade et al. (2018), which also found lower bacterial diversity in silages inoculated with *L. plantarum* and *L. buchneri*. This was attributed to the high abundance of *Lactobacillus* that dominated other bacteria genera during ensiling. A probable explanation for this is that LAB combinations are highly tolerant to acidic environments due to beneficial organic acid production and antibacterial activity, thus fostering antagonism against pathogenic bacteria to promote the development of the beneficial bacteria in silage (Duniere et al., 2017). This is coherent with the results of the LAB counts, where it can be found that LAB number increased faster in LbLp-inoculated alfalfa silages after 60 d than in other treatments, suggesting that Lp and Lb might have a synergistic effect on native LAB growth. *Proteobacteria* decreased after ensiling, and *Firmicutes* dominated the inoculated and uninoculated alfalfa silages after 7, 14, 30, and 60 d. Also, *Lactobacillus and Pediococcus* genera dominated other bacteria during ensiling and inhibited the growth of pathogenic microbes in fresh alfalfa. Similar kinds of research have reported the abundance of *Lactobacillus* as the prominent genus in corn and alfalfa silages (Bai et al., 2020; Nazar et al., 2020, 2021). Although the abundance of *Firmicutes* is linked to proteolysis, which causes high NH_3-N contents in silages, studies have suggested that the most prominent genera *Lactobacillus* provide acidic and anaerobic ensiling conditions that eliminate pathogens (Bai et al., 2020).

The emergence of specific traits that result in substantial changes in microbial abundance is the ideal way to comprehend microbial interaction patterns. In particular, for mutualistic interactions and direct microbial antagonism in feedstocks, Faust and Raes (2012) hypothesized that correlation-based techniques are reliable in inferring potential connections from high-throughput relative abundance data. According to the Spearman correlation analysis, *Lactobacillus* correlated positively with pH, CP, WSC, AA, PA, LA, LA/AA, and LAB in Lb-, Pp-, LbLp-, and LbPp-inoculated alfalfa silages after 60 d. This is in accordance with the study of Wang et al. (2022), which found that the positive correlation between *Lactobacillus* and WSC indicates sufficient fermentable substrates that stimulate the development of beneficial LAB during the early phase of ensiling.

Notably, LAB-inoculated alfalfa silages correlated negatively with molds and yeasts throughout ensiling. Yeasts have been reported to cause aerobic deterioration and DM losses in mature corn silage (Santos et al., 2017). The absence of yeasts in LAB-inoculated alfalfa silages confirms the speculation that the present study achieved good-quality silage by the action of LAB inoculants in facilitating *Lactobacillus* and *Pediococcus* abundance, thereby restricting yeast growth in LAB-inoculated silages. *Lactobacillus* and *Pediococcus* are notable for producing adequate amounts of beneficial organic acids and reduced pH (Hu et al., 2020). Based on the improved fermentation profile and bacterial community dynamics in LbLp-inoculated alfalfa after 60 d ensiling, we speculate that LAB inoculants and the dominant epiphytic LAB species, through a potent interaction mechanism, adhered to the surface of alfalfa to hamper the adverse effects associated with the pathogenic microorganisms' growth.

Although the initial stage of the alfalfa silage fermentation has a very diverse bacterial community, the end stage is critical for assessing the fermentation quality, thus requiring the prediction of the functional profiles to fully understand the impact of LAB inoculants or their interaction with native bacteria on alfalfa silage quality. The first-level pathway showed that metabolism was higher in LAB-inoculated alfalfa silages after 30 and 60 d, while the second-level pathway shows that carbohydrate, membrane transport, and amino acid metabolism were significantly enhanced in LbLp-inoculated silage after 60 d ensiling. The metabolic functions of homo- and heterofermentative LAB are evaluated based on carbohydrate metabolism and fermentation products. According to Pophaly et al. (2012), the membrane transport of amino acids such as cysteine in LAB may contribute to maintaining intracellular redox balance during fermentation. In the third-level pathway, carbohydrate genes involved in pyruvate metabolism were enhanced in LbLp-inoculated alfalfa after 60 d ensiling. Pyruvate is a crucial glucose metabolic intermediate in homofermentative and heterofermentative LAB. The metabolism of galactose, glyxylate, butanoate and dicarboxylate, propanoate, ascorbate and aldarate, C5-branched dibasic acid, citrate cycle, and pentose and glucuronate interconversions were inhibited in LAB-inoculated alfalfa after 30 and 60 d of ensiling. This is in line with the study of Wang et al. (2022), which reported the suppression of similar pathways in sorghum silages. Because the citrate cycle occurs under aerobic conditions, its metabolism could be linked to oxygen consumption. The removal of hydrogen ions makes it unable to begin the respiratory chain to undergo total oxidation without oxygen associated with silage fermentation (Zotta et al., 2017). In addition, a few amino acid metabolism genes were significantly enhanced in LAB-inoculated silages after 7 and 14 d of ensiling. Several of these amino acid metabolic pathways were probably constrained by LAB activity, such as the speedy reduction in pH values in the initial phase of alfalfa fermentation that prevented the protein breakdown by pathogenic microbes.

Furthermore, the relative abundance of functional carbohydrate degradation enzymes that break down complex polysaccharides could help reveal a significant function of the silage microbiome in the breakdown of plant carbohydrates. These enzymes are classified as carbohydrate-active enzymes (CAZymes) and comprise various families due to their structural similarities, amino acid sequences, and functions (Lee et al., 2015). Endo-1,4-β-xylanase was significantly increased in LbLp-inoculated alfalfa silage after 60 d. This could be linked to the increased production of xylose, and arabinose detected in the present study since endo-1,4-β-xylanase belongs to glycoside hydrolase enzymes that accelerate glycosidic bond of

β-1,4-xylosides hydrolysis to degrade the linear polysaccharide xylan into xylose and arabinose and have received much attention recently because of its ability to degrade lignocellulosic biomasses. According to Borges et al. (2014), endo-1,4-β-xylanase has a superior specific activity than other eukaryotic xylanases. This atypical activity might indicate a reduced level of this enzyme complex in enzymatic combinations, which would help reduce the cost. Even though studies on the ability of LAB species to degrade plant carbohydrates are limited, You et al. (2022) identified the abundance of CAZymes responsible for degrading arabinoxylan and cellulose by the dominant bacteria genus, *Lactobacillus* in alfalfa inoculated with *L. plantarum* strain PS-8. β-Glucosidase was enhanced in control and LAB-inoculated alfalfa silages after 30 and 60 d. This enzyme converts cello-oligosaccharides and cellobiose to glucose. However, cellobiose and glucose were significantly reduced after 30 and 60 d, thus suggesting successive glucose consumption ensued by the massive proliferation of homo- and heterofermentative LAB to produce LA, AA, and other beneficial metabolites during prolonged alfalfa silage fermentation. Also, glucose 6-phosphate, an intermediate from glucose metabolism, is transformed into ribulose 5-phosphate, carbon dioxide, and NADPH through the PPP (Wang et al., 2021). β-Galactosidase was higher in Pp-and LbLp-inoculated silage after 60 d. Lee et al. (2015) reported the ability of β-glucosidase to disintegrate α-1,4-glucosidic linkages among simple sugars, de-branching activity, hydrolyzing the α-1,6 linkages in oligosaccharides in *Proteobacteria* and *Actinobacteria*. In addition to the ability of β-glucosidase to break down disaccharides into galactose and glucose, which are ultimately used in glycolysis, this enzyme catalyzes the disintegration of various oligosaccharides present in cellulosic biomass.

Conclusion

This study used metagenomic sequence technology to understand the performance of homo- and heterofermentative LAB on the fermentation, microbial community dynamics, and functional profiles of alfalfa silages after 7, 14, 30, and 60 d of ensiling. A positive relationship between the LAB-inoculants and dominant LAB genera with alfalfa silage fermentation profile revealed the efficacy of LAB interaction in altering the development of pathogenic microbes and improving the silage quality after 30 and 60 d ensiling. The 16S rRNA gene-predicted functional analyses further unveiled the potential of the functional profiles facilitated by *L. buchneri* and *L. plantarum* to degrade polysaccharides in alfalfa after 60 d of ensiling, suggesting the need for further studies to uncover the diverse performance of LAB combinations and their consortium with other natural and artificial additives in various kinds of silages.

Supplementary Data

Supplementary data are available at *Journal of Animal Science* online.

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Conflict of Interest Statement

The authors declare that they have no competing interests.

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