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Original Research Article

Changes in chemical composition, structural and functional microbiome during alfalfa (*Medicago sativa*) ensilage with *Lactobacillus plantarum* PS-8

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ABSTRACT

Improving silage production by adding exogenous microorganisms not only maximizes nutrient preservation, but also extends product shelf life. Herein, changes in the quality and quantity of *Lactobacillus plantarum* PS-8 (PS-8) -inoculated alfalfa (*Medicago sativa*) during silage fermentation were monitored at d 0, 7, 14, and 28 (inoculum dose of PS-8 was 1×10^5 colony forming units [cfu]/g fresh weight; 50 kg per bag; 10 bags for each time point) by reconstructing metagenomic-assembled genomes (MAG) and Growth Rate InDex (GRiD). Our results showed that the exogenous starter bacterium, PS-8 inoculation, became the most dominating strain by d 7, and possibly played a highly active role throughout the fermentation process. The pH value of the silage decreased greatly, accompanied by the growth of acid-producing microorganisms namely PS-8, which inhibited the growth of harmful microorganisms like molds (4.18 vs. 1.42 log cfu/g) and coliforms (4.95 vs. 0.66 log most probable number [MPN]/g). The content of neutral detergent fiber (NDF) decreased significantly (41.6% vs. 37.6%; dry matter basis). In addition, the abundance and diversity of genes coding microbial carbohydrate-active enzymes (CAZymes) increased significantly and desirably throughout the fermentation, particularly the genes responsible for degrading starch, arabino-xylan, and cellulose. Overall, our results showed that PS-8 was replicating rapidly and consistently during early- and mid-fermentation phases, promoting the growth of beneficial lactic acid bacteria and inhibiting undesirable microbes, ultimately improving the quality of silage.

1. Introduction

The rapid development of animal husbandry has led to an increasing demand for animal feed. Alfalfa (*Medicago sativa*) is high-quality forage due to its high protein and mineral contents and good palatability. However, there is a potential problem of alfalfa forage, as it is recognized to favor the growth of spoilage bacteria. This is due to its high buffering capacity and low content of water-soluble carbohydrates (WSC), making it more difficult to lower pH and in turn causing storage problems compared with other forages (Jaurena and Pichard, 2001). Additionally, the high humidity and the rainy climate in southern China further intensifies this storage problem, shortening silage shelf life. Thus, to overcome these issues, it is of interest to develop strategies to effectively suppress spoilage and undesirable microbes during fermentation, improve nutrient preservation, and prolong silage shelf life.

Ensilage is a traditional forage preservation method based on microbial anaerobic fermentation, mainly by lactic acid bacteria (LAB). In this process, WSC in the green raw material are fermented

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to organic acids, such as lactic acid and acetic acid, resulting in pH decline that inhibits undesirable microorganisms (Duniere et al., 2013). Although there is approximately 10% nutrient loss during the silage fermentation process, the organic acids and organic chelate minerals produced during fermentation are absorbed more readily by ruminants (Kalac, 2011). The most frequently added LAB are homofermentative species, such as *Lactobacillus plantarum* and *Pediococcus* spp. Homolactic fermentation is desirable because of the extremely high theoretical dry matter (DM) recovery and efficient production of lactic acid; the release of lactic acid causes a more rapid pH decline than other types of acids. Fermentation is a microbial-driven process, and the quality of silage fermentation is highly dependent on the activities and types of microorganisms involved in the ensiling process (Peng et al., 2018). Beneficial microorganisms that accelerate the rate of utilization of WSC by other microbes and produce organic acids contribute greatly to the silage fermentation process. In contrast, some undesirable microorganisms could cause spoilage (Driehuis and Oude Elferink, 2000). This study selected to investigate the effect of inoculating a homofermentative LAB strain, *L. plantarum* PS-8 (PS-8), on alfalfa silage fermentation. This strain is proven to be beneficial in improving the quality and quantity of silage fermentation and cow milk production in previous studies (Cao et al., 2021; Xu et al., 2017). The contribution of PS-8 can be evaluated by monitoring the succession of the complex epiphytic microbial communities in relation with changes in chemical composition of the silage at different stages of fermentation.

Metagenomics in combination with advanced bioinformatic analysis of high-quality data could be applied to assemble single-sample metagenomes and unveil the functional potential of strain-level bacterial genomes. Pasolli et al. (2019) used such methods to leverage 9,428 metagenomes obtained from human gut sequencing to reconstruct and track 154,723 metagenomic-assembled genomes (MAG), revealing novel microbial functions (Pasolli et al., 2019). In addition, Emiola and Oh (2018) developed an algorithm to calculate bacterial growth rate (i.e., Growth Rate In-Dex, GRiD) of individual strains based on bacterial genome sequencing. Such analysis was advantageous in estimating how much the viable subpopulation of the overall metagenomes contributed to the community phenotype (Emiola and Oh, 2018).

This study hypothesized that adding exogenous LAB could improve the alfalfa ensilage process via modulating the silage microbiome. Although some previous works investigated the application of microbial silage starter prior to the ensilage process, few studies investigated the microbial dynamics in-depth via whole-genome metagenomics approaches, which could help reveal the functional potential and vitality of silage microbiota and PS-8 during different stages of fermentation. Thus, the current work utilises the advent of metagenomic analysis to investigate the contribution of PS-8 as silage starter strain to facilitate silage fermentation. The dynamics of the microbial community structure and function were assessed by using the single-sample metagenomic assembly approach, focusing on the changes in GRiD and carbohydrate metabolism at different stages of alfalfa silage fermentation. Meanwhile, a number of chemical parameters were monitored during the silage fermentation process.

2. Materials and methods

2.1. Study design and sample collection

Alfalfa was harvested at early bloom stage, and fresh forage was taken immediately to the silage site and wilted to approximately 50% moisture content in the sun. The forage was chopped into pieces of approximately 2 cm using paper-cutters. The chopped forage was then sprayed with PS-8 (inoculation dose: 1×10^5 colony forming units (cfu)/g fresh weight; provided by the Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University of China). The PS-8 strain was originally isolated from a naturally fermented yoghurt made by an animal-herding family residing at Wulatezhongqi Grassland of Inner Mongolia of China (Wu et al., 2009). The applied amount was the least effective dose for silage inoculation based on a previous study (Huisden et al., 2009). The PS-8-sprayed alfalfa was thoroughly mixed and was randomly packed into vacuum-sealing polyethylene plastic bags (approximately 50 kg per bag; a total of 40 individual bags were prepared). The packed silage samples were kept at ambient temperature (20 to 25 °C) and without any atmosphere conditioning measures for 28 d. Fresh (labelled as d 0) and ensiled materials were sampled on d 0, 7, 14, and 28 for microbial and chemical analyses. Except on d 0, 10 bags were opened at each time point. A nine-point sampling method was used to sample the upper, middle, and lower parts of each bag after removing the surface layer. All samples were collected in sterilized containers and kept in ice boxes during transportation. The ensiled samples collected at d 0, 7, 14, and 28 (10 samples per day) were selected for bacterial metagenomics and chemical composition analyses. Suitable amount of cryoprotectant (Sample Protector for RNA/DNA; Takara Bio, Inc.) was added and mixed with samples designated for metagenomic sequencing to avoid DNA degradation. This reagent could stabilize nucleic acids (including RNA) in samples during sample transportation and prolonged storage, enabling metagenomic and gene expression analyses.

2.2. Physical and biochemical indicators and microbial counts

Each silage sample (25 g each) collected at d 0, 7, 14, and 28 was vortex mixed with 225 mL of deionized water for 30 min for pH determination via a pH meter (Mettler Toledo, Zurich, Switzerland). For determining the chemical composition, each sample (25 g each) was dried in a forced-air oven at 55 °C for 72 h for the determination of DM content (Ke et al., 2017). Dried samples were milled to pass a 1-mm screen of a laboratory knife mill (Taisite Instrument, Tianjin, China). Milled samples were analyzed for crude protein (CP), soluble protein (SP), acid detergent fiber (ADF), neutral detergent fiber (NDF), WSC, and ammonia nitrogen (NH_3—N). The contents of SP, CP, NDF, and ADF were determined according to Licitra et al. (1996) and Ke et al. (2017). The contents of NH_3—N and WSC were determined according to the methods described by Broderick and Kang (1980) and Thomas (1977). Results were expressed on a DM basis, and all chemical assays were performed in triplicate.

Lactic acid, acetic acid, γ-aminobutyric acid, and phenyl lactic acid were detected using ultra performance liquid chromatography-electrospray ionization-quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOF MS; Waters, Milford, MA, USA). The sample preparation along with the UPLC and QTOF-MS conditions were based on the methods described in Bao et al. (2016).

To determine lactic acid and acetic acid levels, a preparative ZORBAX Elipse AAA C18 column (3.5 μm, 4.6 mm × 150 mm) was used. Solvent A was phosphate buffer solution (pH 2.5), and solvent B was methanol solution. Elution was performed with a gradient of 97:3. Analytical column temperature was 300 °C, and the flow rate was 1 mL/min. Absorbance was detected at 210 nm. To determine γ-aminobutyric acid, 10 mg o-phthalaldehyde (OPA) derivative reagent (99%, Sigma) was dissolved in 0.5 mL methanol, then 30 μL 2-mercaptoethanol and 2 mL 0.4 mol/L borate buffer (HPLC grade) (pH 9.4) were added. Before injecting into the machine, 10 μL of sample solution was mixed with 90 μL OPA derivative reagent, reacting for 1 min. A preparative ZORBAX Elipse AAA C18 column (3.5 μm, 4.6 mm × 150 mm) was used. Solvent A was sodium

hydrogen phosphate buffer solution (pH 7.8), and solvent B was the mixture of methanol, acetonitrile, and deionized water (45:45:10). Elution was performed with a gradient of 97:3. The analytical column temperature was 35 °C, and the flow rate was 2.0 mL/min. A fluorescence detector was employed for detection of the excitation and emission wavelengths of 340 nm and 450 nm, respectively. Determination of phenyl lactic acid was performed with high-performance liquid chromatography (HPLC) on an Agilent 1100 Series LC system. A preparative BEHC18 column (1.7 μm, 2.1 mm × 100 mm, Waters, America) was used. Solvent A was formic acid diluted in deionized water (1:999), and solvent B was formic acid diluted in acetonitrile (1:999) solution. Elution was performed with a linear gradient as follows: solvent B 20% to 50% in 2 min, 50% to 95% in 2.1 to 3 min, 95% to 5% in 3 to 3.1 min. Analytical column temperature was 30 °C, and the flow rate was 0.4 mL/min (Bao et al., 2016).

2.3. DNA extraction and shotgun metagenomic sequencing

Silage samples (25 g per sample) reserved for metagenomic sequencing were diluted 10 times with sterile PBS solution and soaked for 24 h at 4 °C on a shaker, followed by filtration through sterilized gauze. The filtrate was centrifuged, and the precipitate of the bacterial mud was collected. The E.Z.N.A. Soil DNA Kit (OMEGA Bio-Tek, USA) was used for sample DNA extraction following the manufacturer's instructions. The quality of extracted DNA was checked by 1% agarose gel electrophoresis and spectrophotometric analysis (the ratio of optical density at 260 nm to that at 280 nm). All extracted DNA samples were stored at −20 °C for further analysis. DNA libraries of fragments (approximately 400 bp long) were prepared separately for each sample. The samples were sequenced on an Illumina HiSeq Xten instrument (Illumina, San Diego, California, USA). Paired-end reads (151 bp) were generated in both forward and reverse directions.

2.4. Metagenomic reads quality control and taxonomic profiling

Raw whole-metagenome shotgun sequencing reads were trimmed in each sample, based on the length and quality, by using SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle). An average of 7.8 Gb of high-quality paired-end reads were obtained for each sample, making a total of 313 Gb of high-quality data (Supplementary data Table S1). MetaPhlan2 (ver. 2.0) was used to taxonomically profile the microbial composition of each sample at the species-level using default settings with Bowtie2 (ver. 2.2.9) as search engine (Truong et al., 2015; Langmead and Salzberg, 2012).

2.5. Illumina metagenomic assembly

High-quality Illumina metagenomic samples were assembled using metaSPAdes (ver. 3.13.0) (Nurk et al., 2017). The parameters -k 33,55,77,99,111 -meta. QUAST (ver. 5.0.0) (Mikheenko et al., 2016) were used to evaluate the results of metagenomic assemblies. The above assembled scaffolds were used to predict the functional genes with Prodigal (ver. 2.6.3) (Hyatt et al., 2010). Finally, a non-redundant gene catalog was constructed using CD-HIT (ver. 4.8.1) (Fu et al., 2012). The gene abundance in the samples was determined by aligning the reads to the gene catalog using Bowtie2 (Qin et al., 2012).

2.6. Metagenomic-assembled genomes

MetaBAT 2 (ver. 2.12.1) (Kang et al., 2019) was used to bin the assemblies using a minimum scaffold length threshold of 2,000 bp. Depth of coverage required for the binning was inferred by mapping the raw reads back to their assemblies with Bowtie2, following by calculating the corresponding read depth of each individual scaffold with samtools (ver. 1.9) (Li, 2011) together with the jgi_summarize_bam_contig_depths function in MetaBAT 2. The completeness and contamination of each MAG was estimated with CheckM (ver. 1.0.18) (Parks et al., 2015) using the lineage_wf workflow. The taxonomy of each MAG was annotated against the National Center for Biotechnology Information (NCBI) nonredundant Nucleotide Sequence Database (NT) using BLASTn at a threshold of 95% identity over 70% coverage. Subsequently, high-quality MAG (completeness > 80% and contamination < 5%) were selected for downstream analysis. The phylogeny was built using the 400 universal PhyloPhlAn markers based on parameters described in Pasolli et al. (2019). The phylogenetic trees were visualized using iTOL (ver. 5.5.1) (Letunic and Bork, 2019).

High-quality MAG classified into *L. plantarum* were compared with the genome of PS-8 for average nucleotide identity (ANI) analysis using fastANI (ver. 1.1) (Jain et al., 2018). The replication rates of *L. plantarum* genomes were calculated by using the GRiD) (ver. 1. 3) (Seshadri et al., 2018). Then, the MAG were clustered at species-level genome bin (SGB) using dRep (ver. 2.2.4) (Olm et al., 2017) with the following parameters: -pa 0.95 -sa 0.95 -nc 0.30 -cm larger. To estimate SGB abundance, all reads were aligned to contigs in SGB using Bowtie2. The mapped sequence counts, contig lengths and total sequence counts were used to normalise the sequence counts and represent the reads per kilobase per million (RPKM) of each sample to the contigs.

2.7. Functional annotation and metabolic pathway analysis

The annotated amino acid sequences were aligned against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database using Usearch (Kanehisa and Goto, 2000) with the option of -usearch_local -id 0.3 -query_cov 0.7. Genes related with polysaccharide metabolism and organic acid biosynthesis pathways were predicted based on the KEGG orthologue group (KO) key reaction. Carbohydrate-active enzymes (CAZymes) were annotated by the dbCAN2 database using HMMER (Eddy, 2009).

2.8. Statistical analysis

Statistical analysis was performed in R-3.5.1. The vegan package (ver. 2.5-2) (https://CRAN.R-project.org/package=vegan) was employed for alpha diversity analysis and principal coordinate analysis (PCoA) analysis using the Bray–Curtis distance. The ggplot2 package (ver. 3.1.0) was used for data visualization (Wickham, 2011). Linear discriminant analysis (LDA) effect size method based on a normalized relative abundance matrix was used to identify the significant differences between samples on d 0 and 7. Spearman correlation analysis was carried out between chemical parameters and relative contents of microflora, as well as between PS-8 and other species. The heatmap was constructed using the "pheatmap" package (ver. 1.0.12) (Kolde and Kolde, 2015). Spearman rank correlation coefficient-based networks were visualized by Cytoscape (ver. 3.7.1) (Shannon et al., 2003). Significant differences were assessed by Wilcoxon rank sum test (Haynes, 2013); $P < 0.05$ was considered statistically significant.

2.9. Data availability

The entire sequence dataset was deposited in the NCBI Sequence Read Archive (SRA) database (accession number PRJNA495415).

3. Results

3.1. Chemical changes during alfalfa silage fermentation

Monitoring changes in the chemical profile (e.g., chemical composition, microbial population, and pH) of the fermented silage could provide indication for the progress and quality of silage fermentation. During silage fermentation, there was no significant change in DM content. The contents of SP increased with fermentation time, although the change in the SP content was not significant, it showed an obvious increasing trend. NH_3–N content increased significantly during the first 7 d ($P < 0.05$), reaching a stable phase between d 7 and 14, followed by a significant decrease between d 14 and 28 ($P < 0.05$). NDF decreased significantly on d 7 ($P < 0.05$), followed by non-significant decreases thereafter. WSC showed a general decreasing trend (Table 1).

Lactic acid content significantly increased with the fermentation time and reached a maximum at 28 d ($P < 0.05$). Acetic acid content exhibited an increasing trend, showing significant increase at 14 and 28 d ($P < 0.05$). The content of γ-aminobutyric acid began to increase significantly at d 14 ($P < 0.05$) and the content of phenyl lactic acid increased significantly at d 14 and 28 ($P < 0.05$). As the organic acid content continued to accumulate as fermentation progressed, the amounts of mold and coliform were suppressed significantly and reduced to levels below the detection limit (Table 1; Supplementary data Table S2). Consistently, the pH of the alfalfa silage decreased significantly from d 7 ($P < 0.05$).

3.2. Dynamics of silage microbiome during fermentation

To understand the microbial contribution to silage fermentation, the dynamics of PS-8 silage microbiome before (d 0) and after (d 7, 14, 28) fermentation started were analyzed by shotgun metagenomic sequencing. The quantity of sequencing data generated in this work was shown in Supplementary data Table S1. The fermentation process was divided into 2 stages; namely, the microbial (*L. plantarum* and *Pediococcus pentosaceus*) proliferation phase when drastic microbial changes occurred (d 0 to 7) and the stable phase (d 7 to 28) when microbial changes were less obvious.

The alpha-diversity of the silage microbiome increased significantly at the early phase ($P < 0.001$), followed slight fluctuation during the stable phase (Fig. 1A). The PCoA performed based on Bray–Curtis distance also showed that the initial silage microbiota (d 0) were structurally distinct from those of the later time points (Fig. 1B). The Bray–Curtis distances of alfalfa silage microbiota was remarkably lower in the stable phase (d 7 to 28) compared with the baseline level at d 0 (Fig. 1C).

At the species level, 7 major bacterial species were identified across all samples (Fig. 1D). Apparent differences were observed in the microbiota composition before and after fermentation. At d 0, the silage microbiota was largely comprised of *Weissella cibaria* (82.4%) and *Pantoea agglomerans* (3.22%), and sequences representing the epiphytic species of alfalfa, *W. cibaria* were dominated. The silage microbiota composition remained rather stable beyond d 7, consistently dominated by sequences representing 3 species; namely *L. plantarum* (31.4%), *W. cibaria* (31.2%), and *P. pentosaceus* (11.4%). The relative abundance of *W. cibaria* decreased significantly after fermentation started, meanwhile *L. plantarum* and *P. pentosaceus* increased obviously, reaching the highest level at d 28 and 7, respectively.

LDA was performed together with Kruskal–Wallis test and Wilcoxon rank sum test to identify differences in microbial composition between samples of d 0 and 7. The results of LDA revealed that *W. cibaria* and *Pantoea ananatis* were enriched at d 0 ($P < 0.05$), whereas *L. plantarum*, *P. pentosaceus*, *L. sakei*, *L. pentosus*, *L. coryniformis*, *Lactococcus garvieae*, *Pediococcus lolii*, *Weissella paramesenteroides*, *L. curvatus*, *L. brevis*, and *L. farciminis* were enriched at d 7 ($P < 0.05$; Fig. 1E). These differential abundant taxa were the main contributors to the difference in the microbiota structure before and after fermentation.

Frequent correlations were found amongst different chemical parameters of silage and the species-level microbial composition (Fig. 1F). Positive correlations were observed between several species that increased significantly after fermentation started (including *L. plantarum*, *W. paramesenteroides*, *L. brevis*, *L. curvatus*, and *L. farciminis*) and some chemical parameters (including 4 organic acids, especially lactic acid, SP, and NH_3–N). These species correlated negatively with the pH, the quantity of mold and coliform, NDF, and WSC.

3.3. Species-level silage microbiome and changes in the content of PS-8

Changes in the silage microbiota revealed that the level of *L. plantarum* increased significantly and became the dominant species during fermentation. To clarify the role of PS-8 as an exogenous starter in the fermentation process, the relative abundance of PS-8 reads in the whole genome at different time points

Table 1
Changes in physico–chemical parameters, mold and coliform counts during the alfalfa ensilage process.

Item	Fermentation time, d				SEM	*P*-value
	0	7	14	28		
DM, %	47.8^{a}	47.6^{a}	46.1^{a}	46.5^{a}	1.880	0.226
Crude protein, %DM	24.5^{a}	24.9^{a}	24.4^{a}	25.5^{a}	0.865	0.069
Soluble protein, %DM	10.4^{a}	12.7^{bc}	12.5^{b}	13.8^{c}	0.925	<0.001
Acid detergent fiber, %DM	31.1^{a}	29.9^{a}	29.9^{a}	30.9^{a}	1.205	0.148
Neutral detergent fiber, %DM	41.6^{b}	37.6^{a}	38.9^{ab}	38.2^{a}	2.015	0.002
Water-soluble carbohydrates, %DM	6.53^{c}	5.98^{bc}	5.19^{a}	5.68^{ab}	0.567	<0.001
Ammonia nitrogen, %DM	0.67^{a}	1.59^{bc}	1.86^{c}	1.29^{b}	0.290	<0.001
Lactic acid, mg/g	1.50^{a}	2.61^{b}	3.74^{c}	4.97^{d}	0.325	<0.001
Acetic acid, mg/g	0.62^{a}	0.59^{a}	0.59^{a}	0.85^{b}	0.232	0.026
γ-aminobutyric acid, μg/g	104^{b}	69.9^{a}	318^{c}	360^{d}	13.49	<0.001
Phenyl lactic acid, μg/g	0.02^{a}	0.01^{a}	11.5^{b}	24.3^{c}	3.922	<0.001
Mold count, log cfu/g	4.11^{b}	2.19^{a}	1.72^{a}	1.36^{a}	0.265	<0.001
Coliform count, log MPN/g	4.71^{b}	2.35^{a}	1.75^{a}	0.48^{a}	0.715	<0.001
pH value	5.63^{c}	5.10^{b}	5.01^{ab}	4.94^{a}	0.067	<0.001

SEM = standard error of the mean; cfu = colony forming unit; MPN = most probable number.
$^{a-d}$Data of the same parameter obtained at different time points were statistically compared by Kruskal–Wallis tests. Significant differences are indicated by different letters ($P < 0.05$).

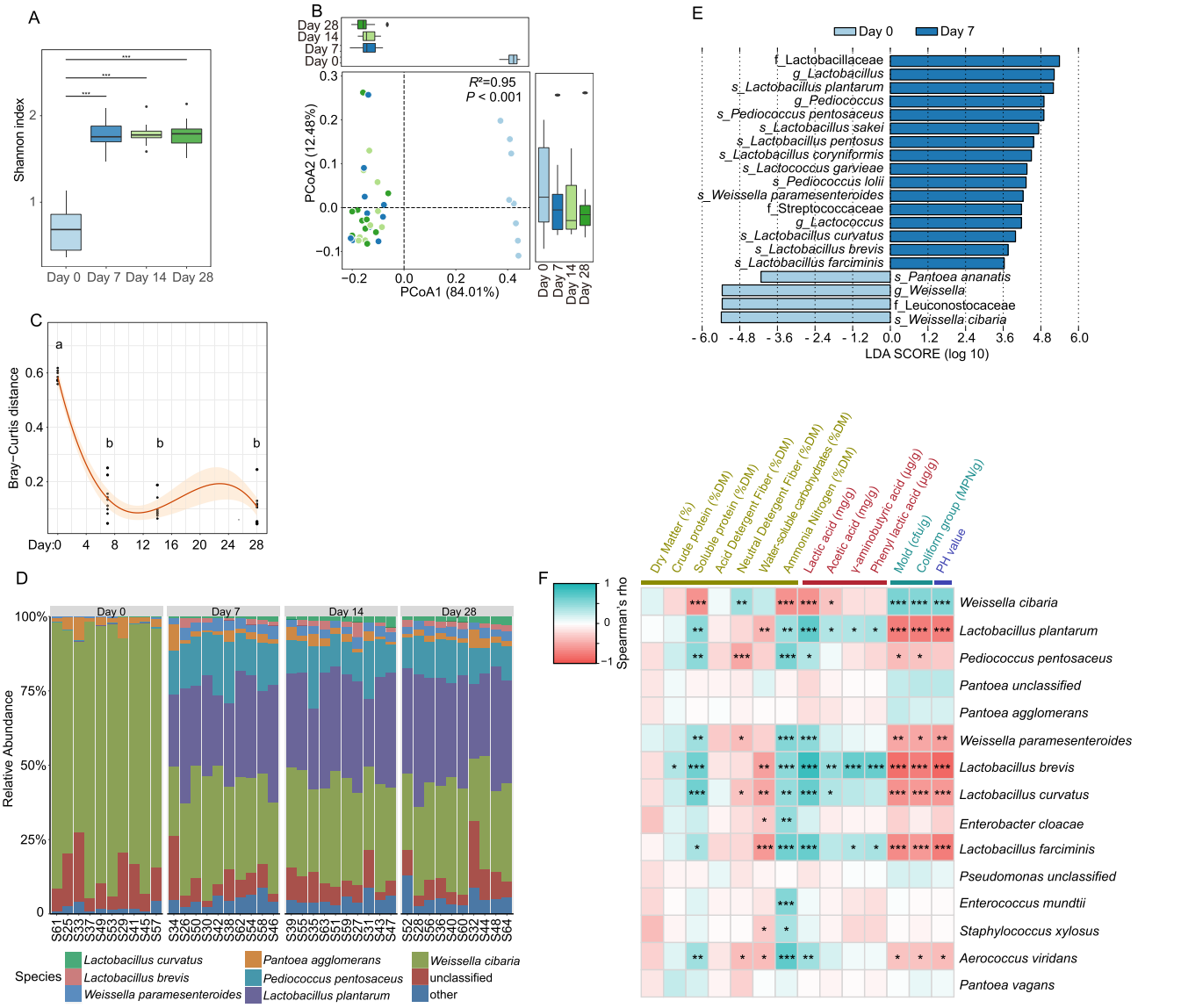

Fig. 1. Relationship between microbial community structure and physicochemical indexes of alfalfa silage at different time points. **(A)** Boxplots showing values of Shannon diversity index. **(B)** Principal coordinate analysis (PCoA; Bray–Curtis distance). **(C)** Bray–Curtis distance calculated by time point. Significant differences are indicated by different letters ($P < 0.05$). The curve was fitted to the data using locally estimated scatterplot smoothing (LOESS); the pink area corresponds to 95% confidence interval of the curve. **(D)** Stacked bar charts showing relative abundances of identified species. **(E)** Linear discriminant analysis (LDA) showing differential abundant taxa between d 0 and 7. **(F)** Spearman correlation heatmap of 15 dominant species and 14 biological/chemical parameters. MPN = most probable number. The color scale represents the Spearman's rho, showing strength of correlation. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

was calculated (Fig. 2A; Supplementary data Table S1). The proportion of PS-8 reads increased significantly from 0 to 7 d, and remained stable from d 7 to 28, ranging from 0.5% to 29%. A total of 1,100 raw bins were obtained initially from the scaffold through metagenomic binning using MetaBAT2. Then, incomplete bins with high contamination were removed and 162 high-quality MAG remained (>80% completeness and <5% contamination; Supplementary data Table S3). These MAG were identified as 22 representative species-level genome bins (SGB) and abundance obtained by RPKM. These high-quality MAG were used to construct a phylogenetic tree with consideration of their taxonomic assignments and sequencing depth (Fig. 2B; Supplementary data Table S3). Based on a cut-off level of 99% with the reference genome of PS-8, 10 of the 162 MAG were identified as PS-8 (Fig. 2C). The proportion of PS-8 in the whole genome and the value of GRiD (representing the replication rate of PS-8) was calculated for each time point. Interestingly, PS-8 had a higher GRiD value, representing a higher viability relative to the overall microbial abundance at d 7 and 14, followed by a drastic decline at d 28 (Fig. 2D; Supplementary data Table S3). In addition, correlations between PS-8 and other identified species in the silage fermentation environment were calculated (Fig. 2E). The relative abundance of PS-8 correlated positively and strongly with other *Lactobacillus* species ($r > 0.6$, $P < 0.001$), while significant negative correlation was found between PS-8 and *W. cibaria*, *Enterobacter agglomerans*, *Erwinia iniecta*, *Serratia marcescens* and *Pseudomonas* sp. ($r > 0.3$, $P < 0.05$).

3.4. Prediction of microbial polysaccharide degradation genes

The quality of silage fermentation depends largely on effective polysaccharide degradation. Thus, the microbial CAZyme genes in the silage microbiome were predicted. The Shannon index calculated based on CAZymes was significantly different between

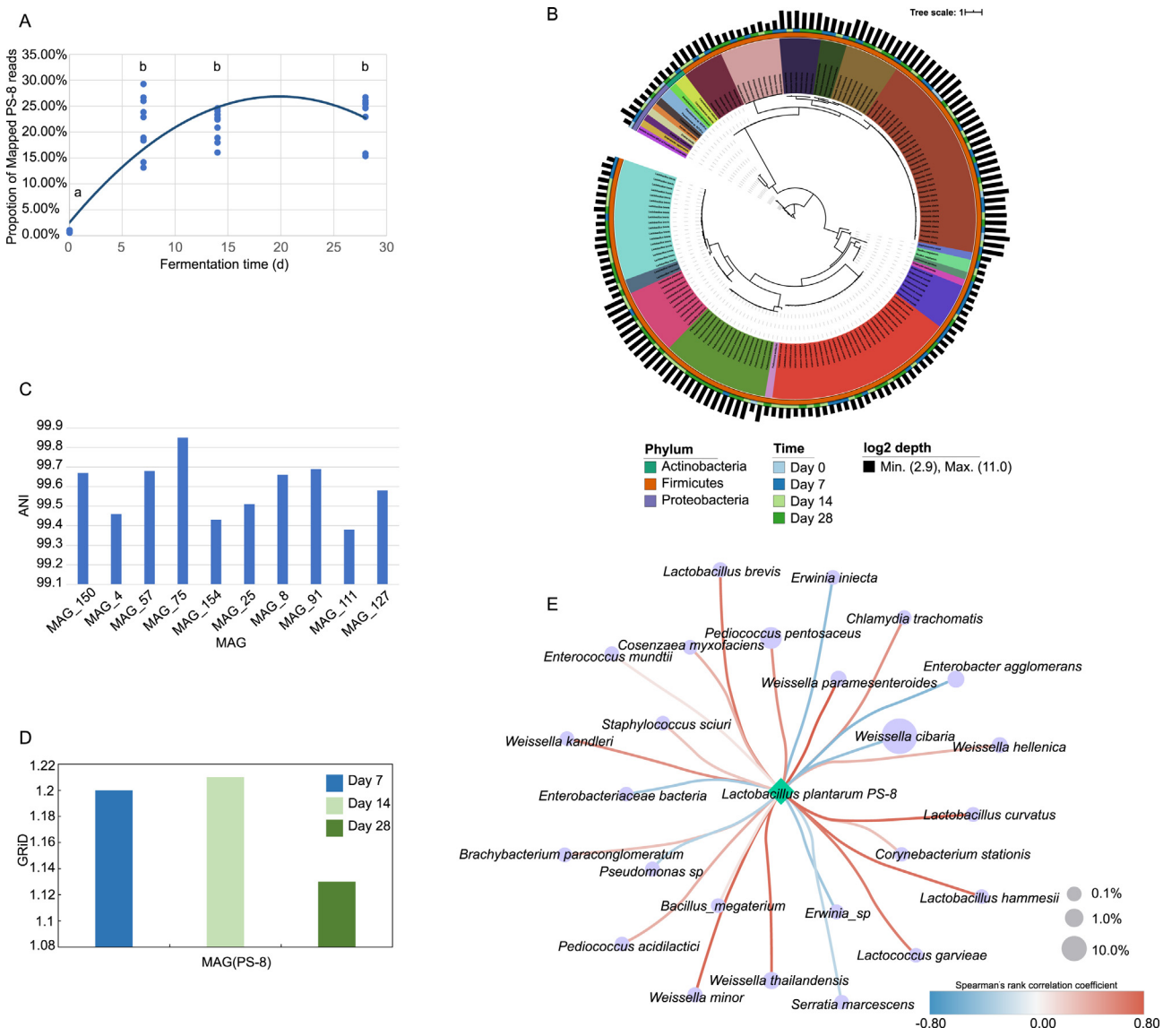

Fig. 2. *Lactobacillus plantarum* PS-8 (PS-8) in alfalfa silage microbiome. **(A)** Proportion of PS-8 reads at different fermentation time points. Significant differences are indicated by different letters ($P < 0.05$). **(B)** Phylogenetic tree constructed based on the metagenomic assembled genomes (MAG). The outermost to the inner layer represents the depth of sequencing, time points, bacterial phyla, and bacterial species, respectively. **(C)** Average nucleotide identity (ANI) between the reference genome of PS-8 and 10 identified *L. plantarum* MAG in the silage microbiome. **(D)** Histogram showing the Growth Rate InDex (GRiD) of PS-8 at d 7, 14, and 28. **(E)** Spearman correlation analysis between PS-8 and other species. Each species is represented by one circle, and the size of the circle represents the relative abundance of that species. The color scale represents the Spearman's rho, showing strength of correlation.

d 0 and time points after initiation of silage fermentation (d 7, 14, 28; $P < 0.05$; Fig. 3A). No significant difference was found in the Shannon index during d 7 to 28. This result was supported by PCoA analysis (Fig. 3B). The right cluster on the PCoA score plot comprised only samples of d 7, while the left clusters comprised samples of d 7 to 28.

The taxa with the genetic capacity for carbohydrate utilization are considered to be important in microbial fermentation process. Thus, CAZymes in the metagenomes were predicted (Supplementary data Table S4). The genomic capacity for degradation of 5 types of carbohydrate (starch, pectin, arabinoxylan, fructan, cellulose) of the 22 representative MAG were predicted (Fig. 3C). A number of *Lactobacillus* and *Weissella* species, including *L. curvatus*, *L. brevis*, *L. plantarum*, *W. cibaria*, *W. hellenica*, and *W. kandleri* carried a higher percentage of CAZyme genes, with great potential for degradation of starch, arabinoxylan, and cellulose. A few bacterial taxa carried genes that degrade pectin and fructan, including relatively high levels of glycoside hydrolases (GH), glycosy transferases (GT), and carbohydrate esterases (CE) (Fig. 3D).

In the fermentation process of microorganisms, polysaccharides within the feed would be decomposed into monosaccharides and further converted to pyruvate enzymatically. The substrates would then enter the aerobic and anaerobic glycolysis pathways to produce 5 fermentation products, namely formate, lactate, acetate, butyrate, and succinate (Fig. 3E). Thus, published literature specific to plant fermentation (substrate utilization and production of specific fermentation end products) was consulted and considered along with the MAG information obtained in this study. The predicted degradation pathways of 5 plant carbohydrates in the metagenomes of the 22 representative MAG belonging to 14 genera are shown in a schematic diagram (Fig. 3E). In addition, the species abundance was calculated, and significant changes were observed

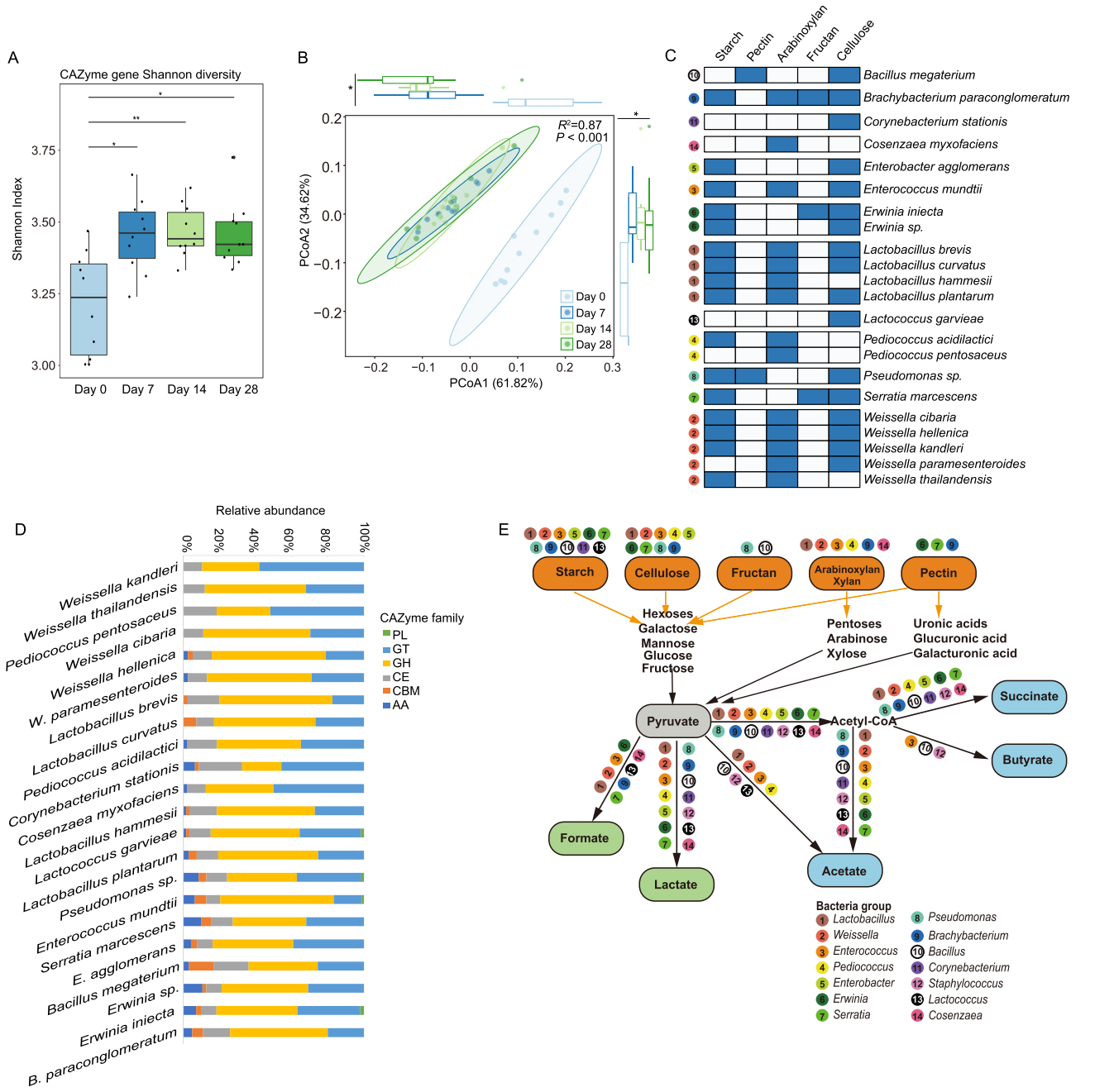

Fig. 3. Predicted polysaccharide degradation genes and pathways in the microbial metagenomes. **(A)** Shannon diversity index of predicted genes coding the CAZymes at different time points (* $P < 0.05$, ** $P < 0.01$). **(B)** Principal coordinates analysis of CAZyme genes predicted at different time points. Permutational MANOVA suggested significant between groups ($R^2 = 0.87$, $P < 0.001$). **(C)** Metagenomic potential of 22 representative metagenomic assembled genomes (MAG) in polysaccharide degradation, reflected by the possession of genes coding carbohydrate-active enzymes (CAZymes). Boxes filled with blue and white represent the presence or absence of specific genes in certain MAG, respectively. **(D)** The distribution of CAZyme coding genes in 22 representative MAG. **(E)** Schematic diagram showing simplified fermentation pathways of plant carbohydrate degradation and metabolism predicted from 22 representative MAG (shown on the genus level) using information from metabolic studies.

in members of the *Lactobacillus* and *Weissella* genera after fermentation (Appendix Fig. 1).

4. Discussion

The quality of silage fermentation depends largely on the amount and type of microorganisms present in the forage (Agarussi et al., 2019) and the availability of soluble carbohydrates. The inoculation of exogenous starter LAB can help increase the ratio of beneficial to harmful microbes in silage raw materials, facilitating the fermentation process (Wang et al., 2019). Although some previous studies investigated the beneficial effects of applying exogenous bacterial additives to accelerate the ensilage process, few studies monitored the dynamics of bacterial metagenomes and exogenous starter bacteria in fermentation (Ogunade et al., 2018). Metagenomic studies based on MAG reconstruction not only provide information of the functional potential of species-level microbial metagenomes (e.g., genes/pathways related to polysaccharide metabolism) at different fermentation stages, but also offer a way to track the viability of the starter and its potential contribution to the process by using an objective index like GRiD, which is calculated from changes in gene abundance of assembled bacterial genomes.

The quality of fermented alfalfa feed is directly reflected by the chemical and microbiota composition of the silage. Microorganisms affect feed characteristics through a series of chemical reactions resulting from plant polysaccharide degradation. Typically, fermented alfalfa silage, particularly those with the addition of LAB starter, would contain a large amount of organic acids (especially lactic acid) resulting from carbohydrate utilization by microorganisms present in the raw silage materials, drastically decreasing the pH during the ensilage process (Yitbarek and Tamir, 2014). Our results found positive correlations between several dominant species (*L. plantarum*, *W. paramesenteroides*, *L. brevis*, *L. curvatus*, and *L. farciminis*) and 4 organic acids, especially lactic acid. A high acidity could help inhibit the undesirable growth of molds and coliforms, potentially increasing silage stability and shelf life. Molds and coliforms always cause a loss of nutrients and an increased chance of toxin contamination (Avila and Carvalho, 2020). The organic acids in the feed not only facilitated long-term storage of the feed, but also improved the intake of dairy cows and thus improved growth performance (Gheller et al., 2020). As the harmful microbial population was reduced, metabolic requirements of the microbes decreased, and the availability of dietary energy and nutrients to the host animal would increase, leading to enhanced growth rate and feed efficiency (Upadhaya et al., 2016). The WSC content was related to bacterial utilization of carbohydrate as substrates for growth and subsequent synthesis of lactic acid. Due to the increase in *Lactobacillus* (*L. plantarum*, *L. brevis*, *L. curvatus*, *L. farciminis*) after fermentation, the content of WSC decreased significantly, which likely accelerated the production of lactic acid. It has been reported that the DM loss of fermentation silage due to lactic acid fermentation would not usually exceed 5%, while the current study observed a stable DM content throughout the fermentation process, confirming that it was mainly lactic acid fermentation (Muck, 2010). The NH_3—N in silage was an indicator of proteolysis during ensiling and typically resulted from plant enzymes and microbial activities (Ogunade et al., 2018). Our results showed that a variety of microorganisms were positively correlated with the NH_3—N content, which could be related with a relatively high rate of NH_3—N accumulation due to the rich protein content in alfalfa. As silage fermentation proceeded, the ammonization of proteins occurred rapidly and decreased in the late stage of fermentation, accompanied by a continuous drop in pH until the late fermentation phase, when microbial activities and NH_3—N release were inhibited. Consistent with McGarvey et al. (2013), some species (including *L. plantarum*, *P. pentosaceus, W. paramesenteroides*, *L. brevis*, *L. curvatus*, and *L. farciminis*) correlated significantly and positively with the level of SP, which would improve animal digestibility of alfalfa silage protein and prevent further soluble ammonia. These species were also associated with the reduction in ADF and NDF contents in the feed during the fermentation process, and the fiber degradation would effectively improve the palatability and digestibility of the feed (Kumar et al., 2008).

Bacterial DNA from dead cells having no effect on fermentation could also be sequenced (Avila and Carvalho, 2020). To overcome this issue, our study calculated the strain GRiD based on assembling single strain genome, which reflected the replication of the exogenous additive PS-8 and allowed tracking of its changes and its role in fermentation. Our results showed that PS-8 grew fast during the first week of fermentation, which was likely due to its competitive advantages over other epiphytic bacteria. A previous study found that an inoculant comprising <10% of the epiphytic LAB population was able to influence the efficiency of silage fermentation (Muck, 2013); and exogenous inoculation of *Lactobacillus* eventually led to the dominance of this genus in the fermented silage, whether the alfalfa was sterilized or not prior to fermentation (Yang et al., 2019). The increase in the GRiD value of PS-8 during the fermentation process in our study suggested that PS-8 was active, especially during the early phases. As the pH continuously declined, the growth of PS-8 was restricted and even decreased at d 28; however, it still comprised a significant proportion in silage microbiota. Guo et al. (2018) reported a similar trend of changes in the relative abundance of *L. plantarum* when it was inoculated in alfalfa silage fermentation (Guo et al., 2018). One possible beneficial mechanism of PS-8 on the silage microbiota could be its promotion of the growth of various acid-producing LAB (Wang et al., 2019). A number of LAB could produce bacteriocin; PS-8 is a probiotic bacterium exhibiting high antibacterial activity (Zhang et al., 2015), supporting its activities in promoting the growth of beneficial bacteria while inhibiting the harmful ones (Vazquez et al., 2005). A good starter usually possesses bacteriocidal and/or bacteriostatic activity, eventually making it a prevalent strain in the silage fermentation process. The reduction in undesirable microorganisms could also improve the quality and DM beyond that attained from an efficient fermentation (Muck, 2013). Dong et al. (2020) found that by adding perennial ryegrass to *Broussonetia papyrifera*, favorable microorganisms such as *Lactobacillus* and *Weissella* would dominate over the entire ensilage time (Dong et al., 2020). Similarly, Wang et al. (2019) reported the highest abundance of *Lactobacillus* when alfalfa was ensiled with *Moringa oleifera* leaves for 60 d (Wang et al., 2019a). He et al. (2020) found that poor fermentation quality of alfalfa silage was related to a low relative abundance of *Lactobacillus* when undesirable microorganisms dominated during ensiling (He et al., 2020). Previous studies found that the majority of bacteria involved in lactic acid fermentation of silage belonged to the genera *Lactobacillus*, *Pediococcus* and *Weissella* (Guo et al., 2018; Li and Nishino, 2013). Our study found that PS-8 was dominant throughout the fermentation process. Such phenomena was likely due to the strong inhibitory activities of PS-8 in suppressing the growth of unfavorable microorganisms, like *Enterobacterium* and *P. agglomerans*, which could produce alcohol during silage fermentation. The inhibition of these microbes could in turn facilitate fermentation (Li and Nishino, 2013). Meanwhile, beneficial bacteria *Lactobacillus* and *Weissella* were promoted after fermentation began; increases in these genera could result in more lactic acid production, reduced pH, and thus an improvement in silage quality.

Alfalfa has low WSC content, high buffering capacity and high fiber content, making it difficult to maintain a high and stable silage quality (Jaurena and Pichard, 2001; Thacker and Haq, 2008; Renteria-Flores et al., 2008); and most polysaccharides in the alfalfa feed would not be able to be utilized directly by the animals. Adding WSC addition and exogenous microbial starter would reduce buffering capacity and facilitate the fermentation of indigestible plant polysaccharides into nutrients to support bacterial growth. Microbial CAZyme genes encode for enzymes having the capacity of degrading macromolecular carbohydrates into small molecules, thereby improving the palatability and digestibility of feed. Our study observed an increase in a variety of CAZyme coding genes, as well as MAG carrying genes relating to degradation of starch, arabinoxylan, and cellulose. The levels of GH-encoding genes were highest among the assembled genomes. Such enzymes could effectively metabolize plant polysaccharides, such as starch and starch-related substrates (Ventura et al., 2007). In addition, they also have a good ability to degrade cellulose and hemicellulose (arabinoxylan), which would require concerted actions of endo-acting-β-1,4-arabinoxylanases and oligosaccharide depolymerizing β-xylosidases (Peng et al., 2016). Our research showed that 52 and 106 bacterial MAG carried endo-acting-β-1,4-arabinoxylanases and β-xylosidases, respectively, indicating good metagenomic potential of decomposition of cellulose and arabinoxylan. One previous study has attempted to elucidate the metabolic products produced during

microbial silage fermentation by measuring the end product fluxes or by inferring from pure or mixed cultures of microorganisms from reference metabolic pathways (Seshadri et al., 2018). This study identified the degradation and metabolism of feed carbohydrates by dominant bacteria extrapolated from assembled single bacterial genome, based on previous metabolic studies on feed fermentation.

5. Conclusion

This study demonstrated that adding *L. plantarum* PS-8 in the ensilage process could improve silage quality by accelerating acidification rates and in turn modulating the silage microbiome, particularly by enhancing the growth of LAB and suppressing undesirable microbes like molds and coliforms. Moreover, PS-8 was replicating rapidly and consistently during early-and mid-fermentation phases. The levels of NDF of PS-8-supplemented silage decreased significantly during the ensilage process. These results together suggested an active role of PS-8 in improving silage quality, nutrient preservation, and potentially extending the silage shelf life. This study provided novel information of the mechanism of silage improvement by an exogenous LAB inoculum, which would help improve the efficiency and quality of the production of commercial silage.

Author contributions

Lijun You: visualization, writing - original draft. **Weichen Bao**: data curation, investigation, formal analysis. **Caiqing Yao**: writing - original draft, software. **Feiyan Zhao**: validation. **Hao Jin**: data curation. **Weiqiang Huang**: bench work. **Bohai Li**: bench work. **Lai-Yu Kwok**: writing – critical evaluation and extensive revision of manuscript. **Wenjun Liu**: conceptualization, methodology, project administration.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix and Supplementary data

Appendix and supplementary data to this article can be found online at https://doi.org/10.1016/j.aninu.2021.12.004.

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