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Effects of different additives on fermentation characteristics, nutrient composition and microbial communities of *Leymus chinensis* silage

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Abstract

Background *Leymus chinensis* (Trin.) Tzvel is a perennial high-quality indigenous grass in China; characterized by high yield, elevated crude protein content, excellent palatability, substantial leaf volume, adaptability, and longevity. This study aimed to examine the impact of *Lactiplantibacillus plantarum* (LP), *Lactobacillus buhneri* (LB), their combination (LPLB), and complex enzyme preparation (CE) on the quality and microbial community of *Leymus chinensis* silage.

Results Throughout silage fermentation, pH levels decreased in all treatment groups relative to the control group. The LPLB group exhibited elevated levels of lactic acid (LA) and water-soluble carbohydrates (WSC) content, alongside reduced concentrations of acidic detergent fibre (ADF), and neutral detergent fibre (NDF) content. It exhibited superior silage outcomes compared to the other groups. *Lactobacillus* predominated in the treatment groups, but *Enterobacter* was predominant in the control group. The microbial diversity was decreased in LP and LPLB within the treatment groups due to the suppression of unwanted bacteria. Functional predictions indicated that glyoxylate and dicarboxylate metabolism, starch and sucrose metabolism, glycolysis/gluconeogenesis, amino sugar metabolism, and nucleotide sugar metabolism were the most significant metabolic pathways, with LP being particularly important in each.

Conclusion The experimental results demonstrated that the incorporation of various additives influenced the bacterial community structure, fermentation quality, and nutrient composition of *Leymus chinensis* silage differently. The LP and LPLB groups decreased pH and ADF levels and amassed a significant quantity of LA during fermentation, while preserving CP and WSC content. The microbial composition exhibited greater stability, which markedly enhanced the quality of *Leymus chinensis* silage, hence preserving its nutrient composition.

Clinical trial number Not applicable.

Keywords *Leymus chinensis*, Fermentation, Microbial community, *Lactiplantibacillus plantarum*, *Lactobacillus buchneri*

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Introduction

Leymus chinensis (Trin.) Tzvel, commonly referred to as alkali grass, is classified within the genus *Leymus Hochst* of the Poaceae family and serves as a significant establishment species in the eastern meadow grassland and arid steppe of the Eurasian steppe zone [1]. This perennial, high-quality natural grass species in China has benefits such as high yield, longevity, robust adaptation, and excellent palatability [2–4]. The species has been shown to endure the dry season when soil moisture is below 6%, indicating its remarkable environmental tolerance [4, 5]. The quality of hay is significantly affected by weather conditions, and rain during the drying process can lead to substantial nutritional losses, an issue that can be efficiently alleviated through silage preparation [6].

Silage enhances nutrient retention, increases palatability, and prolongs the storage duration [7, 8]. Silage fermentation is fundamentally a microbiological process [9]. Consequently, evaluating microbial populations is essential to enhance our comprehension of the role of microorganisms in the silage process [8, 10]. Silage is prone to degradation during utilization since detrimental microbes exploit organic materials, including soluble carbohydrates and proteins, as substrates for their growth and metabolism, consequently diminishing the nutrient content and value of the silage. This results in nutritional depletion and may also induce the fermentation process to generate poisonous and deleterious chemicals, as well as unpleasant volatiles, hence negatively impacting animal nutrition and health [11, 12]. The additives employed in silage fermentation and the duration of fermentation significantly influence silage, animals, and people. Consequently, regulating the additives in the fermentation process is essential for attaining optimal outcomes.

The natural occurrence of lactic acid bacteria (LAB) on *Leymus chinensis* is typically minimal, resulting in inadequate facilitation of effective silage fermentation, while direct silage yields elevated pH levels and decreased lactic acid concentrations [13]. Previous investigations have identified additives as environmentally friendly products that enhance the quality of silage fermentation [14, 15]. *Lactiplantibacillus plantarum* is among the most extensively researched species of the *Lactobacillus* genus due to its occurrence in diverse environmental microhabitats, adaptability, and metabolic capabilities [16, 17]. *Lactobacillus buchneri*, a remarkable and appealing silage-producing microorganism, is extensively found in nature, can be extracted from distinct ecological environments, and has various applications [18]. The *Lactiplantibacillus plantarum - Lactobacillus buchneri* synergy mainly occurs via *L. buchneri's* rapid acid production, which lowers the environmental pH to create a favorable acidic environment for *L. plantarum*. In co - culture systems, *L. buchneri lysis* releases intracellular factors that further promote *L. plantarum* growth and function, thereby enhancing fermented product quality [19, 20]. Numerous research indicate that *Leymus chinensis* enhances fermentation quality and aerobic stability following inoculation with *lactic acid bacteria* (LAB) [21–23]. Complex enzyme formulations rich in cellulase enzymes decompose the fibers of *Leymus chinensis*, significantly enhancing its palatability, as evidenced in numerous silage studies with gramineae plants [24–27]. Many recent studies have focused on lactic acid bacteria (LAB) combinations.Their functional synergy, especially between homofermentative and heterofermentative LAB, has drawn significant attention. For example, combinations of *Lactiplantibacillus plantarum* (homofermentative) and *Lactobacillus buchneri* (heterofermentative) have shown superior performance in lowering pH (3.72) and increasing lactic acid content compared to single-strain inoculants, while effectively inhibiting mold and yeast growth [28]. A triple-strain inoculant combining *Lactiplantibacillus plantarum* A1/LP-21, Enterococcus faecalis, and Pediococcus pentosaceus has also proven highly effective, reducing pH to 4.57 and boosting lactic acid content to 8.00% DM, outperforming single-strain treatments [29]. Additionally, a combination of *Lactiplantibacillus plantarum* ZZU203 and cellulase-producing Bacillus methylotrophicus has been shown to maximize the absolute abundance of lactic acid bacteria within 10 days, highlighting the effectiveness of bacterial-enzyme synergism in promoting substrate release and LAB proliferation [30].

Throughout the silage period, the microbial community demonstrated substantial temporal variations [31]. The alterations in microbial community structure during silage were analyzed to further investigate the primary microorganisms influencing silage quality [32]. Analyzing microbial communities by 16 S rRNA gene sequencing has emerged as a prevalent and economical method for assessing microbial diversity, establishing it as a regular practice in metagenomics [33–35]. Microbiomics was first applied to silage research in the early 21st century but saw rapid advancement post-2015 with the widespread adoption of Illumina high-throughput sequencing and improved bioinformatics. Unlike traditional culture-based methods, modern microbiomics provides unbiased detection of entire microbial communities, including unculturable species. When combined with multi-omics approaches like metabolomics and metagenomics, it enables systematic functional and metabolic network analysis [36]. This shift has allowed researchers to conceptualize silage fermentation as a dynamic microbial ecosystem. KEGG is a curated resource that consolidates 18 databases classified into systems, genetic, chemical, and health information. It also offers KEGG mapping tools that facilitate the comprehension of cellular and

organismal functions derived from genomic sequences and other molecular datasets [37]. This study assessed the nutrient composition and fermentation quality of *Leymus chinensis* silage at various storage stages, comparing the end of silage (60 days) to the bacterial community structure and its KEGG functional profile in silage. An analysis of the interaction between diverse chemicals and microbial populations may serve as a reference for enhancing the nutritional quality of *Leymus chinensis* silage.

This study employed several additives in *Leymus chinensis* silage to examine the alterations in nutrient composition, fermentation characteristics, and bacterial community structure throughout fermentation, aiming to assess the viability of *Leymus chinensis* silage.

Materials and methods

Silage preparation

Leymus chinensis (Zhongke No.1) was cultivated in 2018 at the *Leymus chinensis* planting site of Hoho Horse Farm, located in Ulanhot City, Inner Mongolia Autonomous Region (*Leymus chinensis* Cooperative Project of Xing'an League Academy of Agricultural Sciences, latitude 45°41'53"N, longitude 121°50'30"E). *Leymus chinensis* was harvested using a hand-held scythe on July 28, 2023, during its maturity period, with collection authorized by the Xing'an League Academy of Agricultural Sciences. Harvested *Leymus chinensis* was severed into 1–2 cm segments using a cutting machine and desiccated to 65% moisture content for silage production.

The treatments consisted of: (i) no-addition control (CK); (ii) *Lactiplantibacillus* plantarum (LP, 2×10^{11}CFU/g fresh, Inner Mongolia Hemei Kesheng Biotechnology Co., Ltd, China); (iii) *Lactobacillus buchneri* (LB, 2×10^{11} CFU/g, Inner Mongolia, China); and (iv) *Lactiplantibacillus plantarum* + *Lactobacillus buchneri* (LPLB, 2×10^{11}CFU/ g, Inner Mongolia, China). (v) Composite enzyme preparation (CE, composing enzyme (Xylanase, cellulase,≥18000U/g; cellulase, ≥50U/g; β-mannanase, ≥1800U/g; β-glucanase pectinase≥200U/g; medium temperature α-amylase, ≥1500U/g; neutral protease, 1000U/g, sourced from Shandong Mycobacterium longum biology, China), enzyme activity units of the enzyme complex were measured at pH 6.0 and 37 °C, using the ISO - recommended colorimetric method.

The additives were dissolved in 20 ml of distilled water and uniformly sprayed onto 600 g of material. A total of 600 g of mixed feed was thoroughly combined and sealed in polyethylene bags measuring 30 cm × 40 cm (Shijiazhuang Youlang Trading Co., Ltd., Shijiazhuang, China), which were subsequently vacuum-sealed using a vacuum extraction machine (model: DZ400/2D type, Wenzhou Dafeng Machinery Co., Ltd., Wenzhou, China). A total of 75 bags (5 treatments × 5 open days × 3 replicates) were maintained at ambient temperature (17–25℃). Samples collected at 0, 7, 15, 30, and 60 days of silage were examined for fermentation characterisation and chemical composition, with the 60-day samples, where fermentation had stabilized, chosen for microbial community and function prediction analysis.

Nutritional composition and fermentation characteristics analyses

Nutritional and fermentation characteristics were assessed at 0, 7, 15, 30 and 60 days. The dry matter (DM) content of the samples of the fresh and ensiled material was determined following oven drying at 65℃ for 48 h [38]. The crude protein (CP) level was assessed using the Kjeldahl method [39]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined by using an ANKOM A200i fiber analyzer (ANKOM Technology Corp, Fairport, NY, USA), with hemicellulose content computed as the difference between NDF and ADF. The water-soluble carbohydrate (WSC) content was assessed utilizing Thomas'method [40].

10 g of the sample should be weighed and combined with 90 ml of distilled water. Homogenize using a homogenizing tapper for 2 min, then filter to produce the liquid extract (Model: LC-11 L, Shanghai Jingxin Industrial Development Co., Ltd., China). The pH was assessed using an acidity meter (Model: LEICI PHS-3 C, Shanghai Yitian Scientific Instrument Co., Ltd., China), the content of lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA) were quantified via high-performance liquid chromatography (Model: Waters e2695, MA, United States), and the content of ammonia nitrogen (NH_3-N) was determined through phenol-hypochlorous acid colorimetric analysis [41].

Sequencing and analysis of microbial diversity

Total microbial DNA was isolated and PCR amplified from *Leymus chinensis* samples after 60 days of silage. The primers utilized for amplification in the highly variable region of V3-V4 were 338 F (5′-ACTCCCTACGGGGGAGGCAGCAG-3′) and 806 R (5′-GGACTACHVGGGTWTCTAAT-3′). PCR products were isolated using 2% agarose gel, purified with the Axy Prep DNA Gel Extraction Kit, eluted in Tris-HCl, and analyzed through 2% agarose electrophoresis. QuantiFluor™-ST was employed for quantification, and the purified amplified fragments were utilized to create PE 2*300 libraries in accordance with the standard operating procedures of the Illumina MiSeq platform. Sequencing was conducted utilizing the Illumina MiSeq PE300 platform (Major-bio Bio-Pharm Technology Co., Ltd., Shanghai, China).

Statistical analyses

Two-factor analysis of variance (ANOVA) for the chemical composition and fermentation quality of *Leymus chinensis* utilizing SAS 9.4 software (SAS Institute, Inc., Cary, NC, USA). Duncan's multiple range tests were employed to evaluate differences among treatments. Graphs were generated using Origin 2021 software. Microbiological data analysis was performed using the Majorbio I-Sanger Cloud Platform (www.i-sanger.com).

Results

Fermentation characteristics and nutritional composition of *Leymus chinensis* before and after silage

The fermentation quality of *Leymus chinensis* is presented in Table 1. Significant effects of ensiling duration and treatment interactions on pH, lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA) were observed ($P<0.001$). Ammonia nitrogen (NH_3-N) exhibited a significant effect ($P<0.05$). The pH of the CK declined more markedly in the later half of the silage, while the pH of the inoculated group diminished more swiftly during the initial 15 days of ensiling, followed by a lesser reduction in the latter phase. In comparison to the other groups, the pH level in the LP group (4.12) was markedly lower during the silage process($P<0.05$). The LA concentrations in both the CK and inoculated group exhibited an increase; however, the rate of increase differed across the groups. The LPLB group (10.69 g/kg DM) exhibited significantly elevated LA concentrations compared to the other group at 60 days of ensiling ($P<0.05$). The AA levels in the inoculation groups exhibited a consistent upward trend, whereas the AA levels in the CK group had a declining followed by an ascending trend. Notably, LP (2.88) surpassed the levels observed in the other treatments at the conclusion of the ensiling process. The PA concentration in the inoculated groups rose following 60 days of silage fermentation. Nonetheless, the CK exhibited comparatively lower amounts, whereas elevated PA levels were noted in the LB groups relative to the other groups ($P<0.05$). BA was detected solely in CK following 60 days of silage fermentation. The NH_3-N concentration in the treatments exhibited an initial increase followed by a subsequent decline, and

Table 1 Effect of additives on the fermentation quality of silage at different times

		pH	LA	AA	PA	BA	NH_3-*N*
			g/kg DM	g/kg DM	g/kg DM	g/kg DM	%FW
Fresh forage		6.53	-	-	-	-	-
Day	Treatments						
7	LP	5.75 ± 0.03Ca	2.72 ± 0.01Ad	0.66 ± 0.00Dd	0.48 ± 0.01Cc	ND	0.47 ± 0.02Ba
	LB	6.09 ± 0.03Ba	1.21 ± 0.02Dd	0.74 ± 0.01Cc	0.44 ± 0.00Cc	ND	0.46 ± 0.03Ba
	LPLB	6.06 ± 0.03Ba	1.70 ± 0.01Cd	1.01 ± 0.02Ba	0.43 ± 0.00Dd	ND	0.46 ± 0.04Ba
	CE	6.46 ± 0.03Aa	0.81 ± 0.00Ed	0.81 ± 0.01Cb	0.98 ± 0.01Ab	ND	0.45 ± 0.02Ba
	CK	6.07 ± 0.03Ba	2.35 ± 0.03Bd	2.08 ± 0.05Ab	0.56 ± 0.01Bb	ND	0.56 ± 0.02Aa
15	LP	4.60 ± 0.01Eb	4.12 ± 0.07Bc	0.91 ± 0.01Dc	0.49 ± 0.00Cc	ND	0.48 ± 0.03Aa
	LB	5.01 ± 0.01Cb	4.41 ± 0.07Ac	1.14 ± 0.01Bb	0.43 ± 0.00Dbc	ND	0.49 ± 0.03Aa
	LPLB	4.73 ± 0.05Db	3.84 ± 0.05Cc	1.06 ± 0.02Ca	0.45 ± 0.00Dc	ND	0.50 ± 0.02Aa
	CE	5.18 ± 0.03Bb	3.76 ± 0.02Cc	0.93 ± 0.01Db	0.82 ± 0.01Ac	0.07 ± 0.00	0.47 ± 0.03Aa
	CK	5.90 ± 0.01Ab	3.44 ± 0.10Dc	1.87 ± 0.02Ab	0.59 ± 0.01Bb	ND	0.49 ± 0.01Ab
30	LP	4.28 ± 0.01Bc	6.63 ± 0.05Bb	1.39 ± 0.01BCb	0.51 ± 0.00Cb	0.11 ± 0.08	0.43 ± 0.01Ba
	LB	4.34 ± 0.01Bc	4.82 ± 0.07Cb	1.25 ± 0.07Bb	0.45 ± 0.00Db	ND	0.47 ± 0.01Aa
	LPLB	4.34 ± 0.02Bc	9.78 ± 0.21Ab	1.03 ± 0.05CDa	0.46 ± 0.01Db	ND	0.48 ± 0.01Aa
	CE	4.69 ± 0.03Ac	4.15 ± 0.11Db	0.97 ± 0.03Db	0.97 ± 0.02Ab	ND	0.47 ± 0.01Aa
	CK	4.69 ± 0.03Ac	5.02 ± 0.18Cb	2.58 ± 0.05Aa	0.63 ± 0.01Ba	ND	0.50 ± 0.01Ab
60	LP	4.12 ± 0.04Cd	7.52 ± 0.09Ba	2.88 ± 0.08Aa	0.56 ± 0.00Ba	ND	0.44 ± 0.04Aa
	LB	4.36 ± 0.01Bc	4.99 ± 0.03Da	1.40 ± 0.05Ca	0.48 ± 0.00Ca	ND	0.49 ± 0.04Aa
	LPLB	4.16 ± 0.03Cd	10.69 ± 0.10Aa	1.10 ± 0.02Da	0.56 ± 0.00Ba	ND	0.45 ± 0.00Aa
	CE	4.68 ± 0.03Ac	5.46 ± 0.19Ca	1.23 ± 0.12CDa	1.19 ± 0.04Aa	ND	0.46 ± 0.00Aa
	CK	4.62 ± 0.02Ac	6.85 ± 0.20Ba	2.60 ± 0.11Ba	0.60 ± 0.02Bab	0.08 ± 0.09	0.46 ± 0.01Ab
Levels of significance							
Treatments		***	***	***	***	**	***
Day		***	***	***	***	***	*
T×D		***	***	***	***	***	*

LA lactic acid, AA acetic acid, PA propionic acid, BA butyric acid, NH_3-N ammonia nitrogen, LP *Lactiplantibacillus plantarum*, LB *Lactobacillus buchneri*, LPLB combination of LP and LB, CE composite enzyme, ND not detected, T treatment, D ensiling time, T × D interaction of T and D; *$P<0.05$; **$0.001<P<0.01$; ***$P<0.001$. Values with different uppercase letters (A–D) show significant differences among additives in the same ensiling day; values with different lowercase letters (a–d) show significant differences among ensiling days in the same additive ($P<0.05$)

overall, the influence of silage duration on NH_3-N levels was not significant ($P > 0.05$).

Table 2 presents the nutritional composition of fresh *Leymus chinensis* and its silage. The dry matter (DM) content in LB (56.18) exceeded that of the other treatments after 60 days of ensiling, but the lowest DM content was seen in LP ($P < 0.05$). The CP content in each treatment group exhibited a decreasing-increasing-decreasing trend across the silage duration, peaking at 30 days of silage, with LPLB (8.31) demonstrating the greatest CP content after 60 days of silage ($P < 0.05$). The EE content in the inoculated group exhibited an erratic fluctuation, whereas the control group consistently shown an upward trend, with elevated EE content in LB (2.08) and CK (2.07) at 60 days of silage ($P < 0.05$). The WSC content in nearly all groups exhibited a consistently declining trend throughout the ensiling process ($P < 0.001$), however the LPLB content (2.69) was greater at 60 days compared to earlier stages. The NDF content exhibited a decreasing-increasing-decreasing trend across the silage duration, peaking at 30 days and stabilizing at 60 days ($P < 0.05$). The concentration of ADF exhibited a decline over the duration of silage ($P < 0.05$), whereas treatment did not significantly influence ADF ($P = 0.0557$).

Bacterial diversity of *Leymus chinensis* during ensiling

At the genus level, as illustrated in Fig. 1, the rarefaction curves of all groups converge smoothly when the sequencing data are adequate, with a limited number of novel bacterial core operational taxonomic units (OTUs) remaining undetected. It can be observed from Table 3 that the control group (CK) showed the highest diversity, with OTUs (206.51) significantly more than the inoculated group ($P < 0.05$)and 10.57 times more than the LPLB group (19.54). The Simpson index and Coverage index exhibited no significant differences among treatment groups ($P > 0.05$).

Figure 2 illustrates the genus level. Principal coordinate analysis (PCoA) demonstrated significant clusters among groups, as illustrated in Fig. 2A, with PC1 and PC2 explaining 51.40% and 27.67% of the total variance, respectively. The control group had a greater abundance of OTUs compared to the other groups, with the inoculated group lacking unique species, except for the LP

Table 2 Effect of additives on the nutrient composition of silage at different times

		DM	CP	EE	WSC	NDF	ADF
		%FW	%DM	%DM	%DM	%DM	%DM
Fresh forage		52.87	5.96	1.45	2.03	62.48	37.81
Day	Treatments						
7	LP	56.67 ± 0.20Aa	6.65 ± 0.05Cb	1.78 ± 0.23Aa	2.97 ± 0.05Aa	69.37 ± 0.77Ba	46.32 ± 0.53Aa
	LB	55.83 ± 0.33Aa	10.39 ± 0.12Ab	1.69 ± 0.16Ab	3.02 ± 0.1Aa	68.1 ± 0.84Ba	42.39 ± 0.86Cb
	LPLB	58.98 ± 1.49Aa	6.24 ± 0.05Dc	1.59 ± 0.05Ac	2.78 ± 0.29Aa	69.34 ± 0.32Ba	43.47 ± 0.59BCa
	CE	54.97 ± 3.58ABa	6.42 ± 0.03Db	1.96 ± 0.55Aa	2.89 ± 0.14Aa	72.07 ± 0.75Aa	44.88 ± 0.09ABa
	CK	49.44 ± 0.99Bb	7.51 ± 0.06Bb	1.02 ± 0.02Ac	1.94 ± 0.04Bc	69.82 ± 0.86ABa	45.13 ± 0.44ABab
15	LP	53.48 ± 1.00Ab	5.72 ± 0.05Cc	1.43 ± 0.07Ab	2.75 ± 0.15Aa	68.7 ± 1.06Aa	42.99 ± 0.45Ab
	LB	53.31 ± 1.77Aab	5.69 ± 0.19Cc	1.19 ± 0.05Bc	2.5 ± 0.12Aab	67.84 ± 1.31Aa	45.11 ± 0.21Aa
	LPLB	55.66 ± 1.18Aab	7.18 ± 0.07Ab	1.51 ± 0.05Ac	2.13 ± 0.03Bb	69.02 ± 0.38Aa	43.6 ± 0.17Aa
	CE	52.27 ± 2.89Aa	6.79 ± 0.09Ba	1.49 ± 0.11Aa	2.04 ± 0.02Bb	68.82 ± 0.28Ab	43.27 ± 0.25Abc
	CK	50.18 ± 2.79Ab	6.88 ± 0.06ABc	1.08 ± 0.05Bbc	2.67 ± 0.13Aa	70.34 ± 0.59Aa	44.24 ± 2.25Aab
30	LP	50.75 ± 0.36Bc	15.59 ± 0.15Ba	2.04 ± 0.09ABa	1.67 ± 0.02Bb	70.97 ± 0.41Aa	45.49 ± 0.21Ba
	LB	51.26 ± 1.32ABb	17.01 ± 0.24Aa	1.8 ± 0.06Bab	2.09 ± 0.01Abc	70.43 ± 0.51ABa	43.83 ± 0.3Cab
	LPLB	55.33 ± 0.89Aab	5.93 ± 0.05Dd	2.3 ± 0.1Aa	2.04 ± 0.02Abc	68.6 ± 1Ba	43.5 ± 0.12Ca
	CE	50.94 ± 1.61Ba	6.81 ± 0.17Ca	1.88 ± 0.19Ba	1.73 ± 0.03Bc	68.97 ± 0.22ABb	42.76 ± 0.53Cc
	CK	49.05 ± 1.86Bb	16.91 ± 0.14Aa	1.19 ± 0.06Cb	1.67 ± 0.02Bd	69.13 ± 0.65ABa	46.83 ± 0.55Aa
60	LP	49.93 ± 0.89Bc	5.61 ± 0.04Dc	1.86 ± 0.04Bab	1.47 ± 0.02Db	69.87 ± 0.14ABa	43.45 ± 0.17ABb
	LB	56.18 ± 0.97Aa	6.17 ± 0.04Cc	2.08 ± 0.03Aa	1.95 ± 0.03Cc	70.64 ± 0.41Aa	44.4 ± 0.28Aa
	LPLB	53.04 ± 1.49Ab	8.31 ± 0.1Aa	2.02 ± 0.08Ab	2.69 ± 0.04Aa	67.8 ± 1.26Ba	42.96 ± 0.15Ba
	CE	54.10 ± 0.71Aa	6.78 ± 0.02Ba	1.74 ± 0.05Ba	2.4 ± 0.03Bb	69.27 ± 0.46ABb	44.22 ± 0.25Aab
	CK	55.96 ± 0.52Aa	5.61 ± 0.02Dd	2.07 ± 0.04Aa	1.28 ± 0.03Ee	62.84 ± 0.41Cb	45.02 ± 0.54Aab
Levels of significance							
Treatments		**	***	**	***	**	0.0557
Day		**	***	***	***	**	*
T×D		*	***	*	***	*	**

DM dry matter, CP crude protein, EE ether extract, WSC water-soluble carbohydrates, ADF acid detergent fiber, NDF neutral detergent fiber, LP *Lactiplantibacillus plantarum*, LB *Lactobacillus buchneri*, LPLB combination of LP and LB, CE composite enzyme, ND not detected, T treatment, D ensiling time, T × D interaction of T and D; *$P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$. Values with different uppercase letters (A–E) show significant differences among additives in the same ensiling day; values with different lowercase letters (a–e) show significant differences among ensiling days in the same additive ($P < 0.05$)

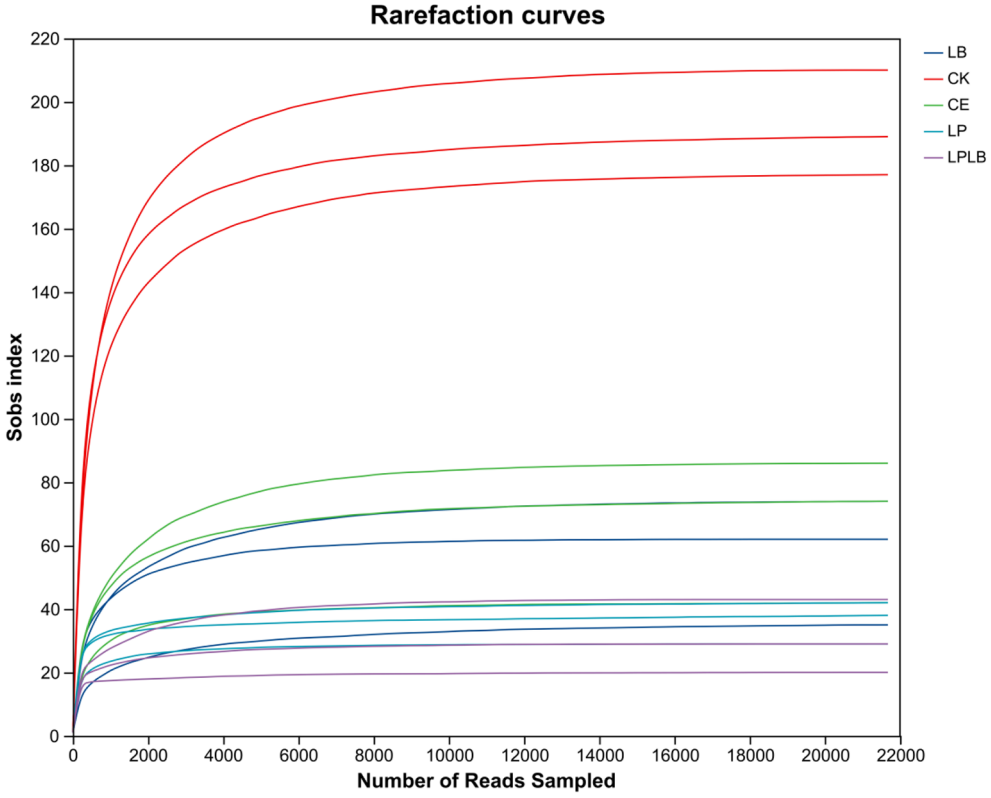

Fig. 1 Rarefaction curves for OTUs number in different treatments during *Leymus chinensis* ensiling. OTUs number of operational taxonomic units, LP *Lactiplantibacillus plantarum*, LB *Lactobacillus buchneri*, LPLB combination of LP and LB, CE composite enzyme; where lines of the same color represent three sets of replicates under the same additive treatment

Table 3 Community diversity and richness of *Leymus chinensis* inoculated and not inoculated at 60 days silage

Sample/Estimators	Sobs	ACE	Chao 1	Shannon	Simpson	Coverage
LP	3.67 ± 0.67B	2.27 ± 2.27B	3.67 ± 0.67B	0.42 ± 0.10B	0.75 ± 0.07AB	1.00 ± 0.00 A
LB	5.00 ± 0.58B	3.67 ± 1.86B	5.00 ± 0.58B	0.40 ± 0.08B	0.78 ± 0.05AB	1.00 ± 0.00 A
LPLB	4.00 ± 0.58B	3.00 ± 1.53B	4.00 ± 0.58B	0.28 ± 0.08B	0.86 ± 0.05 A	1.00 ± 0.00 A
CE	4.67 ± 0.33B	4.67 ± 0.33B	4.67 ± 0.33B	0.43 ± 0.08B	0.76 ± 0.06AB	1.00 ± 0.00 A
CK	11.33 ± 0.33 A	11.33 ± 0.33 A	11.33 ± 0.33 A	0.76 ± 0.07 A	0.62 ± 0.04B	1.00 ± 0.00 A
P-value	***	**	***	**	0.1058	0.58

LP *Lactiplantibacillus plantarum*, LB *Lactobacillus buchneri*, LPLB combination of LP and LB, CE composite enzyme; Values with different uppercase letters (A–B) show significant differences among additives ($P < 0.05$)

group. The treatment groups had a reduced diversity of species. Figure 2B shows that between the treatment groups, there were 8 shared species. The control group had the highest number of unique species, with 41 species significantly more than the inoculated group.

The relationship among the five treatment groups and the ten predominant genera is distinctly illustrated in Fig. 3A. The CK group exhibited a negative correlation with all genera, with the exception of *Lentilactobacillus* and *Lactiplantibacillus*. Each treatment group demonstrated a negative correlation with *Enterococcus*, *Enterobacter*, and *Hafnia-Obesumbacterium*, while showing a positive correlation with the *Lactobacillus* genus. At the phylum level, Proteobacteria and Firmicutes were predominantly identified in the bacterial communities (Fig. 3B). Firmicutes predominated the microbial makeup in the injected group, while Proteobacteria prevailed in the control group. Cyanobacteria were prominently observed in the control group and were scarcely present in the injected group. To further examine the impact of inoculants on the microbial community during silage fermentation, the bacterial composition of *Leymus chinensis* was analyzed at the genus level. Figure 3C illustrates that the epiphytic bacteria in *Leymus chinensis* predominantly comprised *Lentilactobacillus*, *Lactiplantibacillus*, *Enterobacter*, *Achromobacter*, *Hafnia-Obesumbacterium*, *Kosakonia*, *Sphingomonas*, and *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*. In the inoculated

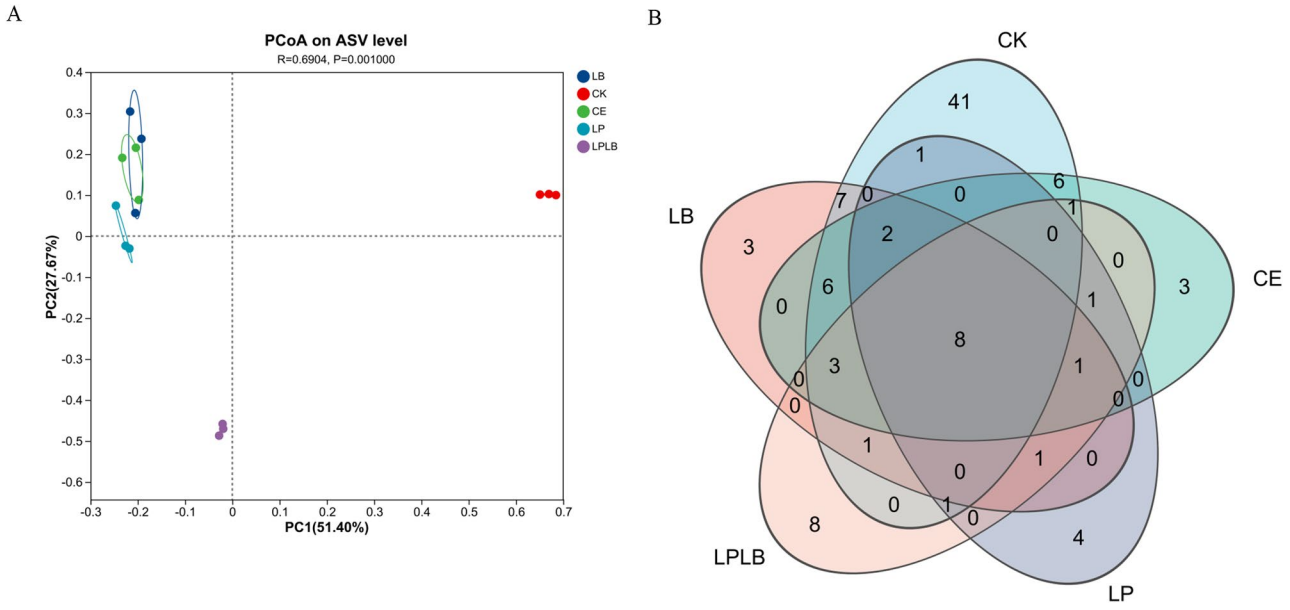

Fig. 2 The community dissimilarities of *Leymus chinensis* under different treatments. **A** Principal Coordinate Analysis (PCoA) of the bacterial community of silages in different treatments during ensiling; **B** Venn diagram depicting unique or shared bacterial OTUs in silages under different treatments. LP *Lactiplantibacillus plantarum*, LB *Lactobacillus buchneri*, LPLB combination of LP and LB, CE composite enzyme, where lines of the same color represent three sets of replicates under the same additive treatment

group, *Lentilactobacillus*, *Lactiplantibacillus*, *Achromobacter*, and *Kosakonia* were predominant, LAB enrichment may significantly improve the aerobic stability and nutrient preservation rate of silage by promoting lactic acid fermentation, inhibiting the growth of harmful microorganisms, and reducing pH, thereby enhancing silage quality. Whereas *Enterobacter* and *Hafnia-Obesumbacterium* prevailed in the control group. This indicates marked divergence in microbial composition between the control and inoculated groups at the genus level, with microbial abundance being greater in the control group compared to the inoculated group. Additionally, within the inoculated group, the abundance of LB and CE exceeded that of the other groups.

Correlation of microbial taxa with silage quality under fermentation conditions

Figure 4 illustrates the connections between bacterial genus levels and fermentation quality during silage fermentation. *Lentilactobacillus* had a significant negative correlation with LA and BA ($P<0.05$). *Lactilactobacillus* had a significant negative correlation with BA ($P<0.05$). *Enterococcus*, *Hafnia-Obesumbacterium*, and *Enterobacter* had a highly significant positive correlation with BA ($P<0.01$). *Achromobacter* had a significant negative correlation with AA ($P<0.05$). There was a significant positive correlation between *Allorhizobium*, *Neorhizobium*, *Pararhizobium*, *Rhizobium*, unclassified o Chloroplast *unclassified_o_Chloroplast* and BA ($P<0.01$). *Kosakonia* had a significant positive correlation with AA, but *Achromobacter* demonstrated a significant negative correlation with AA ($P<0.05$). *Lentilactobacillus* had a significant positive correlation with NDF ($P<0.001$) and ADF ($P<0.01$). *Lactiplantibacillus* had a significant negative correlation with pH ($P<0.05$). *Enterobacter* exhibited a significant positive correlation with EE ($P<0.05$), while demonstrating a significant negative correlation with WSC ($P<0.01$). *Achromobacter* positively associated with WSC ($P<0.05$). *Hafnia-Obesumbacterium* exhibited a significant negative correlation with WSC and NDF ($P<0.01$), and a significant negative correlation with CP ($P<0.05$), while demonstrating a significant positive correlation with EE ($P<0.05$). *Sphingomonas* had a significant positive correlation with pH ($P<0.001$). *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* had a significant positive correlation with pH ($P<0.05$) and a significant positive correlation with EE ($P<0.01$); nevertheless, it demonstrated a significant negative correlation with WSC ($P<0.05$). *Enterococcus* showed a negative correlation with WSC and NDF ($P<0.05$).

Bacterial metabolic functions shift during ensiling

The predicted function of bacterial communities was assessed using PICRUSt in the present study. The predictive function profiles of the 16 S rRNA gene illustrate the first, second, and third tiers of pathways (Fig. 5). At the first pathway level (Fig. 5A), the primary anticipated functional genes in fermentation were categorized into Metabolism, Genetic Information Processing, and Environmental Information Processing activities.

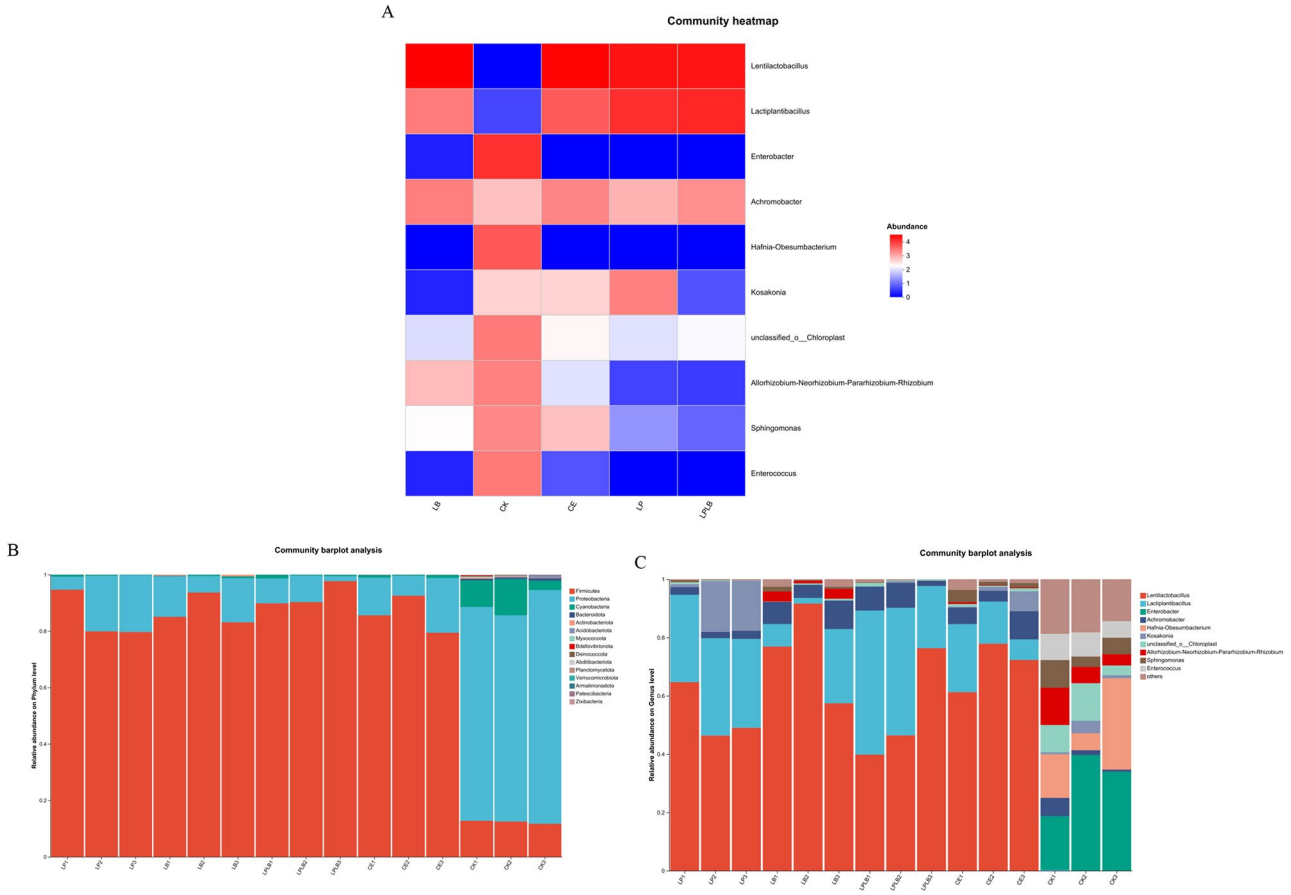

Fig. 3 Bacterial composition of *Leymus chinensis* inoculated and not inoculated at 60 days silage. **A** Heat map of changes in relative abundance of dominant species (Top 10) in the five treatments;**B** Relative abundance of bacterial community at phylum level; **C** relative abundance of bacterial community at genus level.LP *Lactiplantibacillus plantaru*, LB *Lactobacillus buchneri*, LPLB combination of LP and LB, CE composite enzyme, where 1, 2, and 3 represent three replications under the same treatment

The abundance of LPLB was elevated across all metabolic pathways. Figure 5B illustrates the second pathway level, highlighting the 15 pathways with the greatest abundance: Metabolism (8 routes), Environmental Information Processing (2 pathways), Genetic Information Processing (2 pathways), Cellular Processes (2 pathways), and Human Diseases (1 pathway). Metabolism was one of the key functional categories predicted in the analysis. The abundance of LP showed a general upward trend in multiple metabolic pathways. In the third tier of gene function prediction (Fig. 5C), metabolic pathways, biosynthesis of secondary metabolites, microbial metabolism in varied settings, biosynthesis of amino acids, and ABC transporters exhibited greater abundance compared to other pathways. In certain significant metabolic pathways, the abundance of LP was observed to be relatively higher.Beneficial bacteria such as LAB (LP and LPLB) are usually the dominant flora, and the high abundance of their metabolic function indicates that these flora are in an active state and occupy a dominant position during the fermentation process, which can effectively inhibit the growth of harmful bacteria, ensure the smooth progress of fermentation, and make the silage of good quality.

Discussion

The rate and magnitude of pH reduction is regarded as a critical indicator of the silage fermentation process [16]. It is widely recognized that high-quality silage should maintain a pH range of 3.8 to 4.2; in this experiment, LP (4.12) and LPLB (4.16) achieved this standard [42]. The results indicated a sustained decline in pH over time, with a more pronounced fall during the pre-fermentation phase, maybe linked to the fast rise in LA [43]. Nevertheless, the composite enzyme preparation was less effective than the control. This was because the enzymes couldn't completely degrade cellulose in some high - fiber, low - sugar components, so the lactic acid generation was insufficient [44]. Lactic acid (LA) is essential in the silage process, facilitated by LA fermentation [45]. The elevated concentration of lactic acid (LA) in the LP (7.52) and LPLB (10.69) groups resulted from *L. plantarum*, a homofermentative lactic acid bacterium that generates

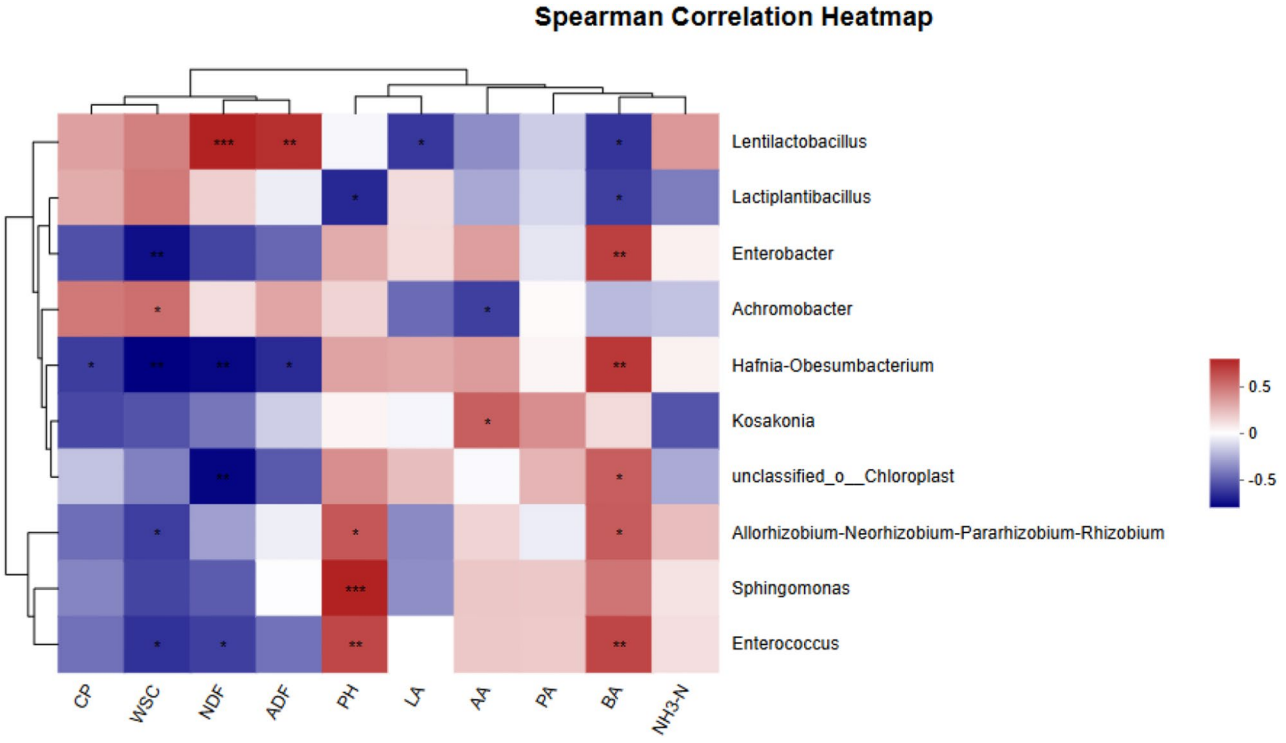

Fig. 4 Effect of the end of silage fermentation on fermentation quality and chemical composition (*$P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$)

LA through the fermentation of hexose carbohydrates, including glucose [46]. AA may impede the growth and reproduction of microorganisms detrimental to silage, including yeasts and molds, diminish their substrate consumption, and create a more favorable environment for lactic acid bacteria fermentation [47, 48]. *L. buchneri*, an obligate heterofermentative lactic acid bacterium, generates lactic acid, ethanol, acetic acid, and carbon dioxide [49]. Rabelo [50] suggested that LB facilitated the conversion of LA to AA; however, this assertion is inconsistent with the current experimental results, likely due to variations in silage ingredients and the types of organic acids generated by their metabolism, as well as the influence of the species of lactic acid bacteria involved. PA exhibits a potent bacteriostatic action, potentially inhibiting silage deterioration and enhancing silage quality [51]. Compared with other treatment groups, the PA content in the CE group was significantly higher ($p < 0.05$), potentially attributable to its elevated pH level. Reduced pH promotes rapid lactobacilli fermentation, inhibiting pathogenic bacterial growth, attenuating protein degradation, and lowering PA levels. Conversely, elevated pH is associated with increased PA accumulation. BA is generated by the degradation and transformation of AA and sugar in silage by detrimental bacteria, and a high concentration of BA indicates significant activity of these harmful bacteria, which is unfavorable for silage processing [52, 53]. BA was infrequently observed during silage and solely in CK at the conclusion of silage, indicating that the metabolic activity of Clostridium was restricted post-fermentation [54]. NH_3-N is generated from the degradation of proteins and amino acids in silage [55]. Throughout the silage period, NH_3-N exhibited an initial increase followed by a subsequent decrease. The LP and LPLB groups demonstrated lower NH_3-N levels and superior fermentation quality compared to the other treatment groups. This phenomenon can be attributed to the low pH environment, which suppresses the proliferation of detrimental microorganisms, including spoilage bacteria and clostridia, thereby minimizing protein degradation [56]. Elevated NH_3-N concentrations adversely impacted nutritional value and diminished palatability [57].

Analysis of chemical constituents during fermentation indicated a significant reduction in dry matter (DM) across all groups, with a slight increase observed in the LB group (56.18). This phenomenon was ascribed to the efficacy of *L. buchneri* in mitigating DM loss during the silage process by suppressing microbial activity [58]. At the conclusion of silage, crude protein (CP) diminished in the majority of treatment groups relative to the initial phase; however, it increased in the CE group. This anomaly may be ascribed to the degradation of cellulose and hemicellulose by cellulase and hemicellulase enzymes present in the composite enzyme preparation, leading to the conversion of resultant xylose and free monosaccharides into CP through microbial metabolism [59,

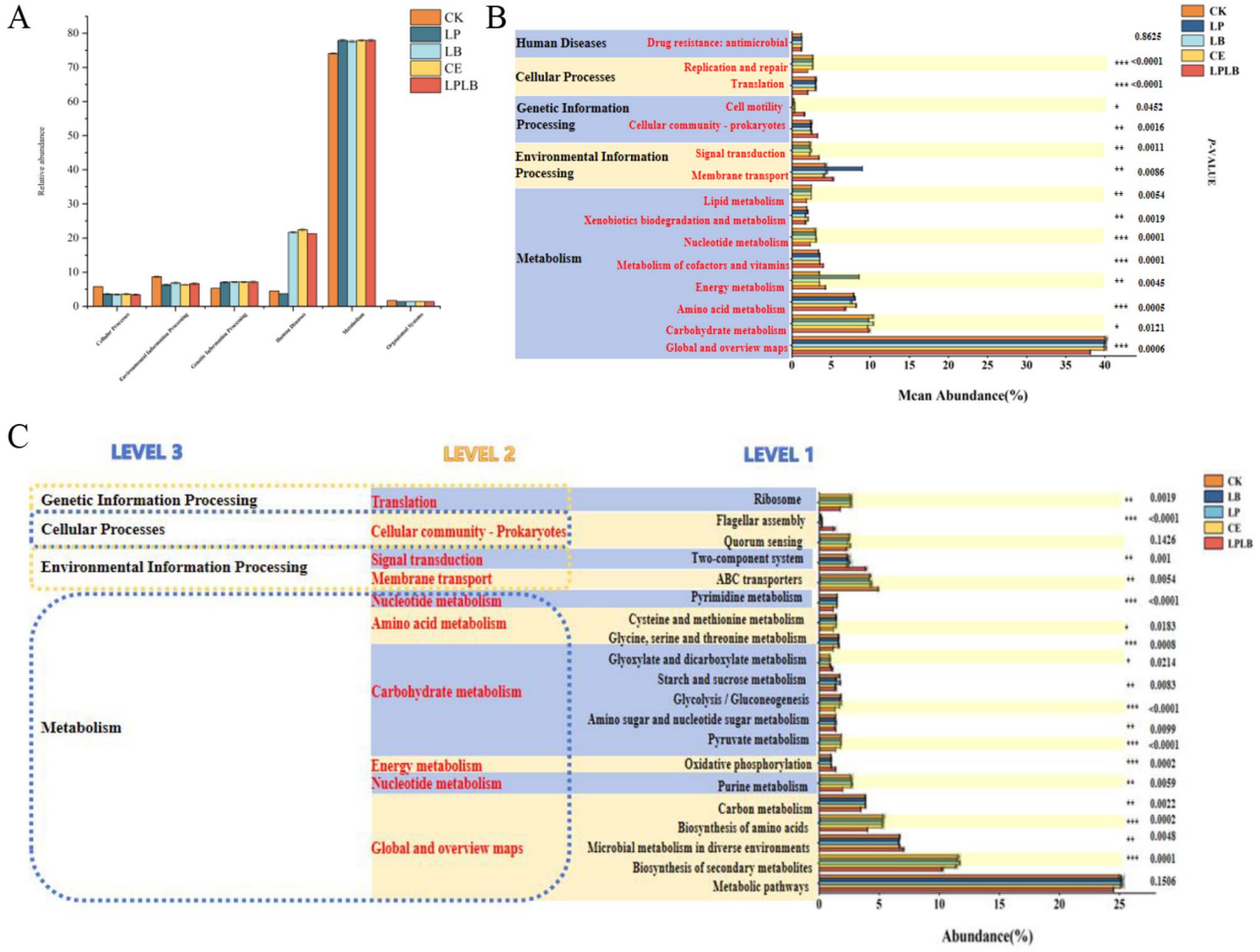

Fig. 5 Bacterial functional profiles of 60-day silage *Leymus chinensis* under different inoculant treatments. (**A**) 16 S rRNA genepredicted KEGG function profiles at pathway level (1) (**B**) 16 S rRNA gene-predicted KEGG function profiles at pathway level (2) (**C**)16 S rRNA gene-predicted KEGG function profiles at pathway level 3

60], Some other studies also support this view. Khantibongse [61] et al. found that the degradation of cellulose and hemicellulose produces monosaccharides, which can be utilized by microorganisms and converted into useful substances such as biofuels. Yang [62] et al. used cellulases and hemicellulases to degrade cellulose and hemicellulose in lignocellulosic waste into fermentable sugars. These sugars can serve as substrates for microbial fermentation to produce a variety of useful products, including single-cell protein. EE rose to different extents in all treatment groups following silage, attributed to the transformation of carbohydrates, decomposed by LAB and yeasts, into fat-soluble compounds such as lactic acid and volatile fatty acids, finally shown in the EE [63]. NDF and ADF are critical indices indicating the nutritional value of feed; lower levels of NDF and ADF correlate with nutrients are maintained during the fermentation process. ADF diminished in all treatment groups during silage fermentation in this experiment, aligning with the findings of Kung [64] and Ju [65] et al. The variation in NDF levels pre- and post-fermentation was not significant. This difference may stem from the fact that NDF digestibility is intricately linked to the composition of the forage itself. Variations in silage input materials, the species of lactic acid bacteria employed, and discrepancies in fermentation duration can all influence this outcome, consistent with Duvnjak's research [66]. Microorganisms convert water-soluble carbohydrates to lactic acid during silage fermentation, thereby lowering pH in anaerobic conditions and enhancing silage quality [67]. Consequently, in this experiment, the WSC of each treatment exhibited varying reductions at the conclusion of fermentation compared to its initial state.

LAB swiftly commences fermentation by employing accessible carbohydrates, lowering pH beneath 4.0 to suppress detrimental microorganisms [68]. Conversely, enzyme complexes alone liberate sugars by fiber degradation, a method that is gradual and inadequate for

maintaining prolonged fermentation in low-sugar *Leymus chinensis* [69]. Excessive moisture and insufficient oxygen during ensiling may further impede enzyme activity [70], resulting in delayed sugar release, a gradual pH decline, and the formation of detrimental metabolites such as butyric acid and ammonia nitrogen, so diminishing the likelihood of a successful fermentation process. In contrast to LAB, which rapidly acidifies to surpass other microorganisms, enzyme complexes exhibit no microbial metabolic activity [71]; thus, fermentation is less successful when utilized in isolation.

The quality of silage mostly relies on the composition of the microbiota, with the bacterial population evolving throughout the silage production and stabilizing upon completion [72, 73]. The OTUs, Chao1, and ACE values of bacteria in the CK group exceeded those in the inoculated group, as illustrated by the Venn diagrams. This suggests that the introduction of inoculant effectively suppresses unwanted bacteria during the silage process, particularly highlighting the pronounced effect of lactic acid bacteria [74]. The PCoA graphic was created to illustrate variations in bacterial community compositions as distances among treatments [75]. The principal coordinates analysis indicated no significant differentiation among the LP, LB, and CE groups. This study found that Firmicutes, Proteobacteria, and Cyanobacteria were present in all treatment groups at the conclusion of fermentation, with Firmicutes being the predominant phylum [76, 77]. The ensiling environment promotes the proliferation of Firmicutes, as they are characteristic bacteria in anaerobic and acidic settings [78]. The notable prevalence of Proteobacteria in the CK group may result from competition between Firmicutes and Proteobacteria during ensilement [79, 80]. This study observed a significant presence of *Lentilactobacillus* in the treatment group supplemented with *Lactobacillus*, a bacterial group essential for the aerobic stability of silage [81]. In the control group, *Enterobacter* and *Hafnia-Obesumbacterium* were the predominant genera, aligning with Zhao's [82] findings in her study on high-moisture alfalfa. *Enterobacter*, as a parthenogenetic anaerobic bacterium, competes with lactic acid bacteria for fermentation substrates and generates metabolites such as NH_3-N, succinic acid, and butyric acid, which influence its nutritional value and diminish palatability [58]. Consequently, the absence of LAB resulted in a considerably elevated presence of *Enterobacter* in the CK group compared to the other inoculation groups.

The diversity of microbial communities can indicate alterations in fermentation properties. Correlation analyses were conducted to clarify the relationship among chemical composition, fermentation parameters, and microbial communities. This study indicates that *Lactiplantibacillus*, due to its robust growth in acidic environments and its ability to collect lactic acid during fermentation, exhibits a negative correlation with pH levels. In this study, the pH of each treatment group significantly decreased and was lower than that of the CK group, whereas the pH of CE was higher than that of CK. *Lactiplantibacillus* exhibited a positive correlation with lactic acid (LA) and acetic acid (AA) as determined by Spearman correlation analysis. This finding aligns with the results of Sun [83], who investigated the fermentation pattern of whole plant corn, and Becerrad [84], who demonstrated that LAB can significantly produce lactic acid during fermentation and generate a certain quantity of acetic acid under specific conditions. This indicates that the metabolic activity of LAB is directly correlated with the synthesis of LA and AA. This further corroborates the significance of lactic acid bacteria in the synthesis of LA and AA. The experiment's results suggested that LP and LPLB had elevated amounts of LA and AA, corroborating the aforementioned conclusions. *Lactiplantibacillus* had a positive correlation with water-soluble carbohydrates (WSC) and ammonia nitrogen (NH_3-N) content. The results indicate that lactic acid bacteria has a significant nutrient-preserving function [85]. *Achromobacter* exhibits a positive correlation with CP. During silage, *Achromobacter* may participate in nitrogen conversion via a comparable mechanism (heterotrophic nitrification-aerobic denitrification capability), influencing the creation and accumulation of crude protein in silage [86, 87]. *Enterococci* is a bacterium that evaluates the health status of animals; an overabundance of *Enterococci* is associated with disease. Furthermore, *Enterococci* exhibit a positive correlation with biogenic amines (BA) due to *Enterobacteria* fermentation, which results in protein degradation and the conversion of lactic acid or glucose into amino acids (AA) and BA under anaerobic conditions. Additionally, *Enterobacteria* and *lactobacilli* compete for limited nutrients, adversely affecting the fermentation quality of the silage mix and nutrient retention [83, 88, 89].

This study utilized KEGG functional mapping of the microbial community, estimated by PICRUSt2, employing 16 S rRNA gene sequences derived from *Leymus chinensis* post-silage. KEGG functional mapping revealed that metabolism is the primary metabolic pathway, with significant changes observed in Global and summary mapping, Carbohydrate metabolism, Amino acid metabolism, and Nucleotide metabolism, as revealed in other research [90, 91]. Amino acids are vital compounds for plants, playing a crucial role in facilitating primary metabolism and protein synthesis. The prevalence of the amino acid metabolism pathway was greater in the CE group compared to the other inoculation groups, as acidic fermentation conditions disrupt amino acid metabolism in forage [92]. Kilstrup et al. indicate that

the majority of metabolic responses pertain to bacterial utilization of nucleotides or the regulation of nucleotides by metabolites. Consequently, the inoculation of groups with LAB leads to augmented nucleotide metabolism, with the LP group being the most predominant [93]. Carbohydrate metabolism, including glyoxylate, dicarboxylate, starch, sucrose, and sugar metabolism, showed variations among groups, possibly due to differences in sugar metabolism gene profiles among *L. plantarum* strains [94]. Alpha diversity indices show that the CK group has the highest species richness. However, in functional prediction, it only exhibits significant advantages in certain metabolic pathways. This is because under normal physiological conditions, the metabolic activities of microbial communities mainly focus on basic Cellular functions and Environmental Information Processing [95]. The aforementioned functional mapping, derived from 16 S rRNA meta-coding with constrained resolution and considerable speculation, necessitates cautious examination and should ideally be corroborated by transcriptomic, proteomic, and metabolomic investigations to clarify the mechanisms of metabolic pathways during silage.

Conclusion

The impact of *Lactiplantibacillus plantarum*, *Lactobacillus buchneri*, and complex enzyme additions on the fermentation characteristics, chemical composition, and bacterial population of *Leymus chinensis* silage was examined alongside the 16 S rRNA gene sequences. The LP treatment led to a significant reduction in pH, measuring 10.82% lower than the CK group; also, the accumulation of AA was markedly enhanced, exhibiting a 4.36-fold increase by the conclusion of the fermentation period compared to the initial stage. The optimal accumulation of LA occurred with the LPLB treatment, achieving 10.69; the preservation of its WSC content was notably stable, exhibiting merely a 3.24% reduction by the conclusion of fermentation relative to the initial phase. The microbial community exhibited more stability. In conclusion, the two categories of additives, LP and LPLB, can markedly enhance silage performance and yield high-quality silage.

Abbreviations

DM	Dry Matter
CP	Crude Protein
ADF	Acid Detergent Fiber
NDF	Neutral Detergent Fiber
WSC	Water Soluble Carbohydrates
LA	Lactic Acid
AA	Acetic Acid
PA	Propionic Acid
BA	Butyric Acid
NH_3-N	Ammonia Nitrogen
LP	Lactiplantibacillus plantarum
LB	Lactobacillus buchneri
LPLB	combination of LP and LB
CE	Composite Enzyme

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Author contributions

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Data availability

All data generated or analyzed during this study are included in this published article within each editable graph.

Declarations

Ethics approval and consent to participate

Experimental research and field studies on plants (either cultivated or wild), including the collection of plant material, were performed in compliance with relevant institutional, national, and international guidelines and legislation. The experiments did not involve endangered or protected species.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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