BMC Plant Biology

RESEARCH

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Effects of temperature and lactic acid Bacteria additives on the quality and microbial community of wilted alfalfa silage

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Abstract

This study investigated the influence of different temperatures (35℃ High temperature and average indoor ambient temperature of 25℃) and lactic acid bacterial additives (*Lactiplantibacillus plantarym*, *Lentilactobacillus buchneri*, or a combination of *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri*) on the chemical composition, fermentation quality, and microbial community of alfalfa silage feed. After a 60-day ensiling period, a significant interaction between temperature and additives was observed, affecting the dry matter (DM), crude protein (CP), acid detergent fiber (ADF), and neutral detergent fiber (NDF) of the silage feed ($p < 0.05$). Temperature had a highly significant impact on the pH value of the silage feed ($p < 0.0001$). However, the effect of temperature on lactic acid, acetic acid, propionic acid, and butyric acid was not significant ($p > 0.05$), while the inoculation of additives had a significant effect on lactic acid, acetic acid, and butyric acid ($p > 0.05$). As for the dynamic changes of microbial community after silage, the addition of three kinds of bacteria increased the abundance of *lactobacillus*. Among all treatment groups, the treatment group using complex bacteria had the best fermentation effect, indicating that the effect of complex lactic acid bacteria was better than that of single bacteria in high temperature fermentation. In summary, this study explained the effects of different temperatures and lactic acid bacterial additives on alfalfa fermentation quality and microbial community, and improved our understanding of the mechanism of alfalfa related silage at high temperatures.

Keywords High-temperature ensiling, Lactic acid bacterial additives, Microbial community, Fermentation quality

Introduction

Alfalfa (*Medicago sativa* L.) is recognized as a crucial forage crop due to its global significance, attributed to its high nutritional value and digestibility [1, 2]. It is widely incorporated as a protein feed in ruminant diets [3]. Silage, an effective long-term feed preservation method, relies on the growth and multiplication of lactic acid bacteria (LAB) in a sealed anaerobic environment. LAB convert water-soluble carbohydrates (WSC) into organic acids, reducing the pH and inhibiting the growth of harmful bacteria [4]. The microbial populations, particularly LAB and other beneficial microorganisms, attached

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to the raw material's surface, play a critical role in the fermentation process and subsequent fermentation quality [5].

However, when medic is ensiled alone, the fermentation outcomes are often suboptimal due to its high buffering capacity and low sugar and dry matter (DM) concentrations [6, 7]. Consequently, the use of LAB inoculants is common in silage production, as they can modify the fermentation pattern and are widely employed to produce high-quality medic silage feed [8]. Based on the mode of fermentation, LAB are usually classified into two types: homofermentative and heterofermentative [9]. Research has demonstrated that inoculating *Lactiplantibacillus plantarym* can dominate lactic acid fermentation, rapidly reducing the pH to hinder the growth of undesirable microorganisms [10]. Inoculating *Lentilactobacillus buchneri* can enhance aerobic stability by fermenting lactate into acetate and 1,2-propanediol [11].

Temperature plays a critical role in storage conditions as it directly impacts the activity and metabolic pathways of microorganisms. The optimal temperature range for the growth of LAB is typically within 20–30 °C, as temperatures exceeding this range are unfavorable for their growth and reproductive capacity [12]. Previous studies have highlighted the adverse effects of high-temperature ensiling on feed preservation. Weinberg et al. [13] observed a decline in feed quality resulting from high-temperature ensiling. Similarly, Kim and Adesogan [14] demonstrated that high-temperature ensiling leads to reduced aerobic stability of the feed. Furthermore, research has indicated that ensiling under high-temperature conditions can induce clostridial fermentation [15]. However, the specific effect of temperature on silage fermentation mechanism has not been fully clarified. Existing studies have shown that high temperature conditions may adversely affect the quality of silage, but these conclusions still need to be verified and supported by further systematic studies and experimental evidence. In addition, the interaction between different fermentation methods and temperature conditions before the silage process has also become an important research direction. The effects of temperature on fermentation microbial activity and metabolite formation and temperature change on final nutrient composition and storage stability of silage can provide theoretical basis and practical guidance for optimizing silage process and improving silage quality. In recent years, the utilization of 16 S rRNA sequencing in several studies has provided valuable insights into the dynamics of the microbial community in ensiled feed, thereby enhancing our understanding of the microbial mechanisms involved in the ensiling fermentation process [16].

To date, there is a dearth of research examining the influence of additives on the fermentation quality and mechanisms of alfalfa ensiling under high-temperature conditions. Consequently, the specific effects of different temperatures and additives on the fermentation processes in alfalfa silage remain largely unexplored. In light of this knowledge gap, our study postulates that distinct fermentation mechanisms emerge in alfalfa ensiling as a result of varying temperatures and additives. With this in mind, we conducted a comprehensive investigation utilizing microbial sequencing techniques to assess the impact of different additives under high-temperature conditions on the quality of alfalfa silage feed. Our primary objective was to elucidate the intricate interplay between high temperature and the fermentation mechanisms of alfalfa ensiling, employing a microbial perspective as our analytical framework.

Materials and methods

Experimental design and mini silos preparation

The material used in the experiment was planted in June 2021 and carried out at a research site in Hohhot, Inner Mongolia Autonomous Region, located in Eurasia, from June to August 2022. The region experiences a temperate continental monsoon climate with precipitation ranging from 335.2 to 534.6 mm.The geographical coordinates of the test site are between 110°46 '- 112°10' east longitude and 40°51 '- 41°8' north latitude, with an average altitude of 1,226 meters. The experimental plot covered an area of 666 m^2 and consisted of saline-alkali soil. The alfalfa variety "WL168" was selected and harvested at the beginning of alfalfa initial flowering stage.The seeds were supplied by Inner Mongolia Zhengda Co., Ltd. and originated in the United States. Harvesting occurred on June 28th, and a portion of the fresh samples were immediately placed in sterilized storage tubes and transported to a laboratory freezer at -80°C using liquid nitrogen for preservation. The remaining grass samples were spread out on clean plastic sheets and air-dried until they reached a moisture content of approximately 60%. Subsequently, the grass samples were shredded into 2–3 cm lengths. After thorough mixing and homogenization, the alfalfa samples were divided into four equal parts. Three of the parts were sprayed with different microbial agents: the first part with *Lactiplantibacillus plantarym* (Commercial variety name: Silage Bang, Production enterprise: Inner Mongolia Hemei Kesheng Biotechnology Co., Ltd; Production address: Hohhot, Inner Mongolia) at a target bacterial concentration of 1×10^6 cfu/g, the second part with *Lentilactobacillus buchneri* (Commercial variety name: Lalsil Fresh, Production enterprise: Lallemand Animal Nutrition UK Ltd.) at a target bacterial concentration of 1×10^6 cfu/g, and the third part with a combination of *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri* (Commercial variety name: Beneficial silage Bang, Production enterprise: Inner Mongolia Hemei Kesheng

Biotechnology Co., Ltd; Production address: Hohhot, Inner Mongolia) at a target bacterial concentration of 1×10^6 cfu/g. The fourth part served as the blank control group and was sprayed with an equal amount of distilled water. During the spraying process, the grass samples were thoroughly mixed to ensure even distribution of the additives. The treated samples were then packed into polyethylene plastic bags with a capacity of 300 g per bag (size: 300 mm × 400 mm; smooth food-grade vacuum bags from Shenyang Huasheng Plastic Packaging Products Co., Ltd.) and sealed using a vacuum sealing machine (model: DZ-500/2E; Hefei Hanjie Packaging Machinery Inkjet Co., Ltd., Hefei, China). Each treatment group was divided into two equal portions (4 treatment groups × 3 replicates × 2 temperatures). One portion underwent room temperature fermentation indoors, while the other portion underwent high-temperature fermentation at 35°C in a constant temperature incubator. After 60 days of ensiling, the quality of the alfalfa silage feed and the composition of microbial communities were assessed for the following groups: high-temperature treatment with *Lactiplantibacillus plantarym* (GLP), high-temperature treatment with *Lentilactobacillus buchneri* (GLB), high-temperature treatment with a combination of *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri* (GLPLB), high-temperature control group (GCK), room temperature treatment with *Lactiplantibacillus plantarym* (CLP), room temperature treatment with *Lentilactobacillus buchneri* (CLB), room temperature treatment with a combination of *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri* (CLPLB), and room temperature control group (CCK). Additionally, 20 g of each sample were placed in storage tubes and stored in a -80°C freezer. These samples will be sent to Majorbio Bio-Pharm Technology (Majorbio Bio-Pharm Technology Co., Ltd., Shanghai, China) for sequencing analysis.

Sample Collection and Measurement

Each sample of fresh alfalfa and silage underwent three parallel determinations, which included assessing their chemical composition, fermentation characteristics, and microbial counts. The dry matter (DM) content was determined by subjecting the samples to a 65 °C oven for 48 h [17]. After drying, the samples were pulverized in a grinder through a 1-millimeter sieve (Product name: CSJ series coarse crusher; Manufacturer: Jiangsu Hongda Powder Equipment Co., Ltd., China) and then transferred into zip-lock bags for storage. A portion of the pulverized samples was taken out and milled using a ball mill (Product name: MM400; Manufacturer: Verder Shanghai Instruments and Equipment Co., Ltd.) for the determination of CP content. The crude protein (CP) content was determined using the Kjeldahl method with a Gerhart Vapodest 50 s instrument from Germany, following the guidelines provided by the Association of Official Analytical Chemists (AOAC 1990). The neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents were assessed using an Ankom A2000i fiber analyzer (A2000i, Ankom Technology, Macedon, NY, United States) [18]. Furthermore, the content of water-soluble carbohydrates (WSC) was determined using a colorimetric method after reacting the samples with anthrone reagent [19].

To evaluate the fermentation characteristics of the silage, a 10 g sample was mixed with distilled water that had undergone high-temperature sterilization. The mixture was vigorously shaken and allowed to settle in a refrigerator at 4 °C. Subsequently, the solution was filtered through four layers of cheesecloth to remove any solid particles. The pH value of the resulting silage extract was determined using a calibrated glass electrode pH meter (STARTED 100/B, OHAUS, Shanghai, China). For the quantitative analysis of lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA), a liquid chromatograph was employed [20] (Product Name: Waters® e2695; Manufacturer: Micromass UK Ltd.) The concentration of ammonia nitrogen (NH_3-N) was determined using the method described by Broderick and Kang (1980) [21].

To determine the microbial population counts, a 10 g sample of silage was thoroughly mixed with 90 ml of distilled water that had been sterilized at high temperature. The resulting mixture was then filtered to obtain the filtrate. Microbial counting was performed by conducting ten-fold serial dilutions of the filtrate using 1 ml of solution. Enumeration of LAB was carried out by placing the corresponding agar plates in an anaerobic chamber (C-31, Mitsubishi Gas Chemical Co., Tokyo, Japan) and incubating them at 37 °C for 48 h. Coliform was counted by a purplish-red bile AGAR (trade name: Eosin methylene blue AGAR medium, manufacturer: Guangdong Huankai Biotechnology Co., LTD., China) incubated at 37 °C for 48 h. Enumeration of yeast and mold was conducted under aerobic conditions by placing the respective agar plates (potato dextrose agar) in a standard incubator (GP-01, Huangshi Hengfeng Medical Equipment Co., Ltd., Huangshi, China) and incubating them at 30 °C for 48 h [22, 23].

Sequencing and analysis of microbial diversity

The E.Z.N.A. R kit (Omega Bio-tek, Norcross, GA, USA), a commercially available DNA extraction kit, was employed for the isolation of total DNA from both alfalfa and silage samples. The concentration and purity of the DNA were determined using a NanoDrop 2000 UV-Visible spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, United States). PCR amplification was carried out by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China) using primers designed to target the

V3-V4 region (338 F: 5'-ACTCCTACGGGGGAGGCAG CAG-3'; 806R: 5'-GGACTACHVGGGTWTCTAAT-3') of the 16 S rDNA gene. The obtained raw fastq files underwent multiplexing, quality filtering using Trimmomatic, and merging using FLASH. Operational taxonomic units (OTUs) were clustered at a 97% similarity threshold using UPARSE, and chimeric sequences were identified and eliminated using UCHIME. The classification of each 16 S rRNA gene sequence was conducted using the RDP classifier algorithm and the Silva (SSU123) 16 S rRNA database, with a confidence threshold set at 70%.

Statistical analysis

The chemical composition, fermentation quality and microbial characteristics of silage were evaluated by SPSS ANOVA program. To determine the significance of the difference, Tukey test was used, and $p<0.05$ was the significance level. Experimental data values are reported as the mean of the repetitions of different treatments and the standard error of the mean. The microbiome data analysis was carried out using Majorbio I-Sanger cloud platform online platform.

Results

Chemical and microbial composition of fresh alfalfa samples

The chemical composition and microbial population of the original alfalfa sample prior to ensiling are presented in Table 1. The DM content was determined to be 42.75%, while the WSC content was found to be 1.59%. The CP, NDF, ADF were measured at 20.52%, 38.29%, and 31.30%, respectively. Microbial counts based on fresh matter (FM) were conducted, revealing the presence of LAB, aerobic bacteria, *Escherichia coli*, yeast, and mold at levels of 5.41, 6.33, 2.77, 5.96, and 3.16 $\log_{10}$ cfu/g, respectively.

Table 1 Nutrient content and microbial population of alfalfa fresh materials

Items	Content
Dry matter (g/kg FW)	425.73 ± 9.81
Crude protein (g/kg DM)	205.16 ± 12.30
Neutral detergent fiber (g/kg DM)	382.94 ± 15.38
Acid detergent fiber (g/kg DM)	312.95 ± 7.13
Water-soluble carbohydrates (g/kg DM)	15.93 ± 0.49
Lactic acid bacteria($\log_{10}$ cfu/g FM)	5.41 ± 1.87
Aerobic bacteria ($\log_{10}$ cfu/g FM)	6.33 ± 0.43
Coliform bacteria ($\log_{10}$ cfu/g FM)	2.77 ± 2.66
Yeasts ($\log_{10}$ cfu/g FM)	5.96 ± 0.19
Mold ($\log_{10}$ cfu/g FM)	3.16 ± 0.28

Note: FM, fresh matter; log, denary logarithm of the numbers; cfu, colony-forming units; results are expressed as mean ± standard deviation.)

Quality of alfalfa silage treated at different temperatures

Table 2 presents the chemical characteristics of the silage subjected to fermentation at different temperatures for 60 days. After the 60-day fermentation period, a significant interaction between temperature and inoculant was observed regarding the content of DM and ADF ($p<0.05$), as well as CP and NDF ($p<0.0001$). Inoculation with the bacterial strain resulted in a reduction in DM content. Furthermore, under ambient temperature conditions, the treated groups exhibited lower CP and ADF content compared to the control group, while under high-temperature conditions, the treated groups showed higher CP and NDF content than the control group. The interaction between temperature and inoculant did not have a significant effect on the content of WSC ($p>0.05$).

Temperature had a highly significant impact on the pH of the silage ($p<0.0001$), with the silage stored under high-temperature conditions displaying lower pH values compared to that stored under ambient temperature. Temperature did not significantly affect the concentrations of LA, AA, BA, and PA ($p>0.05$). Conversely, inoculation with the bacterial strain had no significant effect on LA, AA, and PA ($p>0.05$). The concentration of acetic acid was higher in the silage inoculated with the LB strain compared to the LP strain.

During ambient temperature fermentation, the population of LAB in all treatment groups was lower than that in the control group. However, under high-temperature fermentation, only the LP group exhibited a higher population of LAB compared to the control group ($p>0.05$).

Microbial community diversity analysis of alfalfa silage feed

The bacterial community in both fresh alfalfa material and ensiled feed was analyzed using next-generation sequencing of the full-length 16 S rRNA gene, as presented in Table 3. The sequencing coverage for all samples exceeded 99%, indicating ample sequencing depth for comprehensive characterization of the bacterial community and facilitating effective analysis of its diversity. The mean values of diversity indices for fresh alfalfa samples are as follows: ace is 800.55; chao1 is 764.76; shannon is 3.39; sobs is 728.67; coverage is 0.9976. In comparison to the fresh alfalfa sample, the ace, chao1, shannon, and sobs values of the ensiled feed exhibited a decrease. The chao1 index value of the GCK group was higher than that of the CCK group. Additionally, the chao1 and sobs index values in the GLPLB group were higher than those in the CCK group. Furthermore, ace, chao1, shannon, and sobs values in the room temperature fermentation group were higher than those in the high temperature fermentation group. Moreover, following a 60-day ensiling period, the CLP group displayed the highest ace, chao1, shannon, and sobs values.

Table 2 Effects of different temperatures and lactic acid bacteria additives on the chemical composition, fermentation quality, and microbial composition of alfalfa silage after a 60-day period

Item	G			C				T	L	TxL
	LP	LB	LPLB	CK	LP	LB	LPLB	CK			
Dry matter(g/kg FM)	376.19±0.4b	406.95±1.63a	371.11±7.96bc	421.02±5.15a	352.97±2.58c	367.54±4.75bc	364.10±0.51bc	368.24±4.62bc	<0.001	<0.001	<0.001
Crude protein (g/kg DM)	180.08±8.50c	185.32±4.58c	176.43±1.74c	175.45±3.28c	219.43±4.78b	228.10±4.25ab	168.16±6.77c	245.44±0.82a	<0.001	<0.001	<0.001
Acid detergent fiber(g/kg DM)	368.61±7.87d	381.16±2.77bcd	429.91±6.48a	376.98±5.89 cd	400.16±5.00abcd	409.38±9.04abc	419.53±12.85ab	432.82±9.40a	<0.001	0.001	0.006
Neutral detergent fiber(g/kg DM)	479.48±6.31ab	465.45±6.55a	486.98±6.04ab	421.44±10.42 cd	404.64±9.07d	413.64±4.86 cd	457.91±5.23ab	446.55±5.76bc	<0.001	<0.001	<0.001
Water soluble carbohydrates(g/kg DM)	8.16±0.70a	9.85±0.62a	8.79±0.66a	9.89±0.73a	8.63±0.23a	7.46±1.69a	9.26±1.52a	8.77±0.53a	0.356	0.780	0.399
pH	5.64±0.07b	5.84±0.45b	6.15±0.31ab	5.88±0.36b	7.04±0.04a	6.6±0.08ab	7.04±0.01a	7.07±0.05a	<0.001	0.434	0.540
Lactic acid (g/kg DM)	3.23±0.38a	0.57±0.23b	0.10±0.10b	0.07±0.07b	1.00±0.17b	1.13±0.26b	0.97±0.69b	ND	0.344	<0.001	0.010
Acetic acid (g/kg DM)	ND	0.6±0.15a	0.50±0.15a	0.63±0.03a	0.20±0.00ab	0.37±0.09ab	0.70±0.20a	0.40±0.12ab	0.842	0.003	0.116
Propionic acid (g/kg DM)	0.27±0.15a	0.40±0.15a	0.10±0.10a	0.23±0.03a	0.43±0.07a	0.43±0.15a	0.53±0.27a	0.10±0.05a	0.228	0.368	0.267
Butyric acid (g/kg DM)	ND	ND	ND	ND	ND	ND	ND	0.23±0.12	0.070	0.032	0.032
Ammonia nitrogen(g/kg DM)	4.37±0.07a	4.47±0.03a	4.47±0.12a	4.20±0.06a	4.47±0.19a	4.23±0.03a	4.57±0.12a	4.10±0.06a	0.637	0.012	0.282
Lactic acid bacteria(log_{10} cfu/g FM)	4.29±1.03a	3.35±0.77a	2.85±0.31a	3.76±0.17a	3.48±0.28a	4.21±0.33a	3.76±0.22a	4.79±0.03a	0.178	0.318	0.250
Aerobic bacteria (log_{10} cfu/g FM)	3.65±0.52a	3.20±0.66a	4.37±1.29a	3.21±0.38a	3.34±0.09a	3.46±0.01a	3.50±0.06a	5.05±0.08a	0.570	0.475	0.134
Yeasts (log_{10} cfu/g FM)	5.17±0.95a	3.25±0.54ab	3.28±0.37ab	2.99±0.18b	3.20±0.08ab	3.59±0.04ab	3.47±0.02ab	3.42±0.09ab	0.393	0.127	0.030
Coliform bacteria (log_{10} cfu/g FM)	ND	ND	ND	ND	ND	ND	1.10±0.1a	ND	<0.001	<0.001	<0.001
Mold (log_{10} cfu/g FM)	ND	ND	ND	ND	ND	ND	ND	ND			

Note: T: the significance of different temperatures; L: The significance of different lactic acid bacteria additives; TxL: Interaction between temperature and lactobacillus additives; CK, control group; G, 35 ℃ high temperature; C, room normal temperature; LP, *Lactiplantibacillus plantarym* treatment group; LB, *Lentilactobacillus buchneri* treatment group; LPLB, *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri* mixed inoculant treatment group; log, denary logarithm of the numbers; cfu, colony-forming units; FM, fresh matter; ND, not detected. Values in the same row with different letters are significantly different ($p<0.05$)

Microbial diversity analysis was performed using principal coordinate analysis (PCoA), a method employed to assess dissimilarities between individuals or populations. Each data point represents a sample, with points of the same color indicating membership in the same group. The proximity of points reflects the similarity in microbial community composition. In Fig. 1A, no distinct separation was observed among the bacterial communities of the fresh alfalfa samples. However, clear separation emerged after ensiling, with a more pronounced differentiation in the microbial communities of the feed subjected to high-temperature fermentation compared to ambient temperature fermentation. These findings suggest a temperature-dependent impact on microbial communities. Under different temperature conditions, distinct separations were observed in the bacterial communities of the ensiled feed treated with various additives (Fig. 1B). In comparison to the CCK group, the CLP, CLB, and CLPLB groups exhibited more prominent separations, indicating an influence on microbial communities in the ensiled feed following additive treatment. Notably, during high-temperature fermentation, the GLB and GLPLB groups exhibited a more pronounced separation in their bacterial communities, whereas the separation was less apparent in the GLP group. This means that temperature has a greater effect on the additives to *Lentilactobacillus buchneri* and the compound additives of *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri*.

Quantitative and temporal changes in bacterial community composition of alfalfa silage feed

The dynamics of the bacterial community at the genus level in fresh alfalfa and ensiled alfalfa feed, subjected to different temperatures and additives, were investigated using 16 S rRNA gene sequencing, as illustrated in Fig. 2A. In the fresh alfalfa sample, the predominant genera, apart from unclassified bacteria (41.88%) and others (17.64%), were primarily represented by *Citrobacter* (17.27%), with a minor presence of Lactobacillus (0.67%). Similarly, the CK group exhibited *Citrobacter* as the dominant genus. Notably, the GCK group displayed an increased abundance of Lactobacillus (7.52%) and a reduced abundance of Coprococcus (4.43%) compared to the CCK group. In all experimental groups treated with additives, the abundance of Lactobacillus surpassed that of the CK group, with the CLPLPB group demonstrating the highest abundance at 49.23%. However, under high-temperature fermentation conditions, the abundance of Lactobacillus in all additive-treated groups decreased relative to ambient temperature fermentation, indicating a temperature-dependent impact on the additives.

Figure 2B depicts the dynamics of the bacterial community at the species level between fresh alfalfa and

Table 3 Alpha-diversity of bacterial communities in alfalfa fresh materials and silage

Item	G				C				T	L	T×L
	LP	LB	LPLB	CK	LP	LB	LPLB	CK			
ace	168.74b	271.00ab	267.28ab	304.77a	311.26a	284.70ab	256.43ab	273.51ab	0.159	0.335	0.025
chao1	167.29a	257.34a	261.96a	278.6a	295.7a	280.23a	244.85a	267.10a	0.141	0.466	0.067
shannon	1.48b	2.25ab	2.52a	2.21ab	2.34a	2.44a	1.92ab	2.33ab	0.266	0.108	0.006
sobs	127.67b	221.67ab	217.67ab	223.67ab	251.67a	239.33ab	207.67ab	226.67ab	0.065	0.362	0.05
coverage	0.9987a	0.9989a	0.9989a	0.9988a	0.9992a	0.9989a	0.9989a	0.9986a	0.705	0.574	0.105

Note: T: the significance of different temperatures; L: The significance of different lactic acid bacteria additives; T×L: Interaction between temperature and lactobacillus additives; CK, control group; G, 35 ℃ high temperature; C, room normal temperature; LP, *Lactiplantibacillus plantarym* treatment group; LB, *Lentilactobacillus buchneri* treatment group; LPLB, *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri* mixed inoculant treatment group; Values in the same row with different letters are significantly different ($p<0.05$)

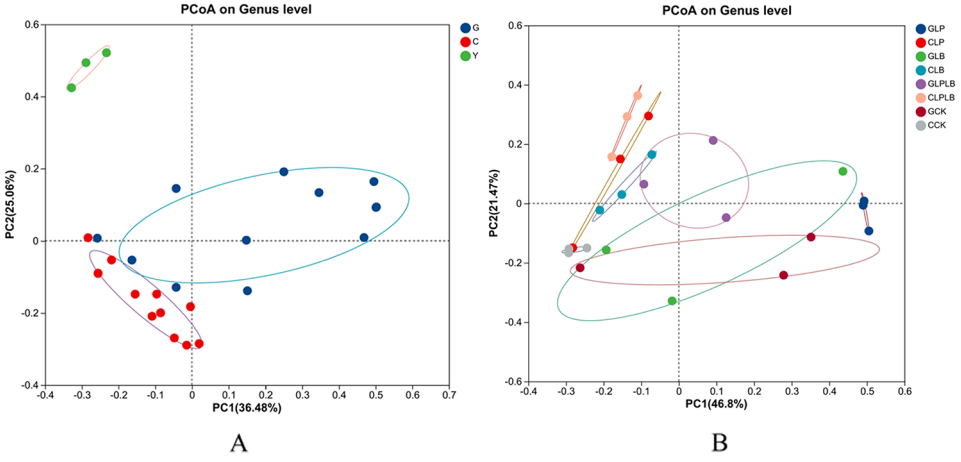

Fig. 1 Principal coordinate analysis (PCoA) maps show changes in community structure at the microbial genus level under different temperature and additive treatments. (**A**) Schematic diagram of microbial community changes in high-temperature fermentation samples, ambient-temperature fermentation samples and fresh materials; (**B**) is a schematic diagram of microbial community changes between treatment groups. G: Samples fermented in high temperature environment; C: Sample fermentation at ambient-temperature; Y: fresh materials; GCK: high-temperature control group; CCK: ambient-temperature control group; GLP: high-temperature *Lactiplantibacillus plantarym* treatment group; CLP: ambient-temperature *Lactiplantibacillus plantarym* treatment group; GLB: high-temperature *Lentilactobacillus buchneri* treatment group; CLB: ambient-temperature *Lentilactobacillus buchneri* treatment group; GLPLB: high-temperature *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri* mixed inoculant treatment group; CLPLB: ambient-temperature *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri* mixed inoculant treatment group

ensiled feed. In the fresh alfalfa sample, the predominant bacteria were characterized by bacterium and unclassified bacteria (others), accounting for 27.02% and 19.09%, respectively. Within the GLP, GLB, and GCK groups, *Enterococcus_sp*. emerged as the dominant species, constituting 65.31%, 23.14%, and 27.00%, respectively. In the CLP, GLPLB, and CLPLB groups, *Lactiplantibacillus plantarum* prevailed as the dominant species, with proportions of 29.15%, 26.37%, and 48.22%, respectively. Similarly, within the CCK and CLB groups, *Citrobacter_sp*. constituted the dominant species, accounting for 27.53% and 35.43%, respectively.

The dynamics of the bacterial community at the phylum level in fresh alfalfa and throughout the ensiling process are depicted in Fig. 3A. In the fresh alfalfa sample, the dominant phyla were unclassified bacteria (42%) and *Proteobacteria* (37%). However, following ensiling, all treatment groups predominantly comprised *Firmicutes* (GLP: 95%, CLP: 74%, GLB: 66%, CLB: 65%, GLPLB: 74%, CLPLB: 81%, GCK: 69%, CCK: 56%) and *Proteobacteria* (GLP: 2.6%, CLP: 19%, GLB: 29%, CLB: 30%, GLPLB: 21%, CLPLB: 16%, GCK: 24%, CCK: 37%). Figure 3B illustrates the differences in bacterial genera between fresh alfalfa and ensiled feed across different treatment groups. In the fresh alfalfa sample, the predominant attached bacteria belonged to the unclassified bacteria category. However, after ensiling, the major attached bacteria in the GLP, GLB, and GCK groups were *Enterococcus*, significantly surpassing other genera ($P<0.01$). Within the CLP, CLB, GLPLB, and CLPLB groups, the major attached bacteria were Lactobacillus, significantly higher than other genera ($P<0.01$). Lastly, in the CCK group, the major attached bacteria were *Coprococcus*, significantly exceeding other genera ($P<0.01$).

LEfSe utilizes Linear Discriminant Analysis (LDA) to estimate the magnitude of the impact of species abundance on observed differences. In this study, we performed statistical analyses from the phylum to the species level, and the cladogram illustrates various treatments. The LDA values were validated by LEfSe, with a threshold

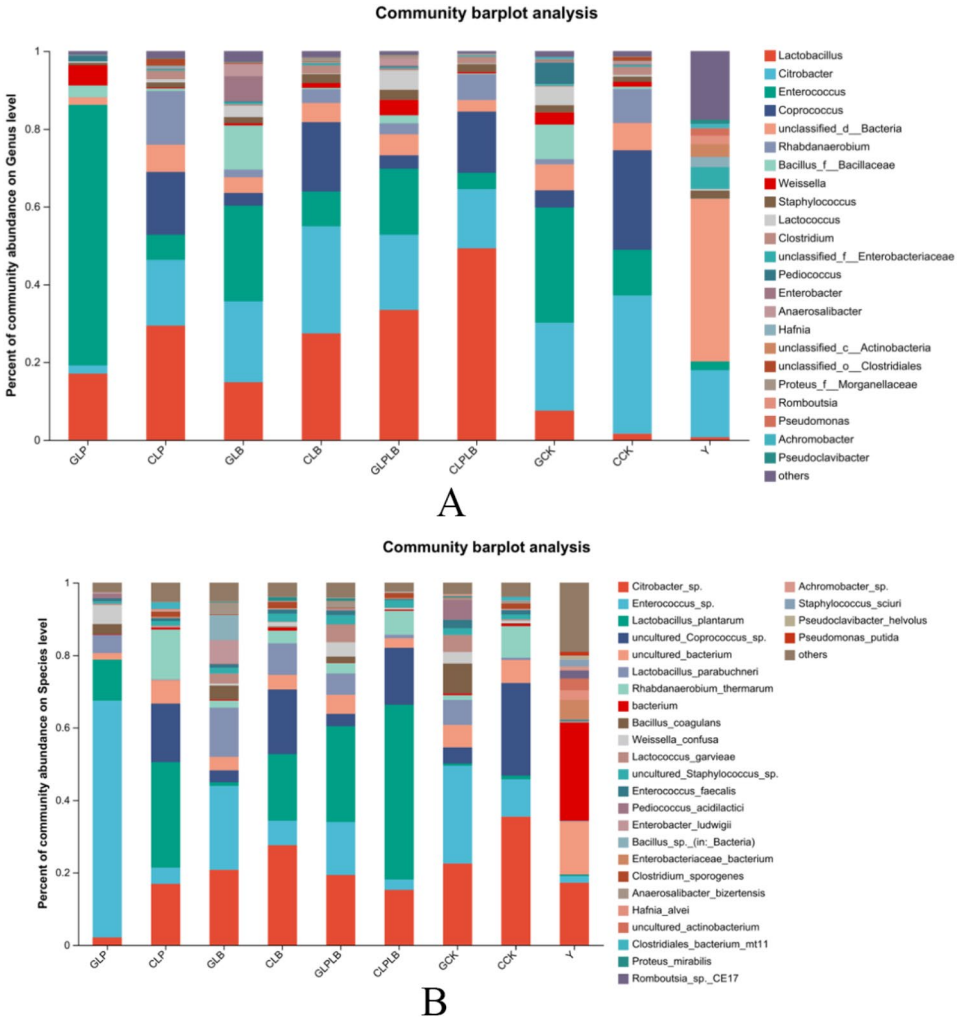

Fig. 2 The bacterial community composition at the genus (**A**) and species (**B**) levels in fresh alfalfa samples and alfalfa silage feed was determined using microbial amplicon sequencing. Y: fresh material; GCK: high-temperature control group; CCK: ambient-temperature control group; GLP: high-temperature *Lactiplantibacillus plantarym* treatment group; CLP: ambient-temperature *Lactiplantibacillus plantarym* treatment group; GLB: high-temperature *Lentilactobacillus buchneri* treatment group; CLB: ambient-temperature *Lentilactobacillus buchneri* treatment group; GLPLB: high-temperature *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri* mixed inoculant treatment group; CLPLB: ambient-temperature *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri* mixed inoculant treatment group. (**A**–**B**)

of 2 or higher (Fig. 4). Within the GLP group, eight bacterial taxa were significantly enriched, and the *c__Bacilli* exhibited the highest LDA score of 5.52. The CLP group displayed significant enrichment of 11 bacterial taxa, with the *s__Rhabdanaerobium_thermarum* achieving the highest LDA score of 4.81. Similarly, the GLB group exhibited significant enrichment of eight bacterial taxa, with the *s__Lactobacillus_parabuchneri* attaining the highest LDA score of 4.70. In the CLB group, 14 bacterial taxa were significantly enriched, and the *s__Lactobacillus_brevis* obtained the highest LDA score of 4.10. The GLPLB group displayed significant enrichment of eight bacterial taxa, with the *f__Streptococcaceae* achieving the highest LDA score of 4.38. Within the CLPLB group, five bacterial taxa were significantly enriched, and the *f__Lactobacillaceae* demonstrated the highest LDA score of 5.31. The GCK group exhibited significant enrichment of nine bacterial taxa, with the *g__Exiguobacterium* obtaining the highest LDA score of 4.49. The CCK group displayed significant enrichment of ten bacterial taxa, with the *c__Clostridia* achieving the highest LDA score of 5.26. These findings indicate that, except for the LPLB group, the number of enriched bacteria under high-temperature fermentation conditions was lower compared to ambient temperature fermentation.

Relationship between chemical composition, fermentation quality, and bacterial community in alfalfa silage

The correlation analysis graph presented in Fig. 5 provides a visual representation of the relationships between chemical components, fermentation parameters, and major fermentation products at the genus

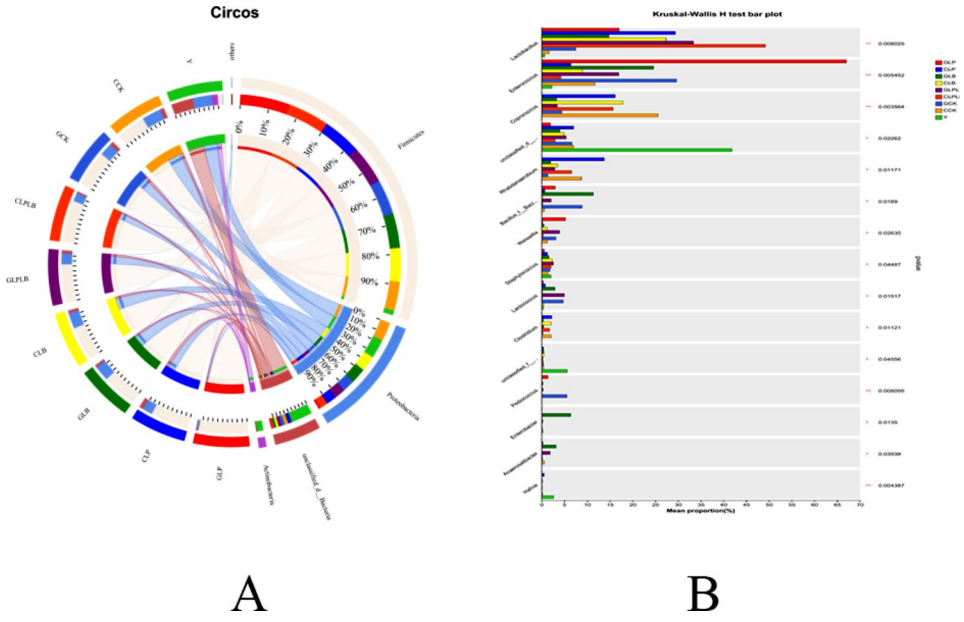

Fig. 3 (**A**)The Circos plot displayed the variations in relative abundance of microbial communities at the phylum level, where the size of each bar represented the relative abundance of that phylum in the samples. (**B**) Kruskal-Wallis H test was employed to analyze the differences at the genus level between fresh alfalfa samples and alfalfa silage feed. *$p \le 0.05$; **$p \le 0.01$; ***$p \le 0.001$. Y represented the fresh material; GCK denoted the high-temperature control group; CCK represented the ambient-temperature control group; GLP indicated the high-temperature treatment group with *Lactiplantibacillus plantarym*; CLP represented the ambient-temperature treatment group with *Lactiplantibacillus plantarym*; GLB denoted the high-temperature treatment group with *Lentilactobacillus buchneri*; CLB represented the ambient-temperature treatment group with *Lentilactobacillus buchneri*; GLPLB indicated the high-temperature treatment group with a mixed inoculant of *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri*; CLPLB indicated the ambient-temperature treatment group with a mixed inoculant of *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri*

level. It reveals several significant associations. Specifically, DM demonstrates a positive correlation with *Cytobacillus* and *Streptococcus*, while displaying a negative correlation with *Hafnia* and *Faecalicatena*. CP exhibits a positive correlation with *unclassified_p__Firmicutes* and *unclassified_o__Lactobacillales*, while displaying a negative correlation with *Frigoribacterium*. Notably, *Coprococcus*, *Rhabdanaerobium*, *Clostridium*, *Hafnia*, *unclassified_p__Firmicutes*, *unclassified_o__Lactobacillales*, and *Leuconostoc* are found to be negatively correlated with NDF, but positively correlated with ADF. Meanwhile, *Faecalicatena*, *Pectobacterium* and *Paracoccus_f_Rhodobacteraceae* were negatively correlated with NDF. Moreover, pH demonstrates a positive correlation with *Coprococcus*, *Rhabdanaerobium*, *Clostridium*, *Hafnia*, *unclassified_p__Firmicutes*, *Faecalicatena*, and *Leuconostoc*, while exhibiting a negative correlation with *Frigoribacterium* and *Pedioccocus*. Additionally, LA shows a negative correlation with *Anaerosalibacter* and *Cutibacterium*. Lastly, NH_3-N displays a positive correlation with Lactobacillus, while revealing a negative correlation with *Pantoea*. These findings provide valuable insights into the interrelationships between various factors and microbial communities, shedding light on the complex dynamics of the fermentation process.

Discussion

The preparation of forage silage is challenging in tropical areas due to the high water content and low soluble carbohydrate content of the forage [24], and the influence of temperature on silage quality should not be underestimated. Typically, a temperature range of 20–30 °C is considered optimal for the fermentation of grass silage [25]. However, during the summer when grass is harvested, temperatures often surpass 30 °C, presenting challenges in producing high-quality silage. Elevated temperatures throughout the ensiling process can have negative effects by hastening aerobic deterioration and inducing the formation of butyric acid and alcohol fermentation byproducts [26]. LAB play a crucial role in the ensiling process, with a LAB content of at least 5.0 log cfu/g FM being typically necessary for successful silage preservation [27]. The attainment of successful silage fermentation is closely intertwined with the environmental conditions prevailing during the fermentation period, underscoring the importance of temperature regulation in ensuring optimal silage quality [28]. It is noteworthy that microorganisms also exert a vital influence in the ensiling process, and this study systematically investigates the quantification and community dynamics of microorganisms in alfalfa silage under varying temperature conditions.

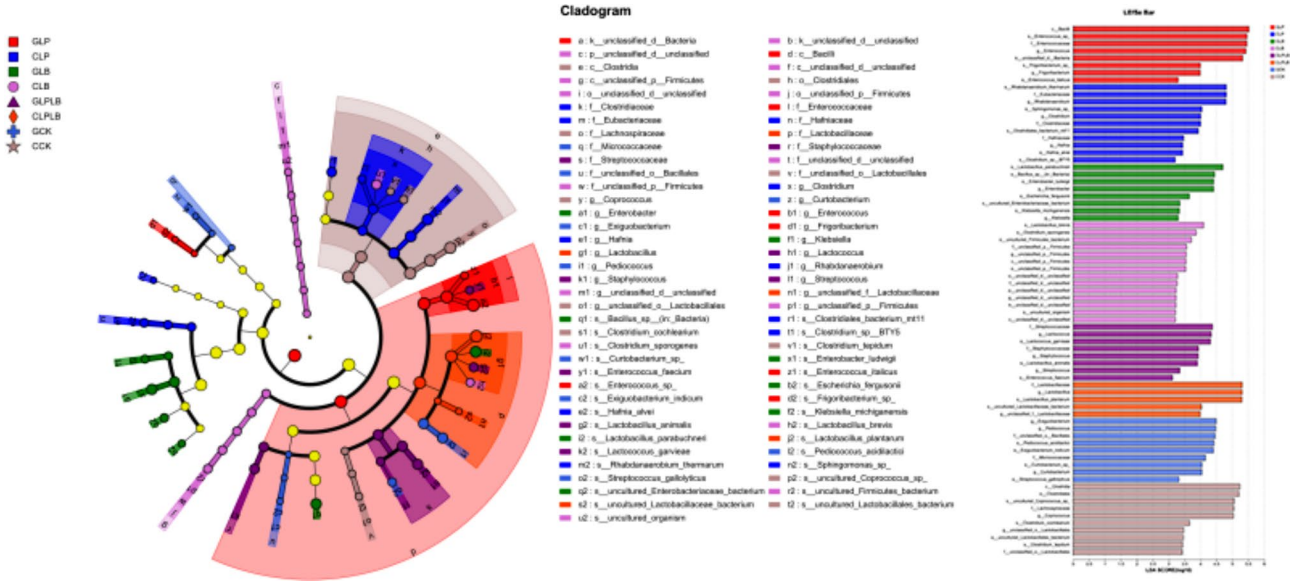

Fig. 4 The cladogram generated using LEfSe to identify specific microbial communities within alfalfa silage. Each node in different colors represents microbial taxa that exhibit significant enrichment in their respective groups and contribute significantly to inter-group differences. Nodes in pale yellow indicate microbial taxa that show no significant differences among the different groups or have negligible impact on inter-group differences. The LDA discriminant analysis results are presented, with different colors representing distinct treatment groups (Panel B). The GCK group represents the high-temperature control group; The CCK group represents the ambient temperature control group; The GLP group corresponds to the high-temperature treatment group with *Lactiplantibacillus plantarym*; The CLP group represents the ambient temperature treatment group with *Lactiplantibacillus plantarym*; The GLB group denotes the high-temperature treatment group with *Lentilactobacillus buchneri*; The CLB group represents the ambient temperature treatment group with *Lentilactobacillus buchneri*; The GLPLB group represents the high-temperature treatment group with a combination of *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri*; The CLPLB group represents the ambient-temperature treatment group with a combination of *Lactiplantibacillus plantarym* and *Lentilactobacillus buchneri*

Analysis of chemical composition and fermentation quality of alfalfa silage feed

Compared to fresh alfalfa, silage exhibits a reduction in WSC content due to microbial utilization of soluble carbohydrates as fermentation substrates, resulting in their conversion into lactic acid [29]. In contrast to the fresh samples, all treatment groups subjected to high-temperature fermentation experience a decrease in CP content. This phenomenon can be attributed to the favorable interaction between plant and microbial enzymes at a temperature of 35 °C during fermentation, which accelerates the conversion of plant protein into non-protein nitrogen (NPN) [30]. The pH value serves as a fundamental parameter for evaluating silage fermentation. To attain high-quality silage, it is essential to have an adequate population of LAB that facilitates a rapid decline in pH [31].In this experiment. The pH value of the high temperature treatment group was lower than that of the normal temperature treatment group, indicating that the high temperature environment may promote the formation of acidic conditions. However, in the detection of the relative abundance of lactic acid bacteria, we found that the relative abundance of lactic acid bacteria in the high temperature treatment group was lower than that in the normal temperature treatment group, so we speculated that the process of high temperature fermentation may be dominated by other microorganisms rather than lactic acid bacteria. The levels of LA and NH_3-N directly reflect the quality of the silage [32]. As anticipated, the addition of LAB inoculants increases the LA content, while the NH_3-N content remains relatively unchanged, suggesting that temperature and additives have minimal influence on NH_3-N content.

Analysis of microbial community diversity in alfalfa silage feed

In this study, we conducted sequencing of bacterial communities in both fresh and ensiled alfalfa to evaluate microbial diversity. It is commonly observed that high-quality silage exhibits lower α-diversity [33]. A higher Shannon index indicates greater microbial diversity within the samples [34]. The Ace and Chao1 indices are utilized to measure species richness, with lower values indicating reduced species richness within the samples [35]. Comparative analysis with the fresh samples revealed a decrease in the Ace, Chao1, Shannon, and Sobs values of ensiled feed, suggesting a decline in the abundance of aerobic bacteria due to the anaerobic environment during ensiling [36]. Our study also found that the α-diversity of silage stored at high temperature was lower than that stored at room temperature. Consistent with these findings, the pH of silage stored at high

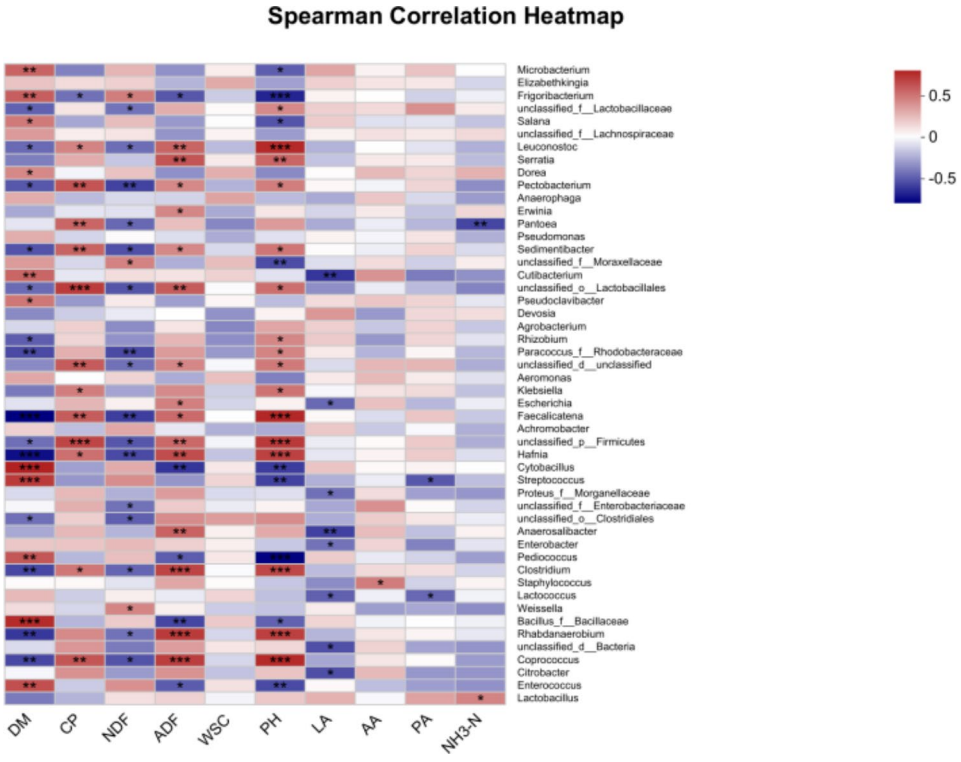

Fig. 5 A correlation heat map was drawn by the correlation coefficients between the dominant microorganisms of topN and environmental factors, effectively illustrating the complex relationship between chemical composition, fermentation quality and bacterial community. The heat map uses a color scheme, with red indicating positive correlation and blue indicating negative correlation. The significance level is shown as follows: $*p \le 0.05$; $P * * 0.01$ or less; $***p \le 0.001$

temperature was lower compared to that stored at room temperature, which aligns with the research conducted by Bai et al. [37]. This observation can be attributed to the inhibition of bacterial growth at 35 °C, limiting the growth of certain bacteria. Concurrently, the metabolic activity of numerous undesirable microorganisms in the raw material is suppressed during anaerobic fermentation, gradually being replaced by LAB, which dominate the fermentation process [38].

Based on the principal coordinate analysis (PCoA), the bacterial diversity of ensiled alfalfa feed fermented at high temperature exhibited distinct separation compared to fermentation at room temperature, indicating a shift in the bacterial population during ensiling. The influence of high temperature on LAB became more evident when additives were employed during ensiling, suggesting varying responses of microbial communities to additives at different temperatures. Notably, the bacterial community supplemented with LP remained relatively stable at high temperature, contrasting with the trends observed for LB and LPLB additions. These alterations in microbial communities could potentially contribute to the disparities in the quality of ensiled feed [39]. To further investigate the impact of different temperatures and additives on the microbial community of ensiled alfalfa feed, an analysis was conducted to assess the dynamic changes in bacterial communities at the genus level.

In the initial alfalfa sample, the genus *Citrobacter*, a Gram-negative facultative anaerobic bacterium, was detected. Subsequent to ensiling fermentation, the dominant genera during the fermentation process were Lactobacillus, *Citrobacter*, and *Enterococcus*. In the absence of LAB additives, the abundance of Lactobacillus was higher in the GCK group compared to the CCK group, indicating more robust growth of Lactobacillus under the 35 °C conditions. Conversely, in the LAB treatment groups, the content of Lactobacillus was lower in the high-temperature treatment group compared to the room temperature treatment group, suggesting a certain influence of temperature on the efficacy of additives, with high temperature inhibiting their effects. Moreover, high-temperature fermentation of ensiled feed suppressed the growth of the genus *Coprococcus*, which actively participates in carbohydrate fermentation during the ensiling process, resulting in the production of butyric acid [40]. Analysis at the bacterial species level revealed that the dominant bacteria in ensiled feed after fermentation were *Lactiplantibacillus plantarym*, *Enterococcus sp*, and *Citrobacter sp*.

In all the treatment groups subjected to high-temperature fermentation in this experimental study, the

abundance of *Enterococcus*, a bacterial genus, was found to be significantly higher compared to fermentation at room temperature. This increase can be attributed to the relatively humid fermentation environment that exists under high-temperature conditions. Previous research has demonstrated the occurrence of *Enterococcus* in high-humidity environments, followed by its subsequent replacement by the more acid-tolerant *Lactiplantibacillus plantarym*, a type of LAB derived from plants [41]. These findings are consistent with the results obtained in this study. During the initial stages of fermentation, *Enterococcus* exhibited rapid production of lactic acid (LA), resulting in a decrease in the pH of the fermentation environment and the creation of an acidic anaerobic environment conducive to the rapid growth of LAB [5]. However, excessive acidity can inhibit the growth of *Enterococcus* [42]. On the other hand, *Lactiplantibacillus plantarym* has the ability to suppress the growth of clostridia and reduce the content of ammonium nitrogen during fermentation, thereby improving the quality of fermentation [43].

In this study, the treatment groups GLP, CLP, GLPLB, and CLPLB, which were inoculated with *Lactiplantibacillus plantarym* and a mixed inoculant, exhibited a significantly higher abundance of *Lactiplantibacillus plantarym*. Similarly, in the CLB treatment group a higher abundance of *Lactiplantibacillus plantarym* was observed. This could be attributed to the fact that *Lentilactobacillus buchneri* enhances the aerobic stability of silage, thereby creating a stable fermentation environment conducive to the growth of *Lactiplantibacillus plantarym*. However, in all the treatment groups that received additives, the abundance of *Lactiplantibacillus plantarym* during high-temperature fermentation was lower than that during room temperature fermentation, indicating a certain inhibitory effect of 35 °C on the growth of *Lactiplantibacillus plantarym*.

Previous studies have demonstrated that *Lactobacillus parabuchneri* can improve the aerobic stability of silage [44, 45]. Higher concentrations of *Lactobacillus parabuchner* were observed in the GLB and CLB treated groups, due to the use of additives. In addition, the abundance of *Lactobacillus parabuchner* during high temperature fermentation was higher than that during room temperature fermentation, indicating that the influence of temperature on *Lactobacillus parabuchner* may be small. Among all the treatment groups, the ones treated with a mixed inoculant demonstrated the best fermentation performance, indicating that the efficacy of a multi-strain inoculant surpasses that of a single-strain inoculant during high-temperature fermentation.MRS Medium is one of the most commonly used media for LAB culture, and the bacteria genera that can be cultured include *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc*. We believe that in the experiment, when the colonies on the medium are counted, not only *Lactobacillus* this bacteria genus, but also other lactobacillus bacteria genera may be included. In the microbial sequencing analysis, the abundance of specific populations was detected, so there may be differences in the number of LAB populations between treatment groups.In the experiment, the relative abundance of lactic acid bacteria was different from the direct microbial count results, which may be due to the interaction of many complex factors. First of all, the microbial sequencing analysis technology adopted in this study has high sensitivity and resolution, which can accurately detect and reflect the abundance changes of different microbial communities. Especially for *Lactobacillus*, sequencing analysis can more accurately reveal their relative abundance. In contrast, the traditional microbial counting method may be affected by the test conditions, such as the selection of medium and the adjustment of culture conditions, which may lead to the growth of miscellaneous bacteria in the process of lactic acid bacteria culture, so that the counting results are not accurate enough. Secondly, in the fermentation process, in addition to the production of lactic acid, it is usually accompanied by the production of other organic acids such as acetic acid, which will reduce the pH value of the fermentation environment.

In this study, the dominant bacterial phyla observed were unclassified bacteria (42%) and *Proteobacteria* (37%), which aligns with previous research findings [46, 47]. However, following the ensiling process, there was an increase in the abundance of the *Firmicutes* phylum across all treatment groups, indicating a transition in the bacterial community from *Proteobacteria* to *Firmicutes*. This shift could potentially be attributed to the heightened prevalence of *Lactobacillus* and *Enterococcus*, known to thrive in the anaerobic environment characteristic of silage fermentation [48].

The introduction of the three types of microbial inoculants resulted in an elevation in the abundance of LAB, with the mixed inoculant demonstrating the most significant impact on the fermentation process. Prior studies have established that the rise in acidity within the fermentation environment subsequent to ensiling facilitates the rapid replacement of *Proteobacteria* by *Firmicutes* [34]. In the present investigation, the group treated with the GLP inoculant exhibited the highest abundance of *Firmicutes*, accompanied by the lowest pH value, thus corroborating this finding.

Silage fermentation constitutes a microbial-driven process crucial to the production of high-quality silage. Correlation analysis has been employed to explore the intricate relationship between chemical composition, fermentation parameters, and microbial communities. Notably, this analysis reveals a consistent pattern wherein

metabolites exhibit positive associations with beneficial microorganisms while displaying negative associations with undesirable bacteria that may emerge during the ensiling process [49]. The findings of this study serve to affirm this established conclusion.

Following a 60-day ensiling period, lactic acid (LA) demonstrated a negative correlation with *Anaerosalibacter* and *Cutibacterium*. *Cutibacterium*, classified as a bacterium within the *Propionibacterium* genus [31], is responsible for the conversion of glucose into propionic acid, rendering it unfavorable in the context of silage. Consistent with previous research, this study observed a positive correlation between NH_3-N and Lactobacillus [50]. Conversely, NH_3-N exhibited a negative correlation with *Pantoea*. It is worth noting that it has been reported that protein degradation can lead to NH_3-N formation when pasture has a high buffering capacity and a low water-soluble carbohydrate content, an observation consistent with the use of alfalfa in this study.Furthermore, research has indicated that an increase in Pantoea abundance possesses the capacity to reduce NH_3-N concentration in silage feed, thus congruent with the outcomes of the present study [51].

Conclusion

The aim of this study was to evaluate the effects of Lactobacillus inoculants on the quality and microbial community of alfalfa silage at different temperatures. The results showed that the increase of temperature in the fermentation process led to the decrease of CP content in alfalfa, but at the same time promoted the formation of acid fermentation environment. In addition, increasing temperatures weakened the diversity of bacterial communities, and microbial populations in high-temperature silage separated significantly. The study found that high temperatures appeared to hinder the growth and proliferation of L. plantarum, and the response of the microbial community to the additive varied with temperature changes.

Based on the relative abundance of microorganisms, it is better to choose mixed bacterials for high temperature silage. From the perspective of silage quality, the choice of Lactobacillus brucei is more appropriate. Considering the relative abundance of microorganisms and the quality of silage, we suggest that appropriate bactericides should be selected according to specific fermentation conditions and objectives to optimize the fermentation effect and nutritional value of silage.

Acknowledgements
We sincerely appreciate the careful review of our manuscript by the editors and reviewers, as well as the valuable feedback they provided. Their expertise and patient guidance have been crucial in supporting our research efforts.

Author contributions
J.Y. L.: Methodology, Conceptualization, Validation, Formal analysis, Writing-Original Draft, Writing-Review & Editing. M.Q.E. Z.: Conceptualization, Investigation, Supervision, Writing-Review & Editing, Resources, Methodology. J.F. H.: Methodology, Validation, Formal analysis. X.Q. Y.: Methodology, Writing-Review & Editing. Z.H. F.: Formal analysis, Writing-Review & Editing. N. Z.: Formal analysis, Validation. Y.S. J.: Conceptualization, Validation. Z.J. W.: Methodology, Formal analysis. G.T. G.: Methodology, Conceptualization, Validation, Writing-Original Draft, Writing-Review & Editing, Supervision, Project administration, Funding acquisition.

Funding
This work was funded by the National Key Research and Development Program of China (No. 2022YFE0111000).

Data availability
Sequencing data for 16 S rRNA gene sequence were stored in NCBI (https://www.ncbi.nlm.nih.gov/) with BioProject accession number PRJNA1063548.

Ethics declarations

Ethics approval and consent to participate
The samples of this study were from the experimental planting base of Grassland and Resources and Environment College of Inner Mongolia Agricultural University. The research complied with all relevant institutional, national and international guidelines and was licensed by the College of Grassland and Resources and Environment, Inner Mongolia Agricultural University.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Received: 5 January 2024 / Accepted: 9 August 2024
Published online: 09 September 2024

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