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Inclusion of Hybrid *Pennisetum* and Probiotics Enhanced Anaerobic Fermentation Quality and Bacterial Diversity of Alfalfa Silage

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Abstract: This study aims to assess the impact of *Bacillus subtilis* (BS) and *Lactobacillus buchneri* (LB) on the fermentation quality, microbial communities, and predicted metabolic pathways in mixed silage made from alfalfa and hybrid *Pennisetum*. We prepared mixed silage from fresh alfalfa and hybrid *Pennisetum* in a 1:1 ratio and inoculated it with BS, LB, or a combination of both (BSLB) or left it untreated as a control. The silage was fermented for 30 and 60 days. The results showed that inoculation with BS, LB, or their combination increased the lactic acid and crude-protein content while reducing the fiber content compared to the control. Additionally, BS and LB inoculation raised ($p < 0.05$) the acetic acid content, and the combination of both strains increased ($p < 0.05$) the ratio of lactic acid to acetic acid. LB alone and the combined inoculation also increased the relative abundance of *Lactobacillus* during the pre-silage period. Functional analysis through the Kyoto Encyclopedia of Genes and Genomes (KEGG) revealed considerable variations among the different probiotic treatments. The silage process reduced nucleotide metabolism but enhanced carbohydrate, amino acid, energy, cofactor, and vitamin nucleotide metabolism. High-throughput sequencing combined with KEGG functional prediction demonstrated significant differences in community composition and functional changes at 30 and 60 days of fermentation. These findings enhance our understanding of bacterial communities and functional changes in mixed silage of alfalfa and hybrid *Pennisetum*, offering valuable insights into the fermentation mechanisms of legume and grass silage and informing practices for producing high-quality mixed silage.

Keywords: *Bacillus subtilis*; mixed silage; probiotic application; microbial communities; functional prediction

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1. Introduction

The rapid growth of the livestock industry and increased demand for animal products have raised the need for animal feed, driving up feed costs and intensifying land competition between human food and livestock feed [1,2]. As a result, there is growing interest in utilizing various forage resources for ruminants [3]. Alfalfa (*Medicago sativa*), a perennial legume known for its high crude-protein content, is widely used in ruminant production [4,5]. However, due to its seasonal harvest and preservation challenges, alfalfa is often ensiled to provide winter fodder [6]. Hybrid *Pennisetum* (*Pennisetum americanum* × *Pennisetum purpureum*), a fast-growing, high-yield, perennial grass, is widely grown in southern China [7,8]. It is adaptable, low-maintenance, and can withstand tough conditions, like drought, flooding, and poor soil quality [9]. While alfalfa's high crude-protein content is beneficial, its high buffer capacity and low carbohydrate content make it difficult to ensile alone. On the other hand, hybrid *Pennisetum* has high soluble carbohydrates but low crude protein, making it less suitable for ruminant growth [10]. However, combining these two forages can solve these issues. Alfalfa's high nutritional value and hybrid *Pennisetum's* carbohydrate content complement each other, improving both the nutritional quality and fermentation process of the silage.

Silage is a common method for preserving feed, and its quality is influenced by factors like forage type, nutrient composition, and processing techniques. Additives are often used to improve fermentation and enhance silage quality and nutritional value [11]. *Lactobacillus buchneri*, a heterofermentative bacterium, is a popular silage inoculant. It produces lactic acid during fermentation and converts it into acetic acid, 1,2-propanediol, and ethanol [12]. Acetic acid, an antifungal compound, helps reduce ethanol production [13] and inhibits secondary fermentation [14]. It also produces bacteriocins that, together with acetic acid, inhibit fungal growth and improve aerobic stability [15]. While *Lactobacillus buchneri* consumes a lot of substrate, reducing nutrient content, it excels at producing acetic acid in later fermentation stages, which helps prevent aerobic spoilage and nutrient loss. Studies by Kristensen et al. [16] and Tabacco et al. [17] showed it improves the aerobic stability of various silages. Some strains of *Lactobacillus buchneri* also produce ferulic acid esterase, which can enhance fiber digestibility [10].

Bacillus subtilis, a Gram-positive bacterium, is known for its high reproductive capacity, low nutrient requirements, and resistance to harsh conditions. It produces digestive enzymes that break down organic matter and metabolic products that enhance silage quality [18]. *Bacillus subtilis* also generates antibiotic substances and bacteriocins that reduce fiber content and improve both aerobic stability and palatability [19]. Bai et al. [20] showed that inoculating corn silage with *Bacillus subtilis* increases soluble carbohydrates and decreases fiber content. *Bacillus subtilis* in alfalfa silage enhances fermentation quality and inhibits protein hydrolysis [21]. However, research shows that adding *Bacillus subtilis* to round bale silage does not improve aerobic stability [22]. There is also limited research on the combined effects of *Bacillus subtilis* and *Lactobacillus buchneri* on silage quality, bacterial diversity, and functional transformation.

This experiment aimed to assess the impact of *Lactobacillus buchneri* and *Bacillus subtilis*, both individually and in combination, on the fermentation quality and bacterial diversity of mixed silage made from alfalfa and hybrid *Pennisetum*. The findings from this study will offer valuable insights for the future production of alfalfa and hybrid *Pennisetum* silage. The hypotheses were as follows: the application of LB, BS, and BSLB achieves high-quality fermentation, decreases nutrient loss, and alters the microflora compared to untreated control.

2. Materials and Methods

2.1. Silage Preparation

Alfalfa was sourced from the Agricultural Meteorological Experimental Station in Dingxi City, Gansu Province (35.47° N, 104.60° E, 2035 m above sea level), which is located in the semi-arid climate zone of the Loess Plateau. The crop was harvested at bud stage of the first cut, with a stubble height of 2–3 cm. Hybrid *Pennisetum* was obtained from the National Engineering Research Center of JUNCAO Technology at Fujian University of Agriculture and Forestry (26.5° N, 119.14° E, 57 m above sea level), situated in a subtropical monsoon climate zone. The crop was harvested at the jointing stage of the first cut, with a stubble height of 8–10 cm. The forage was cut into 10–20 mm lengths, sampled separately for raw-material characterization, and mixed in equal proportions based on fresh weight (FW). After homogeneous mixing, random samples were collected, sealed in bags, and allocated to the respective treatments. Probiotic additives were applied according to the following treatments: (1) equal amounts of sterile water (CK); (2) inoculation with 1.0×10^6 cfu/g of *Bacillus subtilis* (BS, BNCC188080, Beijing Benetronics Institute of Biotechnology, Beijing, China); (3) inoculation with 1.0×10^6 cfu/g of *Lactobacillus buchneri* (LB, BNCC187961, Beijing Benetronics Institute of Biotechnology, Beijing, China); (4) inoculation with 0.5×10^6 cfu/g of *Bacillus subtilis* and *Lactobacillus buchneri* (BSLB). The probiotics were dissolved in 10 mL of sterile water and sprayed onto the fresh forage samples. After thorough mixing, 400 g portions of the silage were packed into polyethylene bags (248 mm × 344 mm). A total of 24 bags were prepared (four treatments × two fermentation durations × three replicates), vacuum-sealed, and placed at room temperature (25 °C ± 1 °C) for fermentation. Samples were collected from each treatment after 30 and 60 days to assess fermentation quality and nutrient content.

2.2. Silage Fermentation Quality and Nutrient Analysis

A 10 g sample from each bag of silage was taken into 90 mL of distilled water, sealed in a refrigerator at 4 °C for 24 h after maceration, and filtered through 4 layers of gauze and qualitative filter paper. Part of the filtrate was taken to determine pH using a pH meter (pHS-3D, Jinan, China), and the other part was filtered through a 0.22 µm filter sieve, partitioned, and stored at −20 °C for the determination of ammonia nitrogen (AN) and organic acid content. Ammonia nitrogen was determined by the phenol-sodium hypochlorite colorimetric method [23], and organic acids were determined by high-performance liquid chromatography (HPLC) [24]. The dry-matter (DM) content of silage was determined by drying the silage at 65 °C for 48 h. The DM content of silage was determined by drying the silage at 65 °C for 48 h. The water-soluble-carbohydrate (WSC) content was determined by anthrone–sulfuric acid colorimetry [25]. Total-nitrogen (TN) and crude-protein content (CP = TN × 6.25) was determined using a Kjeldahl nitrogen analyzer (KDN-103F, Shanghai, China) [26]. NDF and ADF concentrations were measured following the method described by Ke et al. [27]. Lactic acid bacteria (LAB) were counted using MRS medium (Merry Biotechnology Co., Ltd., Fuzhou, China), yeast (Yeast) and mold (Mold) were counted using PDA medium, and aerobic bacteria (AB) were counted using PCA medium.

2.3. Sequencing and Analysis of Silage Bacterial Communities

A 10 g silage sample was taken and mixed with 90 mL of sterile water (sterile phosphate buffer solution, i.e., PBS). The extract was centrifuged at 10,000 rpm for 15 min at 4 °C, and the bacterial precipitate was collected for extraction of total genomic DNA using the E.Z.N.A.® kit (Omega Bio-tek, Norcross, GA, USA). Total genomic DNA was extracted according to the method described by Ling et al. [28] PCR amplification, amplicon purification, and quantification were performed as previously described. PCR products were sequenced on a Misq PE300/NovaSeq PE250 platform according to the protocol of Majorbio (Shanghai, China). The raw sequenced sequences were quality controlled using fastp software (version 0.20.0) and spliced using FLASH software (version 1.2.7). Sequences were OTU clustered and chimeras were excluded based on 97% threshold identity using Uparse software (version 7.1). Sequences were compared and annotated using the Silva 16S rRNA database (v138). Principal coordinate analysis (PCoA) was performed in QIIME (v1.9.1) to assess cluster differences between samples. Communities or species with significant differences between groups were calculated using linear-discriminant-analysis-effect-size (LEfSe) analysis. Communities were predicted in the Kyoto Encyclopedia of Genes and Genomes (KEGG) database by Phylogenetic Investigation of Communities Reconstructed from Unobserved States (PICRUSt2) [29], which predicts the functional abundance of a sample based on the abundance of marker gene sequences in the sample.

2.4. Data Analysis

A two-way ANOVA was performed using a general linear model with fixed effects of days of silage, probiotic agent, and the interaction between the two using SPSS software (version 25.0, Chicago, IL, USA). Differences between means were assessed using Duncan's multiple comparisons, with $p < 0.05$ considered significant and $p < 0.01$ considered highly significant.

3. Results and Discussion

3.1. Characteristics of the Fresh Material Before Ensiling

Table 1 presents the characteristics of fresh alfalfa and hybrid *Pennisetum* before silage. The dry-matter (DM) content was 196.08 g/kg for alfalfa and 164.29 g/kg for hybrid *Pennisetum*. The crude-protein (CP) content, on a DM basis, was 288.60 g/kg for alfalfa and 81.40 g/kg for hybrid *Pennisetum*. Hybrid *Pennisetum* also had a higher WSC content (61.30 g/kg, DM) compared with alfalfa (47.80 g/kg, DM), which is favorable for producing well-preserved silage [30]. The diversity and abundance of microorganisms in the silage feedstocks varied by material and environment. Hybrid *Pennisetum* had fewer lactic acid bacteria (LAB) (10^3 CFU/g, FW) compared with alfalfa (10^5 CFU/g, FW), which is below the minimum effective level for improving silage quality ($<10^5$ CFU/g) and may lead to reduced DM recovery and increased ammonia concentration [31]. The high WSC content in hybrid *Pennisetum* may support the growth of LAB as well as other microorganisms, such as yeasts and molds, which could compete with LAB for available carbon sources, thereby inhibiting LAB growth and resulting in a lower LAB count. Moreover, the higher abundance of yeasts and aerobic bacteria in alfalfa, along with the presence of molds in hybrid *Pennisetum*, suggests that LAB may not dominate the fermentation process rapidly. This highlights the importance of using probiotic additives in mixed silage to ensure effective fermentation. In this study, chemical composition and microbial counts were measured in each forage separately but not in the mixture finally used as the material to be ensiled. Considering the through mixing, it can be reasonably assumed that the composition and microbial counts in the mix would be similar, calculated from those in the individual forages, and that this should not significantly affect the overall assessment of silage fermentation.

Table 1. Characteristics of raw materials of alfalfa and hybrid *Pennisetum*.

Item	Alfalfa	Hybrid *Pennisetum*
pH	6.29	6.18
Dry matter (g/kg, FW)	196.08	164.29
Crude protein (g/kg, DM)	247.95	81.40
Water-soluble carbohydrate (g/kg, DM)	47.80	61.30
Neutral detergent fiber (g/kg, DM)	396.00	652.90
Acid detergent fiber (g/kg, DM)	287.30	353.00
LAB (Log10 CFU/g FM)	5.22	3.29
Yeasts (Log10 CFU/g FM)	4.53	4.11
AB (Log10 CFU/g FM)	6.09	4.42
Mold (Log10 CFU/g FM)	-	2.26

FW, fresh weight; DM, dry matter; LAB, lactic acid bacteria; AB, aerobic bacteria.

3.2. Nutritional Quality of Mixed Silage

Table 2 presents the effects of silage days and additives on the chemical characteristics of mixed silage, including DM, CP, NDF, ADF, and WSC. All groups, except the 60-day BSLB group, showed higher DM content compared to the CK, which is consistent with previous studies [21]. This suggests that different probiotics improved the preservation of DM in mixed silage, likely due to reduced silage pH that inhibits undesirable microbial activity. Silage proteins are frequently hydrolyzed by plant proteases and microbial activities into soluble N and ammonia nitrogen (NH_3-N) [32]; how to better preserve feed proteins is an important issue in silage fermentation. The CP content of mixed silage exhibited a trend similar to that of DM, with probiotic treatments resulting in higher CP levels compared to the control. Notably, the BS group had a significantly higher CP content than the control ($p < 0.05$). This increase is attributed to the enhanced fermentation by *Bacillus subtilis* (BS) and *Lactobacillus buchneri* (LB), which promoted lactic acid bacteria (LAB) activity and organic acid accumulation. This, in turn, inhibits the activity of plant proteases and microorganisms, reducing their consumption of protein from the feed and thereby retaining more crude protein in the silage. A meta-analysis showed that silage treated with LB inoculation had lower WSC content [13]. This is similar to our results, where lower WSC content was observed in all probiotic additives compared to the control group. The BS group exhibited the lowest WSC content, likely due to the proliferation of Enterobacteriaceae and intestinal bacteria, which are known to compete with LAB for the utilization of WSC during silage fermentation [33]. And more WSC was used as a fermentation substrate for Enterobacter. There was a significant interaction between silage days and additive type on WSC content ($p < 0.05$). Probiotic inoculation treatments, with the exception of the BS group at 60 days, resulted in significantly lower NDF content compared to the control ($p < 0.05$). Although ADF content decreased in all treatment groups at 60 days, the reduction was not statistically significant compared to the control ($p < 0.05$). Hybrid *Pennisetum* has a high level of lignin, which can form a complex with hemicellulose to hinder fiber degradation [34]. This may be the reason for the lower level of ADF degradation in this study.

Table 2. Change in nutritional quality in mixed silage.

Items	Days	Treatments				SEM	*p*-Value		
		CK	BS	LB	BSLB		D	T	D × T
DM (g/kg, FM)	30 d	202.30 ^{b}	212.07 ^{a}	204.99 ^{ab}	211.01 ^{Aa}	1.10	0.392	0.006	0.137
	60 d	204.10 ^{b}	213.30 ^{a}	204.77 ^{b}	202.28 ^{Bb}				
CP (g/kg, DM)	30 d	172.60 ^{Ab}	202.38 ^{Aa}	173.00 ^{Ab}	180.82 ^{Ab}	4.64	<0.001	0.001	0.129
	60 d	137.92 ^{Bb}	151.23 ^{Ba}	142.89 ^{Bb}	138.73 ^{Bb}				
NDF (g/kg, DM)	30 d	471.45 ^{Ba}	444.62 ^{Bb}	440.68 ^{Bb}	450.52 ^{Bb}	6.54	<0.001	0.009	0.192
	60 d	518.66 ^{Aa}	514.78 ^{Aab}	506.65 ^{Ab}	502.97 ^{Ab}				
ADF (g/kg, DM)	30 d	318.30 ^{Ba}	298.97 ^{Bb}	293.69 ^{Bb}	302.24 ^{Bb}	5.29	<0.001	0.098	0.155
	60 d	352.86 ^{A}	352.71 ^{A}	351.47 ^{A}	348.06 ^{A}				
WSC (g/kg, DM)	30 d	27.80 ^{Ab}	25.15 ^{Ac}	30.14 ^{Aa}	27.71 ^{Ab}	0.45	<0.001	<0.001	<0.001
	60 d	25.48 ^{Ba}	23.45 ^{Bb}	23.96 ^{Bab}	24.66 ^{Bab}				

CK, control; BS, *Bacillus subtilis*; LB, *Lactobacillus buchneri*; BSLB, *Bacillus subtilis* and *Lactobacillus buchneri*; D, Days; T, Treatment; SEM, standard error of the mean. Different lowercase letters represent significant differences between different treatments ($p < 0.05$). Different capital letters indicate significant differences between different fermentation days under the same treatment ($p < 0.05$).

3.3. Changes in Silage Fermentation Characteristics and Content of Major Microorganisms

Table 3 illustrates the impact of silage days and additives on silage fermentation characteristics and major microbial content. Our findings reveal that both silage days and additives, as well as their interaction, significantly influenced all fermentation characteristics, with the exception of butyric acid (BA) content. Generally, a pH range of 3.6 to 4.2 is ideal for silage, as it effectively reduces the effects of harmful bacteria [35]. In this study, all treatments reduced the pH of mixed silage compared to the control (CK), which correlated with the lactic acid (LA) content observed in each treatment group. The NH_3-N content typically reflects the extent of peptide-bond hydrolysis and the deamination of amino acids or peptides [36]. A lower ratio of NH_3-N to total nitrogen generally indicates better preservation of forage protein. Previous research has demonstrated that *Bacillus subtilis* produces both antifungal and antibacterial substances [37,38], and Lara et al. [39] found that certain strains can improve silage quality after aerobic exposure by generating bacteriocins. In this study, the NH_3-N/TN ratio at 60 days of silage was significantly lower ($p < 0.05$) and the crude-protein (CP) content was significantly higher ($p < 0.05$) in the BS group compared with the other groups. This suggests that the antimicrobial substances secreted by *Bacillus subtilis* contribute to improved fermentation quality and reduced protein hydrolysis, confirming the efficacy of BS in enhancing silage quality. The lower LA content observed in the LB and BSLB groups compared with the BS group at 60 days of silage is likely due to *Lactobacillus buchneri* converting lactic acid into acetic acid, 1,2-propanediol, and ethanol [12]. At both silage time points, the BS group significantly increased LA content ($p < 0.05$) and significantly decreased the lactic acid to acetic acid ratio ($p < 0.05$) compared to the CK group. This suggests that the BS inoculation enhanced homolactic fermentation during the silage process. Additionally, the lactic acid to acetic acid ratio decreased at 60 days compared to 30 days across all treatments, indicating a shift towards more heterolactic fermentation as silage time increased. At 60 days of silage, the AA content in the BS group was significantly higher than in the other groups ($p < 0.05$). This increase may be attributed to *Bacillus subtilis* (BS) enhancing AA content by inhibiting yeasts and improving organic matter digestibility [39]. Additionally, some *Lactobacillus* species can convert 1,2-propanediol into propionic acid (PA) [40], which may explain why the PA content in the LB group was significantly higher ($p < 0.05$) compared with the BSLB group. However, PA concentrations in all groups (6.47, 4.36, 6.22, and 4.77 g/kg DM, respectively) remained within the acceptable level (<10 g/kg DM) [41]. Compared with 30 days, the number of lactic acid bacteria (LAB) decreased in all groups at 60 days, consistent with findings by Wang et al. [42]. This decline may be attributed to the reduction in pH, which inhibited the growth and proliferation of some LAB while affecting other microorganisms.

Table 3. Change in fermentation in mixed silage.

Items	Days	Treatments				SEM	*p*-Value		
		CK	BS	LB	BSLB		D	T	D × T
pH	30 d	4.99 ^{Aa}	4.90 ^{b}	4.87 ^{Ac}	4.88 ^{Ac}	0.02	<0.001	<0.001	<0.001
	60 d	4.89 ^{Ba}	4.84 ^{b}	4.67 ^{Bc}	4.67 ^{Bc}				
NH_3-N/TN	30 d	5.60 ^{Ba}	4.68 ^{Bc}	4.23 ^{Bd}	5.04 ^{Bb}	0.19	<0.001	<0.001	<0.001
	60 d	6.93 ^{Aa}	6.08 ^{Ad}	6.53 ^{Ac}	6.77 ^{Ab}				
LA (g/kg, DM)	30 d	30.73 ^{Ad}	34.02 ^{Ab}	35.29 ^{Aa}	31.68 ^{Ac}	1.17	<0.001	<0.001	<0.001
	60 d	20.37 ^{Bb}	23.24 ^{Ba}	22.58 ^{Ba}	22.52 ^{Ba}				
AA (g/kg, DM)	30 d	1.16 ^{Bc}	1.42 ^{Ba}	1.33 ^{Bab}	1.30 ^{b}	0.04	<0.001	<0.001	<0.001
	60 d	1.41 ^{Ac}	1.77 ^{Aa}	1.69 ^{Ab}	1.34 ^{d}				
LA/AA	30 d	26.43 ^{Aa}	23.99 ^{Ab}	26.54 ^{Aa}	24.41 ^{Ab}	1.18	<0.001	<0.001	<0.001
	60 d	14.50 ^{Bb}	13.12 ^{Bc}	13.35 ^{Bc}	16.76 ^{Ba}				
PA (g/kg, DM)	30 d	6.81 ^{a}	4.63 ^{Ac}	6.66 ^{Aa}	5.60 ^{Ab}	0.19	<0.001	<0.001	0.016
	60 d	6.47 ^{a}	4.36 ^{Bc}	6.22 ^{Ba}	4.77 ^{Bb}				
BA (g/kg, DM)	30 d	0.44 ^{A}	0.72 ^{A}	0.31 ^{B}	0.58 ^{A}	0.06	0.054	0.133	0.002
	60 d	0.13 ^{Bb}	0.06 ^{Bb}	0.70 ^{Aa}	0.49 ^{Ba}				

LA, lactic acid; AA, acetic acid; PA, propionic acid; BA, butyric acid; NH_3-N, ammonia nitrogen; TN, total nitrogen. Different lowercase letters represent significant differences between different treatments ($p < 0.05$). Different capital letters indicate significant differences between different fermentation days under the same treatment ($p < 0.05$).

3.4. Changes in Fermentation Indicators and Major Microorganisms in Mixed Silage After Aerobic Exposure

Table 4 displays the changes in fermentation characteristics and major microorganisms in the mixed-silage fractions after aerobic exposure. The reference method to determine aerobic stability in silage measures the time for a rise in temperature in silage when exposed to air after opening the silo. This was not measured in our study, and thus, the changes in pH, LA, ammonia-N, LAB, yeasts, and aerobic bacteria were used as indicators of the silage deterioration upon aerobic exposure. The pH and NH_3-N content of the silage generally increased across all groups as the duration of aerobic exposure progressed. Significant increases ($p < 0.05$) were observed after nine days of exposure, which coincided with a significant reduction ($p < 0.05$) in LA content. Throughout the study, the pH levels in the probiotic groups were consistently lower than those in the control group (CK), with significant differences ($p < 0.05$). Notably, the BS group exhibited the lowest pH after the completion of aerobic exposure, and its rate of pH change was significantly lower than that of the other groups ($p < 0.05$). This reduction is primarily attributed to the higher lactic acid content in this group. When silage is exposed to oxygen, microorganisms that are normally suppressed by acidic conditions become active. Yeast, as a parthenogenetic anaerobic bacterium, will initiate this process [43]. As the yeast population increases and starts to use lactic acid aerobically, the pH of the silage rises. This change in pH creates a more favorable environment for other aerobic spoilage microorganisms. Consequently, both yeast and aerobic bacteria populations grow as aerobic exposure time increases, as demonstrated in this study (Table 4). NH_3-N levels increased significantly after aerobic exposure, with the BS and LB groups exhibiting lower levels and a significantly slower rate of change compared to the CK group ($p < 0.05$). This suggests that aerobic exposure promotes deamination in silage, likely due to the proliferation of aerobic spoilage bacteria at elevated pH. Probiotic inoculation treatments, excluding the CK group, effectively reduced the levels of yeast and aerobic bacteria (AB). Notably, the BS group had the lowest levels of these microorganisms and the highest LA content. These findings suggest that inoculation with BS more effectively preserves the fermentation quality and microbial structure of silage after aerobic exposure relative to the other treatments. This is consistent with previous studies [44]. This improvement may be attributed to BS's ability to produce antimicrobial metabolites [37].

Table 4. Impact of different inoculation treatments on fermentation characteristics and microbial populations in silage after aerobic exposure.

Items	Treatments	0 Days	9 Days	Slope
pH	CK	4.89 ± 0.01 [a]	9.18 ± 0.01 [a]	0.48 ± 0.00 [b]
	BS	4.84 ± 0.00 [b]	8.43 ± 0.01 [d]	0.40 ± 0.00 [d]
	LB	4.67 ± 0.01 [c]	8.69 ± 0.01 [c]	0.45 ± 0.00 [c]
	BSLB	4.67 ± 0.00 [c]	9.05 ± 0.01 [b]	0.49 ± 0.00 [a]
LA (g/kg, DM)	CK	20.37 ± 0.39 [b]	2.36 ± 0.06 [d]	-2.00 ± 0.04[a]
	BS	23.24 ± 0.80 [a]	13.65 ± 0.59 [a]	-1.07 ± 0.15 [c]
	LB	22.58 ± 0.28 [a]	8.51 ± 0.23 [c]	-1.56 ± 0.05 [b]
	BSLB	22.52 ± 0.51 [a]	9.69 ± 0.44 [b]	-1.43 ± 0.10 [b]
NH_3-N (g/kg, DM)	CK	1.43 ± 0.01 [a]	4.59 ± 0.06 [a]	0.35 ± 0.01 [a]
	BS	1.41 ± 0.01 [b]	3.49 ± 0.02 [b]	0.23 ± 0.00 [b]
	LB	1.38 ± 0.01 [c]	3.05 ± 0.02 [c]	0.19 ± 0.00 [c]
	BSLB	1.45 ± 0.00 [a]	4.53 ± 0.05 [a]	0.34 ± 0.01 [a]
AB (Log_{10} cfu/g FM)	CK	6.26 ± 0.29 [ab]	7.24 ± 0.08 [a]	0.11 ± 0.04
	BS	6.24 ± 0.02 [ab]	7.08 ± 0.06 [ab]	0.09 ± 0.01
	LB	6.17 ± 0.05 [b]	6.93 ± 0.16 [b]	0.08 ± 0.02
	BSLB	6.46 ± 0.03 [a]	7.17 ± 0.11 [a]	0.08 ± 0.01
Yeasts (Log_{10} cfu/g FM)	CK	6.13 ± 0.16 [ab]	8.25 ± 0.07 [a]	0.24 ± 0.01 [a]
	BS	5.88 ± 0.01 [bc]	7.16 ± 0.06 [b]	0.14 ± 0.01 [b]
	LB	5.84 ± 0.07 [c]	7.18 ± 0.40 [b]	0.15 ± 0.04 [b]
	BSLB	6.08 ± 0.02 [a]	8.05 ± 0.02 [a]	0.22 ± 0.00 [a]
AB (Log_{10} cfu/g FM)	CK	6.76 ± 0.09 [a]	8.34 ± 0.07 [a]	0.18 ± 0.01 [ab]
	BS	6.51 ± 0.14 [bc]	7.93 ± 0.06 [b]	0.16 ± 0.02 [b]
	LB	6.43 ± 0.12 [c]	8.28 ± 0.05 [a]	0.21 ± 0.01 [a]
	BSLB	6.67 ± 0.02 [ab]	8.31 ± 0.10 [a]	0.18 ± 0.01 [ab]

Different lowercase letters represent significant differences between different treatments ($p < 0.05$). The slope represents the rate of change for each indicator after aerobic exposure, where positive values indicate increases and negative values indicate decreases.

3.5. Effect of Probiotic Additives on Mixed-Silage Bacterial Communities

The effects of silage duration and additives on the mixed-silage bacterial community were analyzed by clustering OTU sequences at the 3% dissimilarity level (Figure 1A). At 30 days, a total of 525 OTUs were identified, while at 60 days, this number increased to 597 OTUs. Specifically, 117 OTUs were found in the CK group and 142, 133, and 133 OTUs in the BS, LB, and BSLB groups, respectively, at 30 days. At 60 days, the OTU counts were 132 for CK and 149, 146, and 170 for BS, LB, and BSLB groups, respectively, resulting in a total of 66 core OTUs. With the exception of the BS group, the number of core OTUs increased with increasing days of silage. This increase in core OTUs, except in the BS group, could be due to the inhibitory effects of *Bacillus subtilis*.

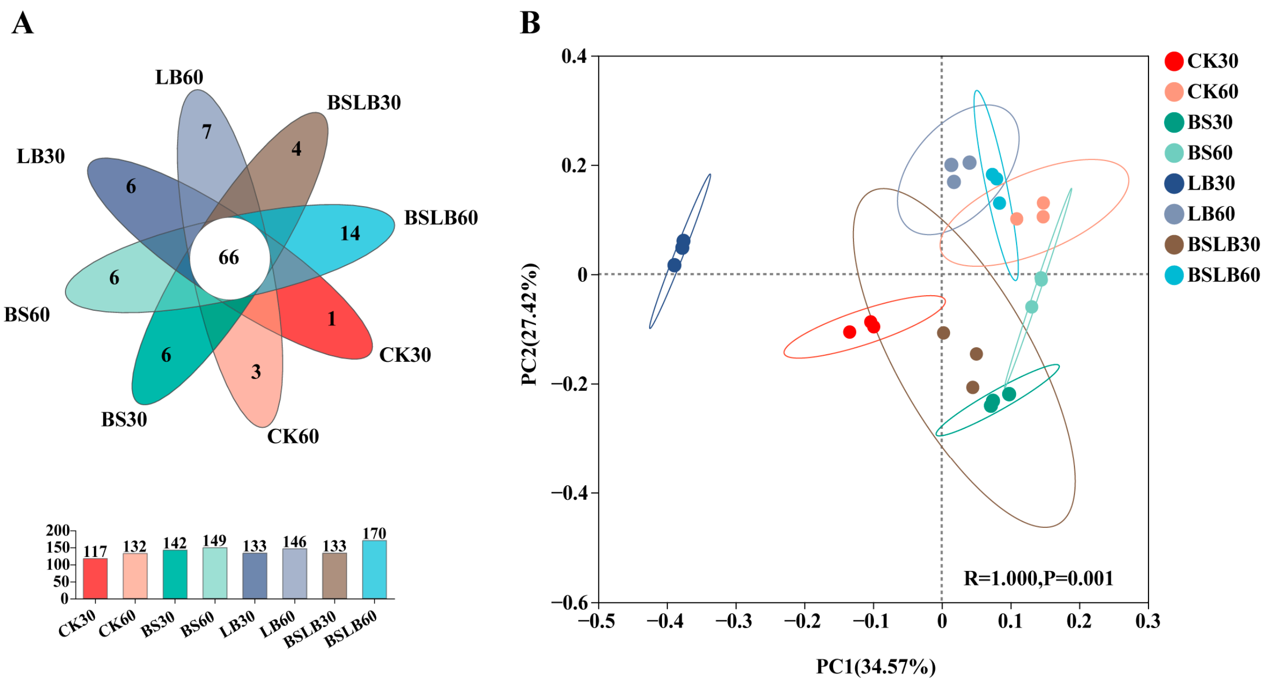

Figure 1. OTU levels of mixed-silage fermentation (**A**) and principal coordinate analysis of bacterial communities at the OTU level (**B**).

3.6. Bacterial Community Diversity in Mixed Silage

Table 5 summarizes Good's coverage and alpha-diversity indices, including Shannon, Simpson, ACE, and Chao1, for the mixed-silage samples. Good's coverage exceeded 0.99 for all samples, suggesting that the sequencing depth sufficiently represents the actual microbial community in the silage samples, thus allowing for a microbial community analysis [45]. Alpha diversity assesses both the abundance of bacterial species and the diversity within a community. This is measured using different indices: the ACE and Chao1 indices focus on bacterial abundance, while the Shannon index reflects the diversity of species [46]. This indicates that, when treated alone or in combination, the BS and LB groups experienced an increase in bacterial community abundance and a decrease in diversity. At 60 days of silage, compared with 30 days, the LB and BSLB groups showed higher species richness, abundance, and diversity, while the BS group had lower species abundance. Additionally, the ACE index was lower in the LB group than in the CK group at 60 days, suggesting that LB inoculation reduced species richness.

PCoA analysis revealed differences between and within groups. The results (Figure 1B) indicated that all treatment groups, except for the BS group, were significantly separated at different days of silage, demonstrating that the duration of silage affects the bacterial community composition. At 30 days of fermentation, the BS, LB, and CK groups were clearly separated, and by 60 days, the BS and LB groups remained distinctly separated. This indicates that the bacterial communities in both the BS and LB groups diverged over time, suggesting that each treatment independently influenced the microbial composition of the silage.

Table 5. Effects of different treatments on alpha diversity of mixed silage.

Items	Days	Treatments				SEM	*p*-Value		
		CK	BS	LB	BSLB		D	T	D × T
Good's coverage	30 d	0.9995 ^{Aa}	0.9992 ^{Ab}	0.9995 ^{Aa}	0.9995 ^{Aa}	0.00	0.137	0.001	0.310
	60 d	0.9995 ^{Aa}	0.9993 ^{Ab}	0.9994 ^{Aab}	0.9993 ^{Aab}				

Table 5. *Cont.*

Items	Days	Treatments				SEM	*p*-Value		
		CK	BS	LB	BSLB		D	T	D × T
Sobs	30 d	73.00 ^{Bb}	86.67 ^{Ba}	84.00 ^{Ba}	82.67 ^{Ba}	2.29	<0.001	<0.001	0.003
	60 d	86.67 ^{Ac}	97.33 ^{Ab}	92.00 ^{Abc}	111.33 ^{Aa}				
Simpson	30 d	0.09 ^{c}	0.16 ^{a}	0.13 ^{Ab}	0.14 ^{ab}	0.01	<0.001	0.092	<0.001
	60 d	0.13 ^{a}	0.10 ^{b}	0.10 ^{Ab}	0.09 ^{b}				
Shannon	30 d	2.63 ^{a}	2.44 ^{c}	2.52 ^{b}	2.44 ^{bc}	0.03	<0.001	0.161	<0.001
	60 d	2.48 ^{c}	2.73 ^{ab}	2.67 ^{b}	2.82 ^{a}				
Chao	30 d	91.21 ^{Bb}	140.91 ^{Aa}	105.27 ^{Bb}	99.69 ^{Bb}	4.18	0.028	<0.001	0.104
	60 d	106.98 ^{Ac}	137.38 ^{Ba}	109.61 ^{Abc}	133.34 ^{Aab}				
Ace	30 d	110.43 ^{Bb}	170.08 ^{Aa}	113.75 ^{Ab}	115.99 ^{Bb}	5.56	0.310	0.001	0.468
	60 d	121.62 ^{Aa}	162.31 ^{Ba}	116.09 ^{Ba}	143.60 ^{Aa}				

Different lowercase letters represent significant differences between different treatments ($p < 0.05$). Different capital letters indicate significant differences between different days under the same treatment ($p < 0.05$).

3.7. Bacterial Community Composition of Mixed Silage

Figure 2A shows the distribution of major microorganisms in mixed silage at the phylum level. Consistent with previous studies [30,47,48], Firmicutes and Proteobacteria were the dominant phyla, with Firmicutes having the highest relative abundance (69.06% to 87.91%). Almost all lactic acid bacteria were classified within the phylum Firmicutes. These bacteria play a crucial role in breaking down biomolecules and produce various enzymes, including lipases, cellulases, and proteases [49], which are vital for generating organic acids like LA and acetic acid (AA) during fermentation. Firmicutes was especially dominant in the 30BSLB silage, where its abundance reached 87.91%. Other prominent phyla included Ascomycetes and Cyanobacteria, with Ascomycetes showing a higher relative abundance in LB silage at 30 d (23.06%) versus 60d (28.99%). Across all treatments, Ascomycetes increased at 60 d compared with 30d, supporting findings from previous studies [45,50,51]. The higher levels of Firmicutes and Proteobacteria contributed to lower pH levels and maintained anaerobic conditions throughout the silage process.

Between 30 and 60 days of silage, *Lactobacillus* was the dominant genus in all groups except 30BS. BS inoculant increased the relative abundances of *Lactococcus* and *Leuconostoc* at 30 and 60 days (Figure 2B,C, respectively), enhancing heterolactic fermentation and AA production, which improves silage resistance to aerobic deterioration [50]. *Lactococcus* abundance decreased from 30 to 60 days, likely due to its growth mainly in the pre-silage period [52]. Both *Enterococcus* and *Lactococcus*, which are capable of fermenting sugars to produce lactic acid, play crucial roles in feed fermentation under anaerobic conditions and can enhance silage quality [53]. However, these bacteria initiate lactic acid fermentation early in the silage process, and Enterococcus is less tolerant to low pH compared to *Lactobacillus* [54]. LB inoculation further reduced *Enterococcus* levels due to lower pH and also increased *Enterobacteriaceae* abundance, contributing to higher acetic acid in the LB group [55]. The higher relative abundance of Enterobacteriaceae observed in the LB group compared with the other groups may account for the increased acetic acid content in this group. Additionally, the relative abundance of *Weissella* in the LB group increased by 9.0% (Figure 2C), suggesting that the antimicrobial capacity of *Weissella* may contribute to the inhibition of undesirable microorganisms in the LB group [56,57].

Differences in bacterial communities among the mixed-silage treatments were analyzed using LEsfe (LAD > 4) (Figure 3). At the phylum level, Firmicutes was predominant in the 30BSLB treatment, while Proteobacteria was more prevalent in the CK60 treatment. At the genus level, the differential species in the BS treatment at 30 days were *Enterococcus* and *Lactobacillus*. The differential species in the LB treatment was *Weissella*. By 60 days, the differential genera in the CK, LB, and BSLB treatments included *Enterobacter*, *Lactobacillus*, and *Pediococcus*.

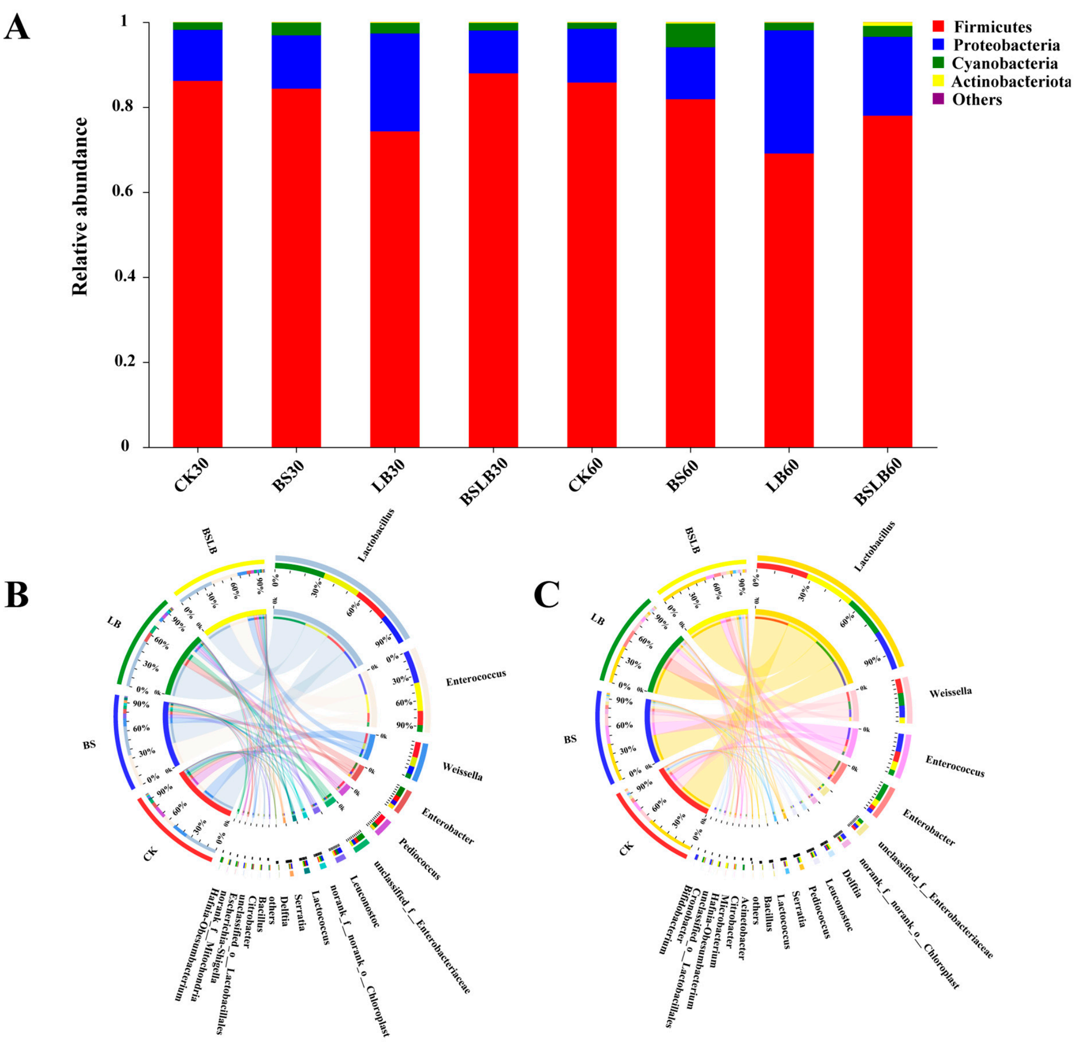

Figure 2. Bacterial community composition of mixed silage at the phylum (**A**) level and Circos plots of the bacterial community of fermentation at 30 d (**B**) and 60 d (**C**) at the genus level.

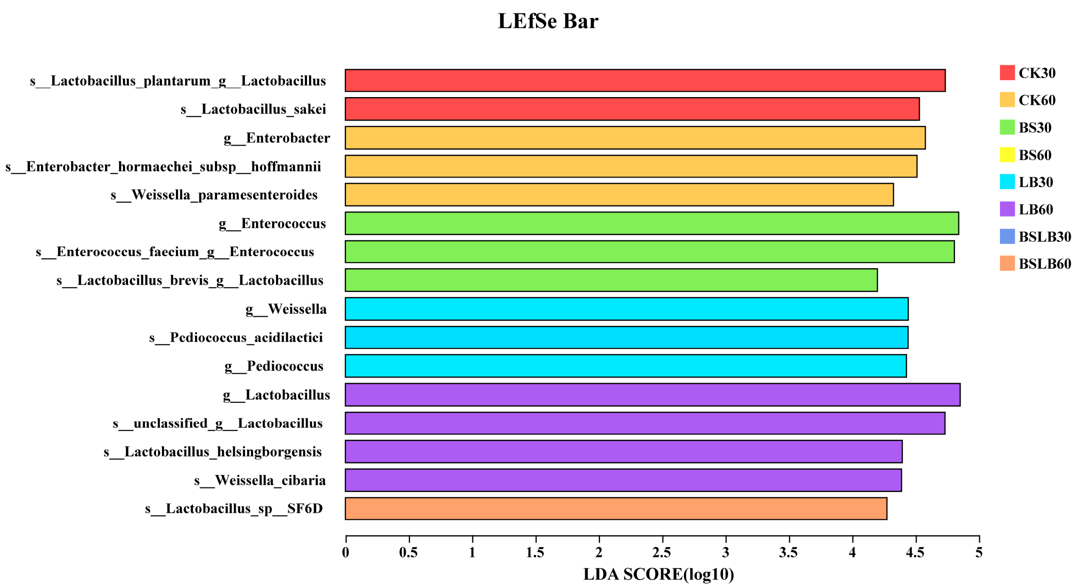

Figure 3. Linear discriminant analysis effect size (LEfSe) of mixed silage of alfalfa and hybrid *Pennisetum*.

3.8. Correlation Analysis Between Mixed-Silage Characteristics and Bacterial Communities

Figure 4 illustrates the correlation between bacterial genera in mixed silage and silage quality. The NH_3-H/TN ratio exhibited a highly significant positive correlation ($p < 0.001$) with the relative abundance of *Delftia* (0.674) and a significant positive correlation with *Weissella* (0.467), *Acinetobacter* (0.458), *Lactobacillus* (0.451), and Citrobacter (0.455) ($p < 0.05$). This suggests that these bacteria may be associated with poorer silage fermentation and may act synergistically, especially given the low relative abundance of bacteria such as *Delftia* and Citrobacter. Conversely, NH_3-H/TN showed a significant negative correlation ($p < 0.01$) with the relative abundance of *Leuconostoc* (−0.550) and significant negative correlations with *Lactococcus* (−0.455) and *Lactobacillus* (−0.455) ($p < 0.05$). Moreover, *Lactococcus* (−0.679) and *Escherichia-Shigella* (−0.650) displayed a highly significant negative correlation. These findings suggest that these genera may either inhibit NH_3-N production or be inhibited by NH_3-N.

The content of NDF showed a significant negative correlation with the relative abundance of *Lactococcus* and a highly significant negative correlation with the relative abundance of *Escherichia-Shigella* ($p < 0.001$). Similarly, ADF content was significantly negatively correlated with *Lactococcus* ($p < 0.05$) and highly significantly negatively correlated with *Escherichia-Shigella* ($p < 0.01$). These correlations may be attributed to the fact that both *Lactococcus* and *Leuconostoc* engage in heterolactic fermentation, which metabolizes various organic compounds to produce acetic acid [58]. This process acidifies the fibers, affecting the NDF and ADF content.

The relative abundance of *Lactococcus* was highly significantly positively correlated with pH (0.776), PA (0.819), and CP (0.754) content ($p < 0.001$). It also showed a significant positive correlation with WSC (0.467) and LA (0.609) content ($p < 0.05$). In contrast, *Lactococcus* abundance was negatively correlated with NH_3-H/TN (−0.679), NDF (−0.490), and ADF (−0.508) content ($p < 0.05$). This may be because the acid production by *Lactococcus* inhibits the degradation of crude protein into ammoniacal nitrogen and the utilization of WSC by undesirable microorganisms while promoting the degradation of crude fiber. Additionally, the relative abundances of *Delftia*, *Bacillus*, and *Hafnia-Obesumbacterium* were highly significantly negatively correlated with pH ($p < 0.001$). Furthermore, *Acinetobacter* and *Cronobacter* also showed significant negative correlations with pH.

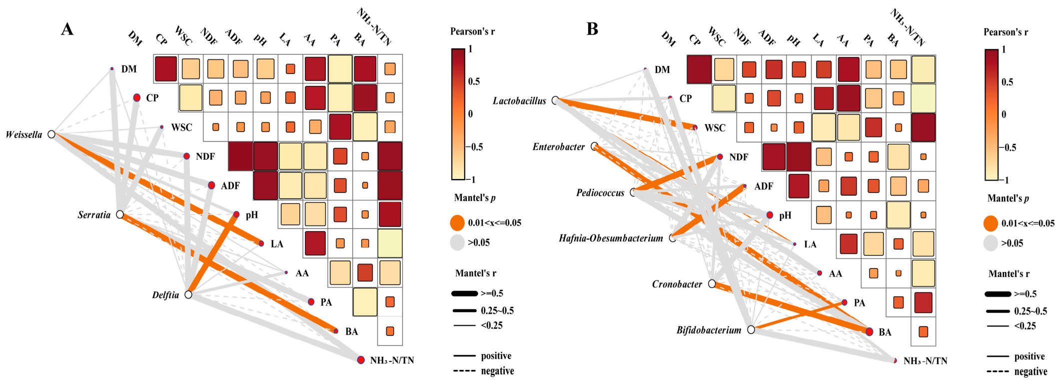

Figure 4. Correlation between bacteria community composition and various indicators in silage feed after 30 (**A**) and 60 (**B**) days of ensiling. The triangles in the upper-right corner represent the correlation between different indicators, and the different colors indicate the Pearson correlation coefficients. The lines in the lower-left corner represent the correlations of bacterial and fungal community compositions, with each indicator obtained through partial Mantel tests. The thicknesses of the lines indicate Mantel's r statistic for the corresponding distance correlation, and the colors of the lines represent statistical significance on the basis of different permutations.

3.9. Functional Prediction of Bacterial Communities During Anaerobic Fermentation of Mixed Silage

Figure 5 illustrates the differences in predicted functions of 16S rRNA genes at the first and second pathway levels. At the first pathway level, the relative abundance of

"Metabolism" was significantly higher compared with other pathways (Figure 5A). This suggests that bacterial activity, through various metabolic pathways, primarily regulates the silage fermentation process by converting fermentable substrates into different metabolites. Probiotic additives enhanced "Environmental Information Processing" and "Cellular Processing" in silage compared to the CK. At the second pathway level within "Metabolism", "Carbohydrate Metabolism" was significantly more abundant than other pathways (Figure 5B). This indicates that carbohydrate metabolism plays a crucial role in affecting fermentation quality. The abundance of "Carbohydrate Metabolism" increased in all groups over time, likely due to carbohydrates being the primary raw material in silage, with their metabolism contributing to a decrease in silage pH. Key energy metabolism pathways in *Lactobacillus* during silage include malate decarboxylation, amino acid decarboxylation, and arginine decarboxylation, which promote the production of lactic acid [2]. In this experiment, all probiotic additive groups enhanced "Energy Metabolism" compared to the CK, which likely explains the increased lactic acid content observed in these groups. Additionally, previous research has shown that LAB inoculants can directly stimulate the production of vitamins, such as α-tocopherol and β-carotene, in silage [59]. Consistent with these findings, the LB group in our study also promoted the production of these vitamins. In the present study, the LB group significantly increased the "Metabolism of Cofactors and Vitamins" compared to the CK. This suggests that LB inoculation enhances the metabolism of cofactors and vitamins in mixed silage of alfalfa and hybrid *Pennisetum*. Additionally, the relative abundance of "Amino Acid Metabolism" increased to varying extents across all additive treatment groups. In contrast, other metabolic pathways showed only minor fluctuations during the silage process. The addition of BS specifically impacted several tertiary metabolic pathways in the ensiled material. Notably, it decreased activity in lipid metabolism, especially in fatty acid biosynthesis, while simultaneously enhancing the metabolism of cysteine, methionine, starch, and sucrose.

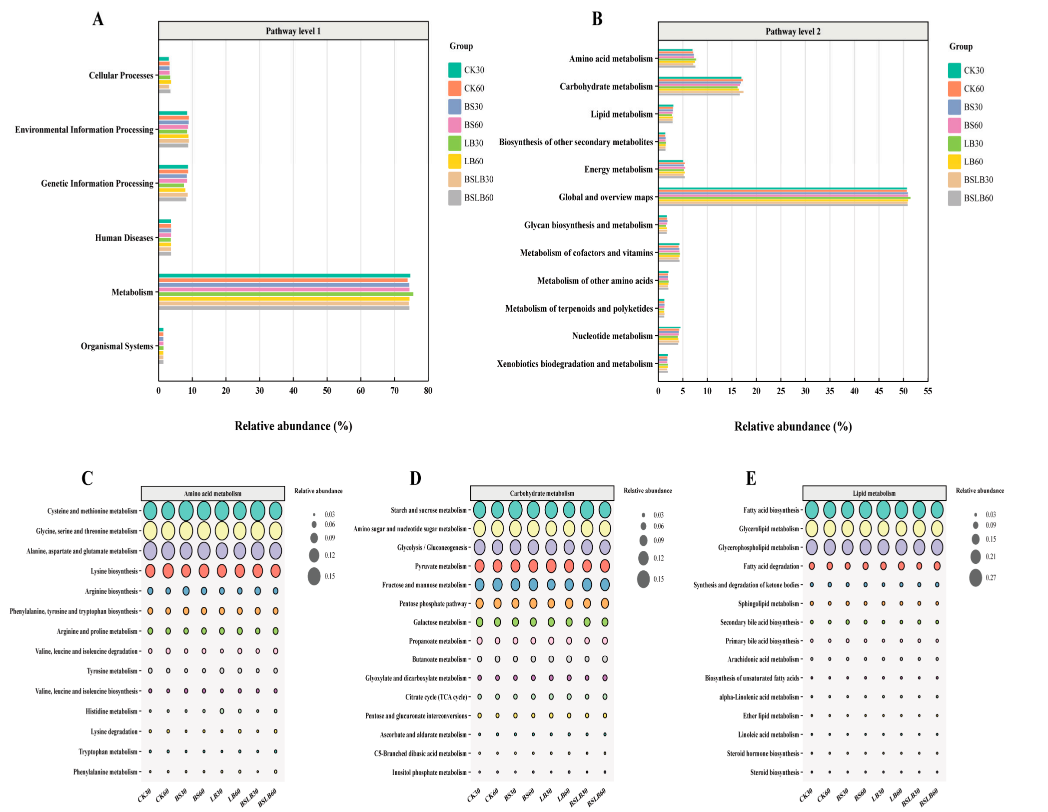

Figure 5. Predicted KEGG metabolic profiles at pathway level 1 (**A**) and pathway level 2 (**B**) based on high-throughput sequencing data. Different colors are used to represent different predicted tertiary metabolic pathways. (**C**) Carbohydrate metabolism; (**D**) lipid metabolism; (**E**) amino acid metabolism.

4. Conclusions

All inoculation groups resulted in higher crude-protein and lactic acid levels while decreasing pH, NDF, and ADF content, thereby reducing nutrient loss during the silage process. BS and LB inoculations increased AA content, whereas the combination of BS and LB elevated the LA/AA ratio, indicating a shift towards homolactic fermentation. This shift ensures improved fermentation quality and stability of the silage. BS inoculation specifically increased the relative abundances of *Enterococcus* and *Leuconostoc*, while LB and the BSLB treatment enhanced *Lactobacillus* levels during the early stages of silage fermentation. These changes improved the bacterial community composition of the mixed silage. Additionally, the addition of various probiotic agents enriched metabolic pathways related to energy metabolism, facilitating the conversion of available sugars into lactic acid through active bacterial colonization. Overall, the use of BS and LB probiotics effectively enhanced the quality of mixed alfalfa and hybrid *Pennisetum* silage, presenting a promising approach for improving the quality of legume and grass silage mixtures.

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