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Inoculation of *Lactobacillus parafarraginis* enhances silage quality, microbial community structure, and metabolic profiles in hybrid *Pennisetum*

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Abstract

Background This study investigated the effects of inoculating *Lactobacillus parafarraginis* alone or in combination with citric acid on the silage quality, microbial community structure, and metabolic characteristics of hybrid *Pennisetum*. The experiment included three treatments: (1) addition of 10 ml distilled water (CON); (2) addition of 1×10^6 cfu/g *L. parafarraginis* (LP); (3) addition of 1×10^6 cfu/g *L. parafarraginis* and 1% citric acid (LCA). The fermentation was maintained at 25 ℃ for 60 days.

Results The addition of *L. parafarraginis* increased the dry matter, water-soluble carbohydrates, and crude protein content of the silage and decreased the fiber contents. Moreover, lactic acid content was notably higher, and pH values were lower in the *L. parafarraginis* group, with higher lactic acid bacteria (LAB) compared with the CON. The microbial community analysis indicated that adding *L. parafarraginis* promoted the proliferation of beneficial LAB and inhibited spoilage bacteria, such as *Clostridium*. In the LCA, amino acid metabolism was improved, particularly with an increase in L-tyrosine concentration, along with significant enrichment of pathways related to tryptophan metabolism.

Conclusions The addition of *L. parafarraginis* improved the fermentation quality of the silage, reduced undesirable microorganisms, and increased the content of organic acids, indicating its potential to enhance the flavor of the silage. Compared with individual treatments, the combination of *L. parafarraginis* and citric acid improved amino acid metabolism and enriched pathways related to tryptophan metabolism, further enhancing the quality of the silage.

†Yijia Liu, Wenqing Ling and Yan Li contributed equally to this work.

DM, Dry Matter; TN, Total Nitrogen; CP, Crude Protein; WSC, Water-Soluble Carbohydrate; NDF, Neutral Detergent Fibre; ADF, Acid Detergent Fibre; NH_3-N, Ammoniacal Nitrogen; LAB, Lactic Acid Bacteria; LA, Lactic Acid; AA, Acetic Acid; PA, Propionic Acid; ITS, Internal Transcribed Spacer.

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These findings highlight the potential of *L. parafarraginis*, especially in combination with citric acid, as an effective additive for producing high-quality, nutritious hybrid *Pennisetum* silage.

Keywords *Lactobacillus parafarraginis*, Hybrid *Pennisetum*, Microbial community, Silage quality

Background

Animal-derived foods are a crucial source of protein for humans and account for 16% of the global food supply. The demand for livestock feed has increased alongside the rapid expansion of the animal husbandry industry, further exacerbating the challenge of balancing the production of livestock feed and food crops [1]. Hybrid *Pennisetum* (*Pennisetum Americanum* × *Pennisetum purpureum*) has shown great potential as a forage crop owing to its high biomass yield [2–3]. However, its preservation and efficient utilization are complicated by several factors, including high water content in fresh hybrid *Pennisetum*, unfavorable climatic conditions for fresh grass storage in major production areas, low water-soluble carbohydrate content, and high lignin content when processed into hay [4–5].

Silage fermentation is a common method for preserving the nutritional value of green forages [6]. However, the complex microbial composition on the surface of fresh forages, characterised by a low proportion of lactic acid bacteria (LAB), can lead to the excessive proliferation of undesirable microbes during fermentation [7]. This results in nutrient loss and compromises silage quality. Moreover, after fermentation, the reintroduction of oxygen promotes the growth of aerobic microorganisms, which consume nutrients, increase silage temperature and pH, and foster the growth of undesirable microbes, leading to mould and rot. These issues result in economic losses and pose food safety concerns [8]. Therefore, minimising nutrient losses during fermentation and subsequently maintaining the silage quality are major challenges in silage production.

To mitigate these issues, additives such as LAB and chemical agents can be used to promote lactic acid (LA) fermentation, lower silage pH, and suppress undesirable microbes, thereby improving silage quality [9–10]. Homofermentative LAB are typically used to enhance fermentation, whereas heterofermentative LAB can produce acetic acid (AA) and 1,2-propanediol during fermentation. In the later stages of ensiling, with both PA and AA can inhibit the growth of undesirable microbes such as yeast after aerobic exposure, thus improving aerobic stability [11–13].

Lactobacillus parafarraginis is a heterofermentative LAB certified as a food additive by the European Union in 2020 [11]. Compared with the commonly used *L. buchneri*, *L. parafarraginis* requires lower temperatures and increases AA production in silage at both 30 °C and 15 °C, whereas *L. buchneri* has a reduced acid-producing capacity at lower temperatures and exhibits inferior aerobic stability at 15 °C [14]. Accordingly, *L. parafarraginis* exhibits a broader application range and is more suitable for overwinter silage storage.

Citric acid is a food additive considered safe and recognized as an antioxidant. Citric acid, as an additive promotes, silage fermentation, inhibits the growth of undesirable microorganisms, and enhances aerobic stability [15]. As an organic acid, citric acid is more environmentally friendly than traditional inorganic acid additives. Therefore, it is widely used in practical silage production. Both *L. parafarraginis* and citric acid can improve silage quality; however, few studies have explored their combined effects on silage microorganisms, fermentation quality, and metabolite changes.

This study aimed to evaluate the individual and combined effects of *L. parafarraginis* and citric acid as silage additives on nutrient composition, fermentation quality, microbial diversity, and metabolite profiles of hybrid *Pennisetum*, with the goal of enhancing silage quality. Our findings provide a theoretical foundation for the production of safe, high-quality hybrid *Pennisetum* silage.

Methods

Feedstock and additives

The hybrid *Pennisetum* used in this study was sourced from the nursery of the China National Engineering Research Centre of JUNCAO Technology at Fujian Agriculture and Forestry University, located in Fuzhou City, Fujian Province, China. Fresh hybrid *Pennisetum* was cut into 1–2 cm sections using a guillotine cutter, mixed thoroughly, spread evenly in a ventilated, cool building, and air-dried overnight. The experiment was divided into the following groups based on the additives: (1) Control (CON): 10 mL distilled water was added to 400 g of hybrid *Pennisetum*; (2) *L. parafarraginis* group (LP): 1×10^6 CFU of *L. parafarraginis* was added per gram of hybrid *Pennisetum*; (3) *L. parafarraginis* and citric acid group (LCA): 1×10^6 CFU of *L. parafarraginis* and 1 g citric acid (1% w/w) were added per gram of hybrid *Pennisetum*. *L. parafarraginis* was purchased from Beijing Yuwei Technology Co., Ltd. The treatment groups had the additives dissolved in distilled water and added up to a total of 10 mL. The treated material was vacuum-sealed in silage bags (24 cm × 35 cm), with each bag containing approximately 400 g of the mixture. Six replicate bags were filled for each treatment, totalling 18 bags for this study. The sealed silage bags were stored at 25 °C and allowed to ferment for 60 days.

Nutritional composition and fermentation index determination

After 60 d of fermentation, six bags from each treatment group were opened, the contents of each bag were mixed well, and 10 g of each bag were removed and added to 90 mL of distilled water and soaked at 4 °C for 24 h. A portion of the extract was filtered through gauze and qualitative filter paper for pH determination, which was determined using a pH meter; another portion of the extract was filtered through a 0.22 μm filter sieve to determine ammoniacal nitrogen (NH_3-N), and organic acid content. Dry matter (DM) was determined by drying the samples at 65 °C for 72 h and then weighing them. Total nitrogen (TN) was measured with an automatic nitrogen analyser (K9840, Hanon, Jinan, China), and crude protein (CP) was calculated as TN × 6.25. Water-soluble carbohydrates (WSC) were determined following the protocol described by Nelson [16]. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) contents were determined following the protocol described by Van Soest et al. [17]. NH_3-N was measured using the phenol–sodium hypochlorite method [18]. Organic acids were quantified using an Agilent 7890 A gas chromatograph (Agilent Technologies Inc., Santa Clara, USA; column: DB-FFAP capillary column, 30 m × 0.32 mm × 0.25 μm, Agilent Technologies Inc., Santa Clara, USA; detector: flame ionization detector, Agilent Technologies Inc., Santa Clara, USA); carrier gas: high-purity nitrogen, constant pressure 100 kPa; temperature program: initial temperature 60 °C, ramped at 12.5 °C/min to 190 °C, held for 1 min; injector temperature: 220 °C, detector temperature: 280 °C; split ratio: 30:1).

Measurement of indicators during aerobic exposure

A portion of silage samples collected from the silage bags opened after 60 d of ensiling were spread on a sterile workbench and covered with a breathable sterile gauze for aerobic exposure. Samples were collected on days 0 and 9 of aerobic exposure for aerobic stability evaluation. pH and LA and ammoniacal nitrogen contents were determined as described above. LAB were cultured using the MRS medium. Yeast was cultured using maltose dipping powder agar (potato dextrose agar (PDA)), and aerobic bacteria were cultured on an agar medium (plate count agar (PCA)). At the end of the incubation, the number of microorganisms was counted. Colony-forming units (CFU) were calculated as the number of microorganisms per gram of fresh matter (FM): microbial count (CFU/g FM) = colony number × dilution factor / sample weight (μL).

Microbial community analysis

Microbial communities in the 60-day silage samples were analysed. Total DNA was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.). The 16 S rRNA gene and internal transcribed spacer (ITS) region were amplified using the following primer pairs: 27 F (5′-AGRGTTYGATYMTGGGCTCAG-3′), 1492R (5′-7CGACTT-3′), ITS1F (5′-CTTGGTCATTTAGAG GAAGTAA-3′), and ITS4R (5′-TCCTCCGCTTATTGA TATATGC-3′). Thereafter, libraries using the amplified products were constructed with the SMRTbell® Express Template Prep Kit 2.0 and sequenced on the PacBio Sequel II System. The raw sequence data were submitted to the National Center for Biotechnology Information under BioProject PRJNA1054343.

Metabolite analysis

Metabolomic analysis of the 60-day silage samples was conducted using LC-MS/MS on an Ultra-High Performance Liquid Chromatography Tandem Fourier Transform Mass Spectrometry (UHPLC-Q Exactive HF-X) system. Chromatographic separation was achieved using an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm; Waters, Milford, USA). The mobile phases were as follows: Solvent A comprised 95% water + 5% acetonitrile (containing 0.1% formic acid), and Solvent B comprised 47.5% acetonitrile + 47.5% isopropanol + 5% water (containing 0.1% formic acid). The column temperature was maintained at 40 °C, with a flow rate of 0.40 mL/min and an injection volume of 3 μL. Raw data were processed using Progenesis QI (Waters Corporation, Milford, USA) to generate data matrices, including retention times, mass-to-charge ratios, and peak intensities. Metabolite identification was performed by comparing the data against public metabolic databases, including the Human Metabolome Database (http://www.hmdb.ca/) and Metlin (https://metlin.scripps.edu/).

Statistical analysis

Data were organised using Excel, and a two-factor analysis of variance was used to assess the significance of fermentation time, treatment effects, and their interactions using SPSS 20.0 software. Multiple comparisons were conducted using Duncan's method, and the results are expressed as the mean and SEM.

Spearman correlation analysis was performed between the bacterial and fungal communities, key quality indices, and metabolite contents in hybrid *Pennisetum* silage.

Results

Nutrient and fermentation characteristics

The pH of fresh hybrid *Pennisetum* was 5.73, and the DM, WSC, and CP were 173.02, 78.47, and 101.20 g/kg, respectively. Before ensiling, the average ADF and NDF fractions of hybrid *Pennisetum* were 358.32 and 664.50, respectively, in the present study, comparable to the values reported previously. Compared with the CON,

Table 1 Characteristics of hybrid *Pennisetum* before and after ensiling for 60 D

Item[1]	After ensiling[2]			SD[3]	SEM[4]	*P* value[5]
	CON	LP	LCA			
pH	4.14	3.72	3.68	0.23	0.05	**
LA (g/kg DM)	9.97	15.93	16.09	3.4	0.80	**
AA (g/kg DM)	1.66	2.06	0.97	0.61	0.16	**
PA (g/kg DM)	0.79	1.03	0.29	0.40	0.10	**
BA(g/kg DM)	1.57	0.62	0.69	0.48	0.12	***
NH_3-N (g/kg DM)	10.31	7.26	7.27	1.6	0.37	**
DM (g/kg)	155.01	169.05	166.17	7.4	1.75	**
WSC (g/kg DM)	6.32	8.39	7.04	1.8	0.42	0.12
CP (g/kg DM)	67.29	76.67	71.06	4.5	1.05	**
NDF (g/kg DM)	552.35	529.66	530.11	12.5	2.93	**
ADF (g/kg DM)	328.99	279.93	291.57	27.2	6.40	**

[1]DM, dry matter

[2]CON, distilled water; LP, *L. parafarraginis*; LCA, *L. parafarraginis* and citric acid

[3]SD, Standard Deviation

[4]SEM, standard error of means

[5]***: $P < 0.001$;**: $P < 0.01$;*: $P < 0.05$

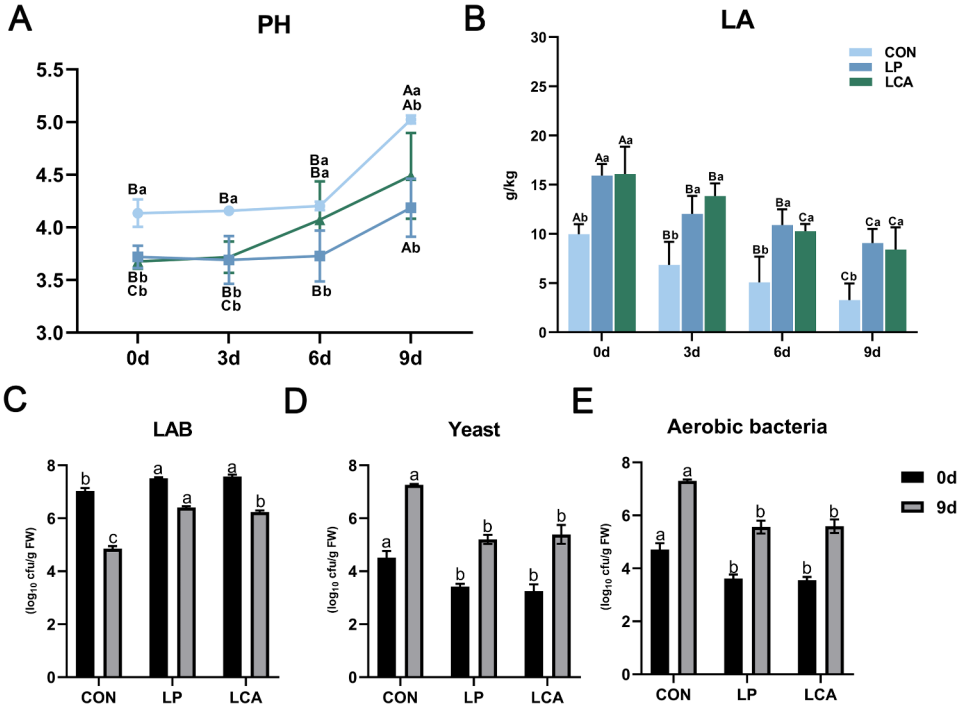

Fig. 1 Changes in quality of hybrid *Pennisetum* silage under aerobic conditions. (**A**) Changes in pH under aerobic conditions. (**B**) Changes in lactic acid (LA) under aerobic conditions. (**C-E**). Microbial (lactic acid bacteria [LAB], yeast, and aerobic bacteria) changes under aerobic conditions. a-c: Different lowercase letters in the same column indicate significant differences between different treatment groups at the same time point ($P < 0.05$). A-C: Different uppercase letters indicate significant differences between different time points in the same treatment group ($P < 0.05$)

after fermentation, ADF and NDF concentrations significantly reduced to 278.93, 291.57 and 529.66, 530.11, respectively, along with increased DM, WSC, and CP in all treatment groups. LP had greater CP, WSC concentrations and lower ADF and NDF concentrations than CON and LCA (Table 1).

LA content was notably higher in both the LP and LCA compared with CON. The LCA exhibited slightly higher LA levels than the LP. AA and PA contents were highest in the LP, followed by the CON, and lowest in the LCA.

Feed quality changes under aerobic conditions

The pH of all treatments increased with prolonged aerobic exposure (Fig. 1A). The pH of LP and LCA was lower ($P < 0.01$) than CON on days 0 and 3 of aerobic exposure. The pH of LP remained lower than CON ($P = 0.005$) and

LCA ($P = 0.033$) by day 6 and lower than CON ($P < 0.01$) by day 9 of aerobic exposure with LCA intermediate.

LA content decreased with increasing days of aerobic exposure across all treatments (Fig. 1B). LA concentrations of LP and LCA remained higher ($P < 0.001$) than CON throughout aerobic exposure. Further, the LA concentrations decreased with advancing days of aerobic exposure across treatments.

The LAB content in the silage declined with aerobic exposure. By day 9, LP retained the highest LAB content, whereas that in the CON was the lowest. Conversely, the populations of yeasts and aerobic bacteria in all groups exhibited an increasing trend during aerobic exposure, and the CON had the highest (Fig. 1C-E) ($P < 0.01$).

Microbial ecosystem of hybrid *Pennisetum* silage

Analysis of the microbial ecosystem in hybrid *Pennisetum* silage revealed significant changes in bacterial α-diversity. The ACE and Chao1 diversity indices were significantly lower ($P < 0.05$) in LP compared to LCA, with the CON showing intermediate values (Table A.1). The bacterial composition of the silage is depicted in Fig. 2A-C. At the species level, the dominant taxa in the CON were *Clostridium guangxiense* (26.48%), *Levilactobacillus brevis* (12.58%), *Lactiplantibacillus plantarum* (10.64%), *Rahnella aquatilis* (9.03%), and unclassified *Raoultella* (8.70%). In the LP, the dominant species were *Lentilactobacillus parafarraginis* (36.46%), *L. plantarum* (34.80%), *L. brevis* (12.93%), and *Furfurilactobacillus rossiae* (7.74%). The dominant species in the LCA were *L. plantarum* (52.28%), *Paucilactobacillus vaccinostercus* (14.74%), *Lactococcus lactis* (5.93%), and *C. guangxiense* (5.30%). Notably, the relative abundance of *L. plantarum* and *Lentilactobacillus parafarraginis* in LP and *L. plantarum* in the LCA increased ($P < 0.05$), whereas that of *C. guangxiense* decreased ($P < 0.05$).

Fungal abundance and diversity were higher in the inoculated groups than in the CON (Table A.2). After 60 days of silage (Fig. 2D-F), the dominant fungal species in the CON were *Erythrobasidium hasegawianum* (12.68%), unclassified *Erythrobasidium* (12.47%), *Sporobolomyces koalae* (4.72%), and unclassified *Cryptococcus* (4.26%). In the LP, the dominant fungal species were *Apiotrichum domesticum* (10.10%), *Saitozyma paraflava* (7.54%),

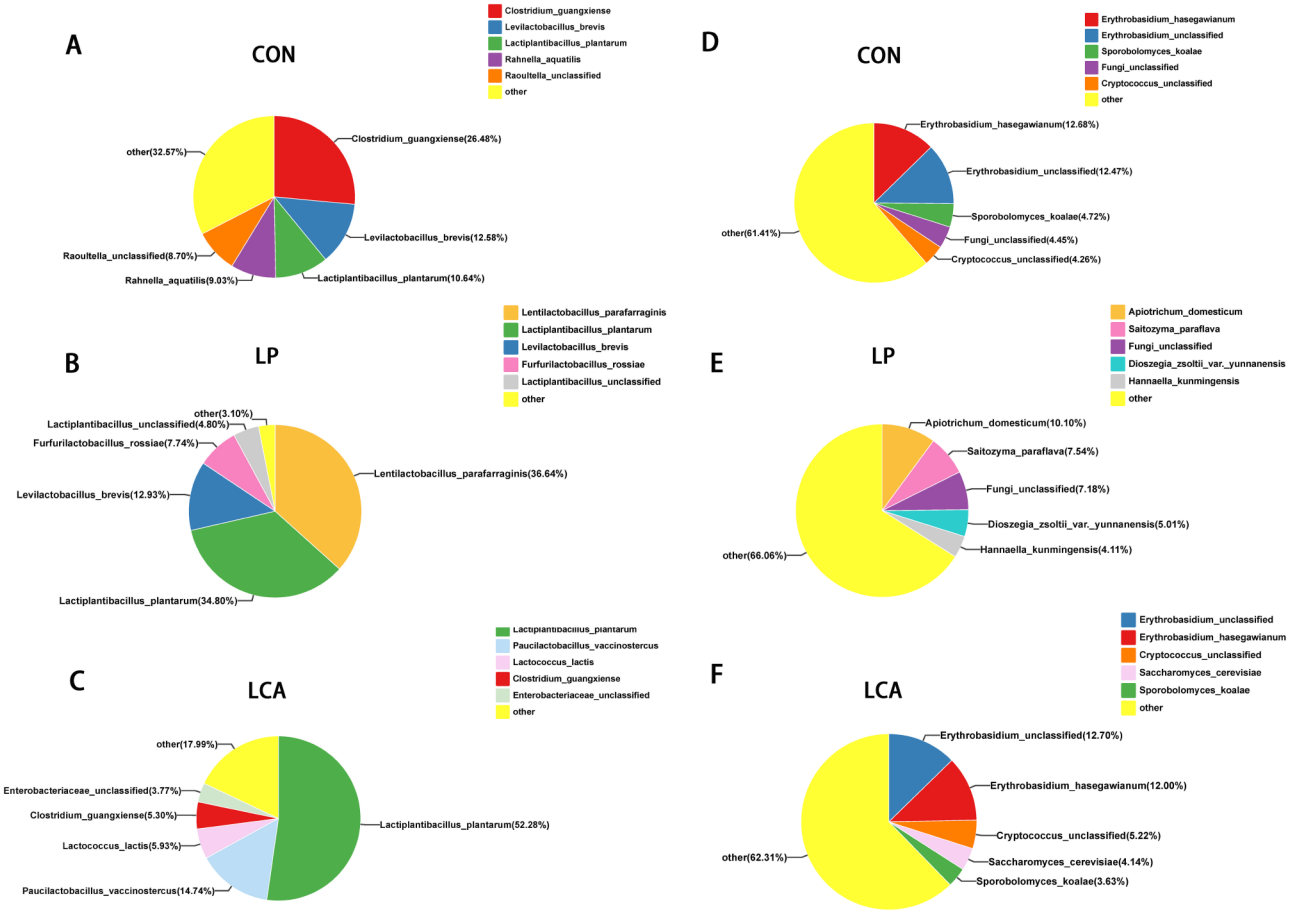

Fig. 2 Microbial community composition of hybrid *Pennisetum* ensiling. (**A-C**) Bacterial abundance at the species level. (**D-F**) Fungal abundance at the species level

Dioszegia zsoltii var. *yunnanensis* (5.01%), and *Hannaella kunmingensis* (4.11%). In the LCA, the dominant fungal taxa were unclassified *Erythrobasidium* (12.70%), *E. hasegawianum* (12.00%), unclassified *Cryptococcus* (5.22%), *Saccharomyces cerevisiae* (4.14%), and *S. koalae* (3.63%). In terms of fungal community composition, the LCA exhibited greater similarity to the CON than the LP.

Metabolomic profiles of the hybrid *Pennisetum* silage

LC-MS/MS analysis identified 2,712 metabolites in the hybrid *Pennisetum* silage (Fig. 3A). According to KEGG compound classification, these metabolites were categorised into nine functional groups, including organic acids, lipids, carbohydrates, and others, with carboxylic acids (a subset of organic acids) being the most abundant (Fig. 3B). Principal component analysis revealed the presence of three distinct clusters, indicating significant variation in the metabolomic profiles across treatments (Fig. 3C).

A comparative analysis of differential metabolites among the three treatment groups highlighted several notable changes (Fig. 3D). In both the LP and LCA, concentrations of lipids, organic acids, peptides, and nucleic acids were higher compared with the CON. Conversely, the levels of carbohydrates, nucleic acids, and certain peptides (such as S-adenosylmethioninamine, pyroglutamic acid, and D-ala-D-ala) were lower in the LP and LCA.

Owing to the importance of organic acids, peptides, and carbohydrates in determining silage quality, these

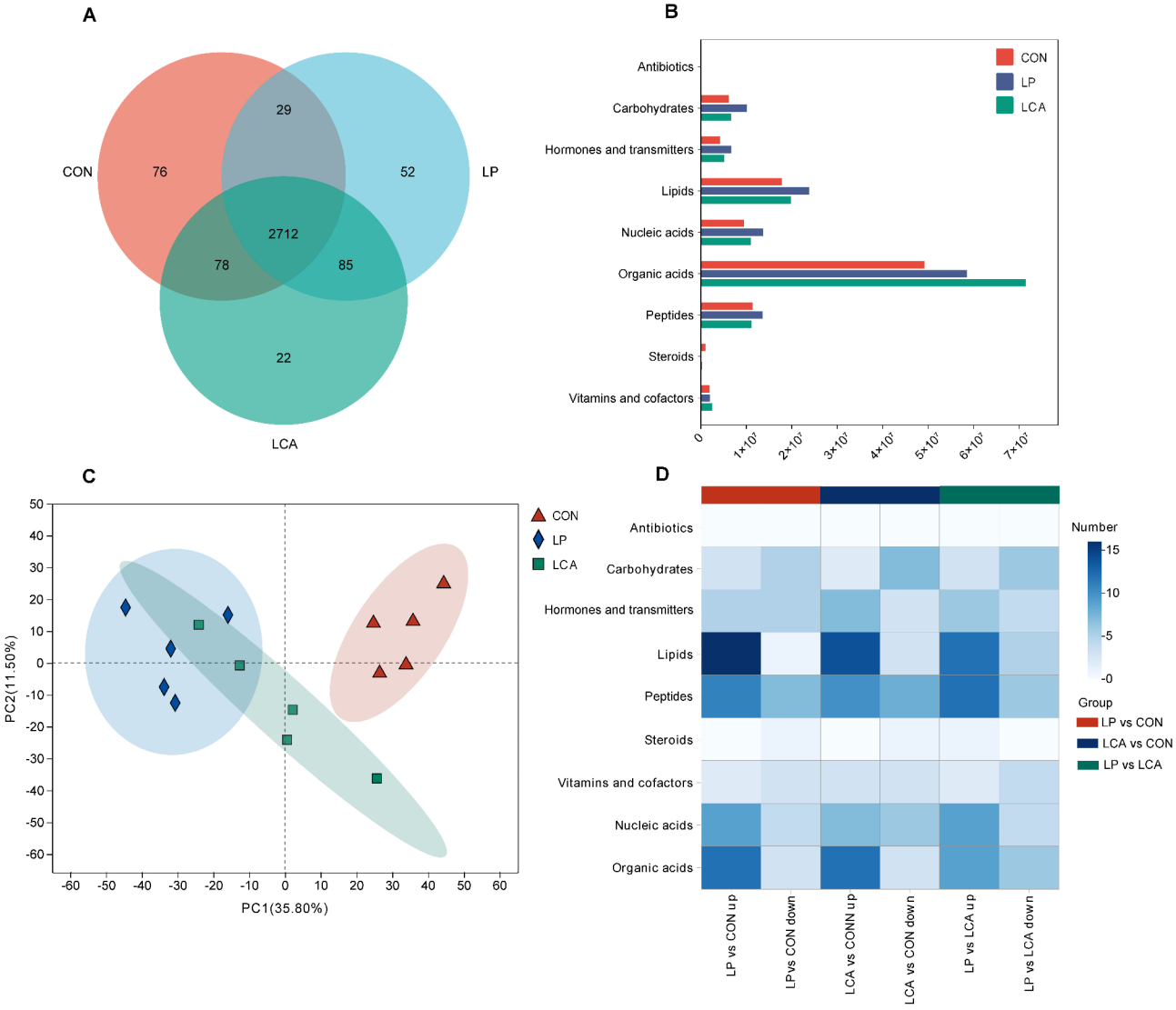

Fig. 3 Metabolomic analysis of hybrid *Pennisetum* silage. **(A)** Venn diagram of metabolites. **(B)** Number of different types of metabolites. **(C)** Principal component analysis (PCA) of metabolic profiles. **(D)** Differential metabolite heatmap, with up-regulation indicated by upward arrows and down-regulation by downward arrows

Table 2 Differential carboxylic acid of ensiled hybrid *Pennisetum* with different treatments

Metabolite	Category	Treatments[a]			log_2 FC[b]		
		CON	LP	LCA	LP/CON	LCA/CON	LP/LCA
Succinic Acid	Carboxylic acids	40,814,130	44,909,271	60,019,032	0.14	0.56	-0.42
Oxalic acid	Carboxylic acids	4,937,581	5,846,775	5,902,645	0.24	0.26	-0.01
Trans-Aconitic Acid	Carboxylic acids	1,761,668	4,149,902	3,100,001	1.24	0.82	0.42
Isocitric Acid	Carboxylic acids	488,110	1,982,103	819,704	2.02	0.75	1.27
Malonic Acid	Carboxylic acids	250,246	503,930	330,263	1.01	0.40	0.61
Glutaric Acid	Carboxylic acids	345,495	267,470	373,314	-0.37	0.11	-0.48
Suberic Acid	Carboxylic acids	101,702	163,390	134,944	0.68	0.41	0.28
Citric Acid	Carboxylic acids	79,043	89,953	89,506	0.19	0.18	0.01
3-Methyl-2-Oxovaleric Acid	Carboxylic acids	103,506	92,511	187,274	-0.16	0.86	-1.02
Adipic Acid	Carboxylic acids	61,076	67,773	94,502	0.15	0.63	-0.48
Heptanoate	Carboxylic acids	39,692	54,457	61,043	0.46	0.62	-0.16
Oxoglutaric acid	Carboxylic acids	16,464	15,698	5088	-0.07	-1.69	1.63
Malic Acid	Carboxylic acids	3092	2994	1320	-0.05	-1.23	1.18
Caproic acid	Carboxylic acids	125,844	298,035	257,585	1.24	1.03	0.21
Butyric Acid	Carboxylic acids	11,615	13,767	11,172	0.25	-0.06	0.30

[a]CON, control; LP, *L. parafarraginis*; LCA, *L. parafarraginis* and citric acid

[b]FC, fold-changes. Positive value means upregulation; negative value means downregulation

Table 3 Differential carbohydrates of ensiled hybrid *Pennisetum* with different treatments

Metabolite	Category	Treatments[a]			log_2 FC[b]		
		CON	LP	LCA	LP/CON	LCA/CON	LP/LCA
D-Gluconic acid	Monosaccharides	4,524,197	4,200,096	4,423,689	-0.11	-0.03	-0.07
L-Erythrulose	Monosaccharides	141,899	204,581	116,061	0.53	-0.29	0.82
N-Acetylneuraminic acid	Monosaccharides	212,066	180,010	141,675	-0.24	-0.58	0.35
N-Acetylmannosamine	Monosaccharides	657,676	354,204	442,608	-0.89	-0.57	-0.32
Deoxyribose	Monosaccharides	183,595	139,206	174,727	-0.40	-0.07	-0.33
D-Mannitol	Monosaccharides	159,053	3,968,746	1,135,862	4.64	2.84	1.80
Rhamnose	Monosaccharides	60,594	27,919	44,069	-1.12	-0.46	-0.66
N-Acetyl-D-galactosamine	Monosaccharides	44,584	22,478	27,080	-0.99	-0.72	-0.27
D-Tagatose	Monosaccharides	8490	22,508	3917	1.41	-1.12	2.52
Neuraminic acid	Monosaccharides	1610	7263	0	2.17	-	-
D-Sorbitol	Monosaccharides	1086	516,512	111,499	8.89	6.68	2.21
Trehalose	Oligosaccharides	46,744	140,800	11,920	1.59	-1.97	3.56
Sucrose	Oligosaccharides	73,170	293,408	2953	2.00	-4.63	6.63

[a]CON, distilled water; LP, *L. parafarraginis*; LCA, *L. parafarraginis* and citric acid

[b]FC, fold-changes. Positive value means upregulation; negative value means downregulation

metabolite categories were examined in greater detail. LP exhibited increased levels of various organic acids, including succinic acid, oxalic acid, trans-aconitic acid, isocitric acid, malonic acid, suberic acid, citric acid, adipic acid, heptanoate, caproic acid, and butyric acid (BA) compared with the CON (Table 2). In the LCA, all of these organic acids, except for malic acid and BA, were present at higher concentrations than the CON.

In the LP, *L. parafarraginis* inoculation elevated the levels of carbohydrates, such as D-mannitol, D-sorbitol, alginate, and sucrose (Table 3). The LP exhibited increased concentrations of L-alanine, N-formyl-L-methionine, L-glutamine, L-arginine, 4-hydroxyproline, L-serine, aspartic acid, L-aspartic acid, L-glycine, and L-dopa than the CON. However, pyroglutamic acid, D-ala-D-ala, citrulline, L-tyrosine, and L-glutamic acid were downregulated in the LP (Table 4). Interestingly, the amino acid profile in the LCA generally showed lower concentrations than the CON, except for L-tyrosine, which was upregulated compared with the LP.

Pathway enrichment analysis revealed that most detected metabolites were involved in metabolic pathways, with amino acid metabolism being the most prominent (Fig. 4). Compared with the CON, 'linoleic acid metabolism' was the most enriched pathway in the LP, with seven differential metabolites annotated to this pathway ($P < 0.05$) (Fig. 5A). In the LCA, the most enriched pathways were 'tryptophan metabolism' (with seven differential metabolites) and 'linoleic acid metabolism' (with four differential metabolites) compared with

Table 4 Differential amino acids of ensiled hybrid *Pennisetum* with different treatments

Metabolite	Category	Treatments[a]			log_2 FC[b]		
		CON	LP	LCA	LP/CON	LCA/CON	LP/LCA
Pyroglutamic Acid	Amino acids	3,477,355	3,189,024	2,697,421	-0.12	-0.37	0.24
L-Alanine	Amino acids	2,575,054	2,670,858	2,481,429	0.05	-0.05	0.11
N-Formyl-L-Methionine	Amino acids	118,885	270,103	232,139	1.18	0.97	0.22
L-Glutamine	Amino acids	19,849	330,428	116,169	4.06	2.55	1.51
L-Arginine	Amino acids	12,995	167,648	107,600	3.69	3.05	0.64
D-Ala-D-Ala	Amino acids	191,542	66,257	109,985	-1.53	-0.80	-0.73
Citrulline	Amino acids	75,647	19,904	29,763	-1.93	-1.35	-0.58
4-Hydroxyproline	Amino acids	13,245	19,658	19,373	0.57	0.55	0.02
L-Tyrosine	Amino acids	9870	3709	13,936	-1.41	0.50	-1.91
L-Serine	Amino acids	11,537	27,590	56,736	1.26	2.30	-1.04
L-Proline	Amino acids	0	119	0	-	-	-
Aspartic Acid	Amino acids	1,127,605	3,376,890	1,784,171	1.58	0.66	0.92
L-Aspartic Acid	Amino acids	306,055	1,210,584	752,899	1.98	1.30	0.69
L-Glutamic Acid	Amino acids	759,595	518,384	508,148	-0.55	-0.58	0.03
L-Glycine	Amino acids	170,524	231,771	215,075	0.44	0.33	0.11
L-Dopa	Amino acids	180,367	193,826	182,396	0.10	0.02	0.09
S-Adenosylmethioninamine	Amines	1,059,448	428,147	501,711	-1.31	-1.08	-0.23
Serotonin	Amines	264,392	128,201	285,456	-1.04	0.11	-1.15
Dopamine	Amines	996,521	702,878	1,074,943	-0.50	0.11	-0.61
Histamine	Amines	8904	22,917	10,601	1.36	0.25	1.11

[a] CON, distilled water; LP, *L. parafarraginis*; LCA, *L. parafarraginis* and citric acid

[b] FC, fold-changes. Positive value means upregulation; negative value means downregulation

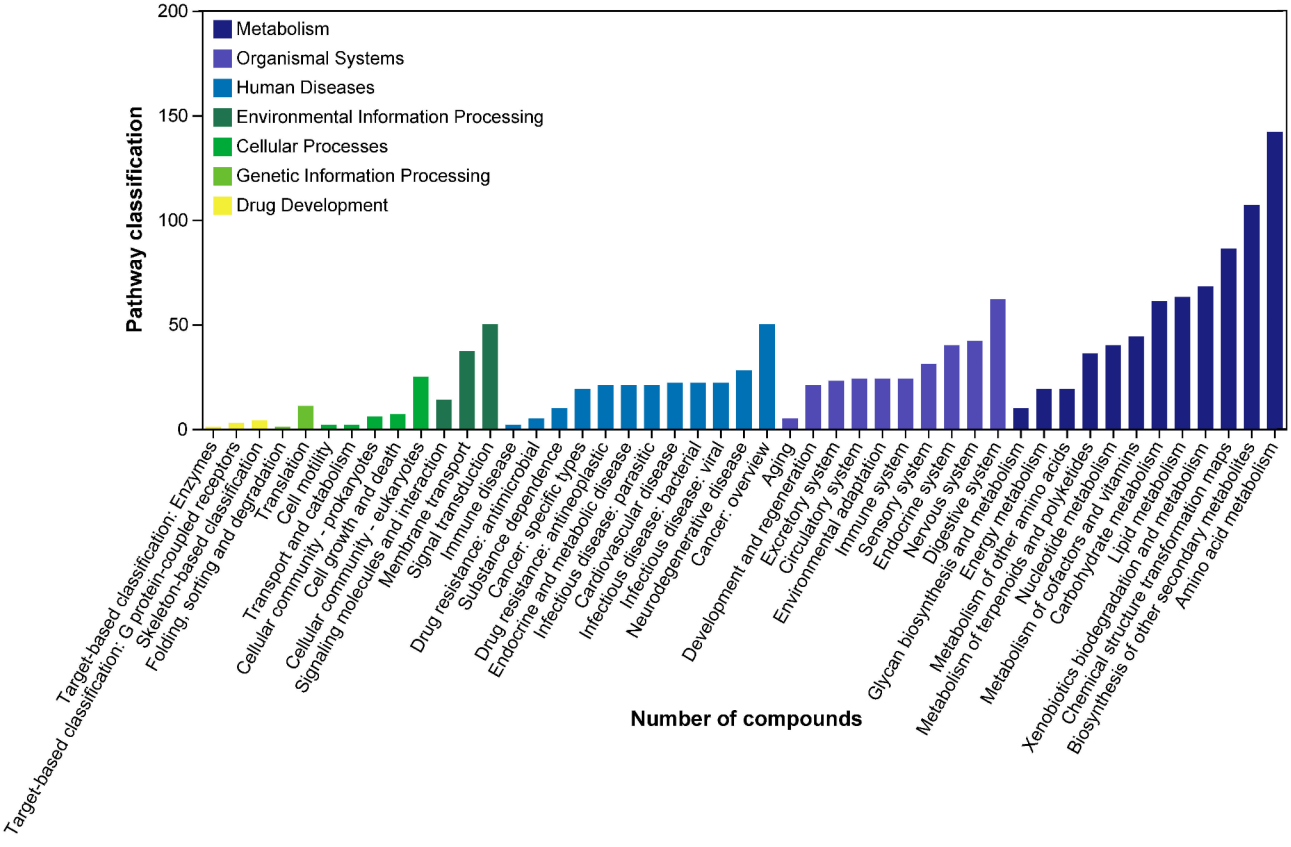

Fig. 4 Metabolite enrichment analysis

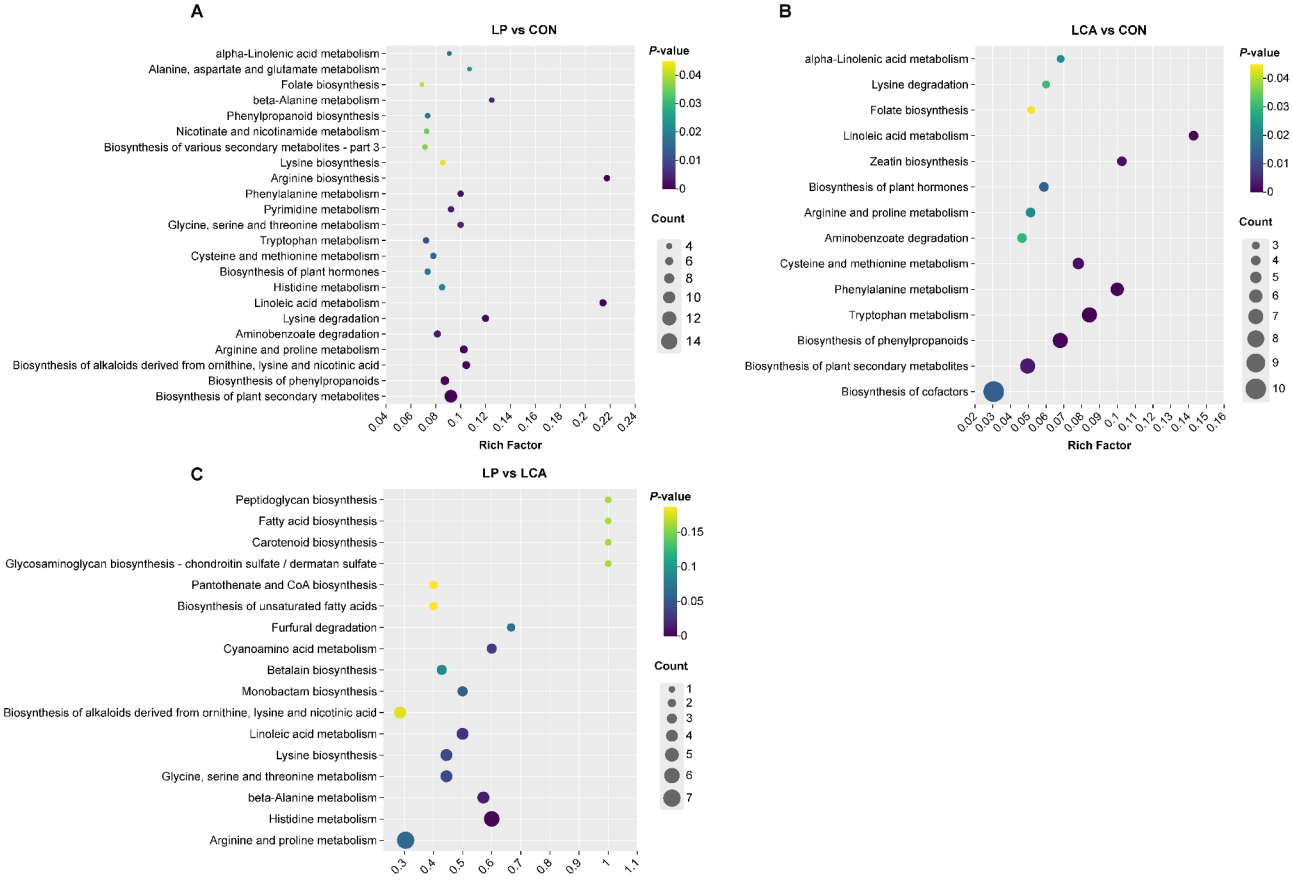

Fig. 5 Metabolite enrichment pathway analysis. **(A)***L. parafarraginis* versus CON. **(B)** Comparison of the mixed additions of *L. parafarraginis* and citric acid with CON. (**C**) Comparison of the *L. parafarraginis* alone and *L. parafarraginis* with citric acid mixed addition groups. Count (dot) represents the number of metabolites annotated to pathways

the CON ($P<0.05$) (Fig. 5B). When comparing LP with LCA, the main enriched pathways in LP were 'histidine metabolism' (six differential metabolites), 'beta-alanine metabolism', 'linoleic acid metabolism', 'glycine, serine, and threonine metabolism', 'lysine biosynthesis' (four differential metabolites), and 'cyanoamino acid metabolism' (three differential metabolites) ($P<0.05$) (Fig. 5C).

Correlation of metabolites with silage indicators and microbial communities

In silage fermentation, the activities of microorganisms are reflected in terms of macro-level silage quality indicators. DM and WSC contents were negatively correlated with the concentration of 4-pentenoic acid ($P<0.05$) (Fig. 6). Conversely, BA content exhibited a positive correlation with 4-pentenoic acid ($P<0.05$). Further analysis revealed that 4-pentenoic acid levels were positively affected by several microbes, including *Raoultella*, *L. lactis*, *Companilactobacillus*, *Enterobacteriaceae*, and *E. hasegawianum* ($P<0.05$).

Similarly, the contents of CP, WSC, and LA were negatively correlated with the concentration of 2-ethyl-2-hydroxybutyric acid ($P<0.05$), whereas the levels of NH_3-N, BA, ADF, and NDF were positively correlated with this metabolite ($P<0.05$). Additionally, 2-ethyl-2-hydroxybutyric acid concentration was positively correlated with *Clostridium*, *Enterococcus*, and *E. hasegawianum* ($P<0.05$).

Ethyl vanillin showed significant positive correlations with the contents of BA, ADF, and NDF ($P<0.05$). It was positively correlated with *L. lactis* and *E. hasegawianum* and negatively correlated with *Malassezia globosa* ($P<0.05$).

LA content was positively correlated with the concentrations of norleucine and cinnamoylglycine ($P<0.05$). Norleucine was positively correlated with unclassified genera of *Lactiplantibacillus* and *Saitozyma parafava* ($P<0.05$) and negatively correlated with *Caproicibacter fermentans* ($P<0.05$). Cinnamoylglycine concentration was positively correlated with *S. parafava* and unclassified genera of *Symmetrospora* ($P<0.05$) but negatively correlated with *Lacticaseibacillus paracasei* ($P<0.05$).

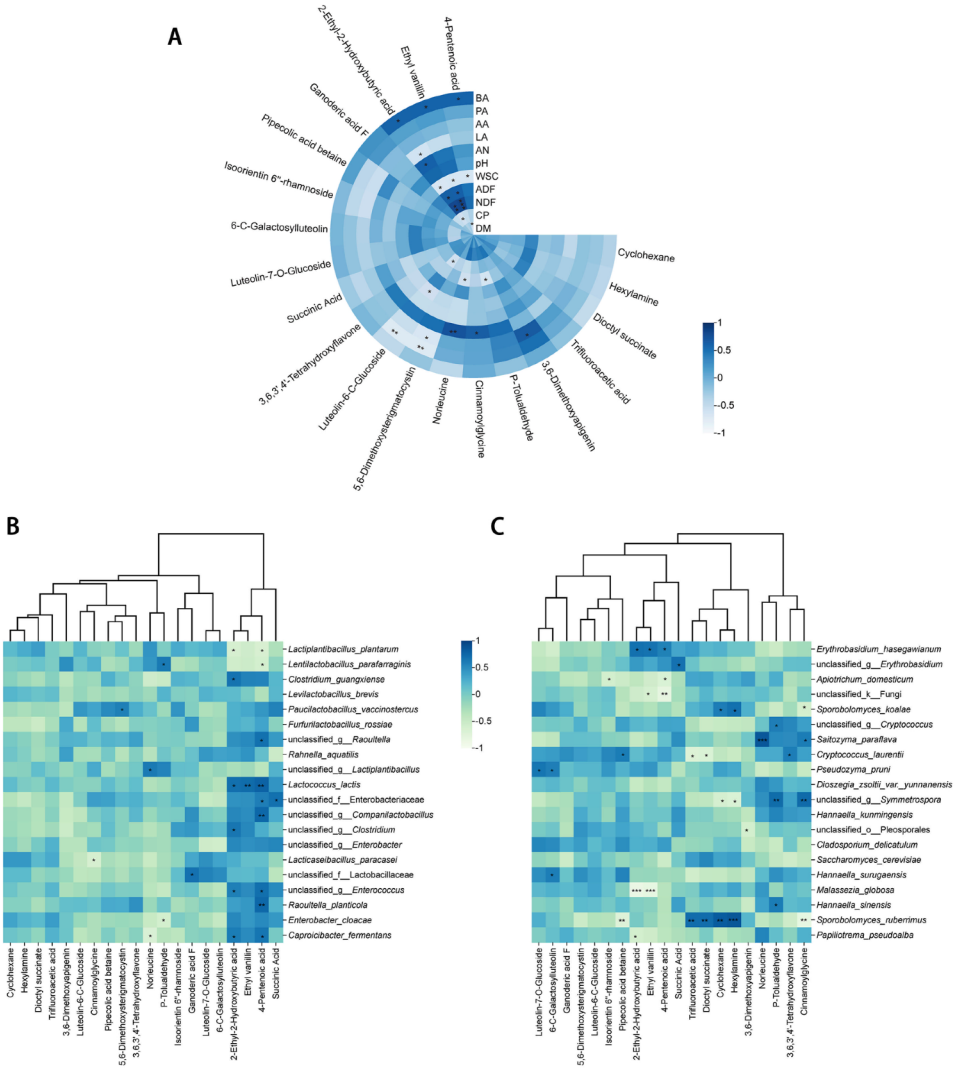

Fig. 6 Analysis of correlation. (**A**) Correlation between silage characteristics and metabolites. (**B**) Correlation between bacteria and metabolites. (**C**) Correlation between fungi and metabolites

Discussion

Nutrient and fermentation characteristics

Forage surfaces harbour various microorganisms, and residual oxygen promotes the proliferation of aerobic spoilage bacteria in the early stages of silage fermentation. These microorganisms consume soluble sugars and proteins, leading to losses in protein, DM, and energy. However, undesirable microbes are highly sensitive to pH changes, and their activity is inhibited as the silage pH decreases. The pH reduction is primarily driven by LAB, although the initial number of LAB in fresh forage is often insufficient for them to dominate the fermentation process immediately. Consequently, in this study, the CON experienced the greatest nutrient loss and exhibited the lowest DM content. In contrast, the addition of *L. parafarraginis* stimulated LA fermentation, which helped lower the silage pH and inhibited the growth of undesirable microbes, thereby minimising nutrient loss [19]. WSC facilitates microbial fermentation during ensiling. Carbohydrates are crucial for silage fermentation, and low carbohydrate content can result in an insufficient substrate for LAB fermentation. The acidic environment generated by *L. parafarraginis* promoted the enzymatic digestion of cellulose, releasing additional WSC, which increased the fermentation substrate and reduced the NDF and ADF content in the silage [20]. Consequently, LP exhibited the lowest NDF and ADF levels and the highest nutrient retention. As a heterofermentative LAB, *L. parafarraginis* produces both AA and 1,2-propanediol, which is converted into PA during the later stages of fermentation (30–60 days). PA and AA have antifungal properties and inhibit the growth of harmful microorganisms such as yeast, thereby enhancing the aerobic stability of the silage after opening [12].

Effect of aerobic environment on the silage quality

Following aerobic exposure, silage comes into contact with aerobic microorganisms, such as moulds and yeast, which proliferate and degrade LA, leading to an increase in pH. This rise in pH fosters the growth of additional harmful microorganisms, further diminishing the nutritive value of the feed [21–22]. Therefore, the pH level after aerobic exposure is a key indicator of silage storage quality. In this study, the pH values of all groups increased with the duration of aerobic exposure. However, the CON consistently exhibited higher pH values compared with the other two groups across all exposure periods. This can be attributed to the increased content of AA, PA, and LA in the silage treated with *L. parafarraginis*, as these acids help stabilise the pH after the silo is opened.

After 9 days of aerobic exposure, the pH was significantly higher in all groups, but lower in the LP than in the other two groups. The LP had the greatest AA and PA than the other two treatments (Table 1), both fungicidal. This likely inhibited yeast and mould growth during aerobic exposure, thereby maintaining a lower pH. As a criterion for assessing aerobic stability, a pH increase of > 0.5 indicates aerobic deterioration [23]. In this study, the pH increase in both the CON and LCA exceeded 0.5, suggesting that *L. parafarraginis* alone was more effective in preserving silage.

The LA content continuously decreased with prolonged aerobic exposure, but the groups treated with additives (LP and LCA) maintained significantly higher LA levels than the CON. Although the LP exhibited higher LA content than the LCA after 6 and 9 days of exposure, the difference was not statistically significant. The addition of *L. parafarraginis* increased the AA content in the hybrid *Pennisetum* silage. AA is crucial in stabilising pH under aerobic conditions. Harmful microorganisms, such as *Clostridium*, can utilise LA as an energy source for metabolism, leading to the consumption of LA, which decreases the acidity of the silage and compromises its quality and stability. In contrast, AA effectively inhibits the growth of these harmful microorganisms [24], reducing LA consumption and helping maintain LA levels, thereby enhancing the aerobic stability of the silage.

After 9 days of aerobic exposure, the additive groups (LP and LCA) exhibited higher LAB counts and fewer yeasts and aerobic microorganisms than the CON. The best results were observed in the treatment LP, suggesting that this additive can reduce the number of harmful microorganisms in silage under aerobic conditions. In addition to producing LA, *L. parafarraginis* generates AA and PA, both of which have inhibitory effects on fungi. Notably, PA has a stronger antifungal effect than AA and effectively prevents the proliferation of undesirable microbes once the silo is opened, minimising its impact on silage quality [25]. Similar results have been reported for corn and oat silage, where the addition of *L. parafarraginis* improved aerobic stability and significantly reduced the number of aerobic bacteria and yeasts after exposure to air [26].

Microbial community analysis in hybrid *Pennisetum* silage

The decrease in bacterial alpha diversity indices (ACE and Chao1) in LP suggests that inoculation with *L. parafarraginis* reduced bacterial diversity in the silage. This effect likely resulted from *L. parafarraginis*, which promoted the dominance of LAB and inhibited the growth and reproduction of other microorganisms. Notably, overall microbial diversity tends to decrease when LAB dominates the silage microbiome [15, 27]. However, when *L. parafarraginis* was combined with citric acid (LCA), LAB growth was inhibited by the acidic conditions. Consequently, silage treated with *L. parafarraginis* alone (LP) exhibited a more stable microbial composition, allowing beneficial bacteria to quickly dominate and suppress the proliferation of other microorganisms. In contrast, the LCA showed a more diverse microbial composition, leading to an increase in the overall diversity of the bacterial community. Similarly, Feng et al.. reported that the ACE diversity index decreased in *L. plantarum*-inoculated silage, whereas a mixture of *L. plantarum* and calcium propionate led to a higher diversity in hybrid *Pennisetum* silage [28].

In this study, undesirable microorganisms were primarily detected in the CON. *Clostridium*, a harmful microorganism in silage, can convert WSC and proteins into BA and NH_3-N, resulting in nutrient loss and reduced fermentation quality [29–31]. *C. guangxiense*, identified in this study, can utilize glucose, maltose, fructose, galactose, and serine as energy sources, producing H_2, CO_2, AA, and BA through fermentation [32]. Additionally, *R. aquatilis* detected in CON may compete with LAB for energy sources, such as WSC [33]. Furthermore, *R. aquatilis* and *Raoultella* have been associated with human diseases and the transmission of antibiotic-resistance genes [34–35]. In contrast, silage samples treated with *L. parafarraginis* were dominated by LAB, effectively inhibiting harmful microorganisms, such as *C. guangxiense*, *Rahnella*, and *Raoultella* species. In the LP, *L. parafarraginis* successfully grew and occupied more than one-third of the microbial niche, demonstrating its suitability as an inoculant for hybrid *Pennisetum* silage.

The relative abundance of *L. plantarum* was high in the LP. As a facultatively heterofermentative LAB, *L. plantarum* primarily produces LA, which helps lower pH and enhances the overall fermentation process in hybrid *Pennisetum* silage [36]. Another beneficial bacterium detected in the LP was *F. rossiae*, a heterofermentative LAB that produces LA as well as 1,2-propanediol

and AA, which may improve the aerobic stability of the silage, reducing spoilage during feed-out [10, 37]. *L. brevis*, another beneficial bacterium produces various metabolites that contribute to unique characteristics in fermented food. The synergistic effects of LAB, including *L. parafarraginis*, *L. plantarum*, *F. rossiae*, and *L. brevis*, resulted in enhanced nutrient retention and improved overall fermentation quality in hybrid *Pennisetum* silage [38].

Notably, after 60 days of ensiling, the bacterial composition in the LCA differed significantly from the LP. *L. parafarraginis* was no longer the dominant bacterium in the LCA, suggesting that the combination of *L. parafarraginis* with citric acid may not be optimal, and the microbial changes in the LCA were likely driven by the addition of citric acid.

Fungi play a major role in aerobic spoilage in silage, and understanding fungal diversity can offer insights into the ability of additives to improve silage stability [39]. In this study, the addition of *L. parafarraginis* alone or in combination with citric acid had little effect on the alpha diversity of the fungal community.

Regarding fungal community composition, CON and LCA were more similar, particularly in terms of dominant species and their relative abundance. In contrast, the fungal community in LP was distinct, reflecting the differences observed in the bacterial community. In CON and LCA, dominant fungal species included *E. hasegawianum* and unclassified *Erythrobasidium*. These fungi, which mainly inhabit leaf surfaces, can utilise various carbon and nitrogen sources, potentially affecting nutrient availability for other microorganisms [40]. Additionally, *Cryptococcus* was detected in CON and LCA. This fungus, primarily found in soil, can cause cryptococcal disease in humans and animals. Cryptococcal infections are often opportunistic, particularly in immunocompromised individuals, and can lead to severe conditions, such as meningitis [41].

In the LP, the relative abundance of *Hannaella* increased, consistent with the findings of the study by Drouin et al.., wherein they reported an increased relative abundance of *Hannaella* in corn silage following LAB inoculation [42]. *Hannaella* is associated with high carbohydrate content, although its specific role in silage fermentation and spoilage requires further investigation [43–44]. Similar to the observations in LP in this study, Xiao et al.. reported that LAB treatment increased the abundance of *Apiotrichum* in oat silage [45]. The genus *Apiotrichum* is associated with the production of amino acids, aldehydes, ketones, and phenols during food fermentation. However, the role of *Apiotrichum* in silage production remains underexplored and warrants further study.

Metabolomics analysis of hybrid *Pennisetum* silage

Carboxylic acids, or organic acids, are key metabolites produced by microorganisms during silage fermentation [46]. These acids, including LA, AA, and PA, are often used as indicators of silage fermentation quality and aerobic stability [8]. Higher levels of certain organic acids, such as AA, can enhance aerobic stability by inhibiting spoilage microorganisms when the silo is exposed to air [19]. Xu et al. also reported that organic acids, particularly LA, AA, and PA, were the most abundant metabolites during the ensiling process [47]. In this study, the LP and LCA exhibited significantly higher organic acid content than the CON, aligning with the findings of Xu et al.. The LCA had the highest levels of organic acids, likely due to the addition of citric acid.

Among the three groups, succinic acid was the most abundant organic acid. Succinic acid is an important metabolite produced by various anaerobic bacteria, including LAB [48]. Ruminants can efficiently absorb and utilise succinic acid as an energy source, leading to improved production performance, such as increased feed intake and digestibility [49–50]. The higher concentration of succinic acid in the LCA may be attributed to LA and citric acid fermentation [51]. Both transaconitic acid and citric acid exhibit antioxidant properties, whereas isocitric acid acts as a regulator of energy metabolism with anti-stress, anti-hypoxia, and anti-oxidative effects [13, 52]. Isocitric acid is used for treating iron deficiency anaemia and certain metabolic disorders [53]. Malonic acid, another detected metabolite, is an intermediate in the production of vitamins and amino acids [54]. Citric acid was present even in the CON and LP, despite no citric acid addition, likely due to its formation by microorganisms during both aerobic and anaerobic stages of fermentation. This suggests that microorganisms can produce various organic acid intermediates, including citric acid, under complex anaerobic conditions, even in the absence of additives [55].

Amino acids and carbohydrates in silage provide essential energy for microbial growth, promoting LA production. A high content of these nutrients also enhances digestibility for ruminants [56]. Sucrose, used as a silage additive, supports LAB growth, promoting pyruvate metabolism and LA formation [57]. The conversion of carbohydrates to LA is crucial for fermentation, and a low carbohydrate content can hinder this process. *L. parafarraginis* inoculation increased sucrose levels, compensating for the reduction in carbohydrates after ensiling [57]. Mannitol, a product of isolactic fermentation, is commonly formed in anoxic and sugary conditions and has antimicrobial and antioxidant activity, contributing to improved silage fermentation quality and aerobic stability [58–59]. Trehalose, a approved food additive found in plants, animals, and microorganisms, stimulates the

growth of beneficial bacteria and enhances bacteriocin production [60]. D-tagatose, a rare sugar with biological and prebiotic activities, is widely used in the food industry [61–62]. The elevated amino acid levels in this study indicate enhanced nutritional value for ruminants consuming hybrid *Pennisetum* silage [63].

Amino acid analysis showed that *L. parafarraginis* inoculation, both alone and in combination with citric acid, modulated the amino acid profile of the silage. Interestingly, L-glutamine concentrations were lower in the CON than in the LP and LCA, whereas L-glutamic acid levels were higher in the CON. During microbial metabolism in silage fermentation, glutamine and glutamate are interconvertible. Glutamine, a versatile amino acid, is critically involved in nutrient metabolism and protein synthesis and is the most abundant free α-amino acid in humans [64]. In mammals, glutamine is essential for maintaining intestinal epithelium homeostasis [65]. Consistent with our results, Xia et al. reported that L-arginine levels increased in LAB-inoculated silage [66]. In contrast, LCA exhibited lower concentrations of other amino acids, although L-tyrosine was upregulated compared with LP. L-tyrosine and its derivatives exhibit antioxidant, anticoagulant, and anti-inflammatory properties, making them highly valuable in various industries [67]. The findings of this study suggest that silage fermentation could offer a cleaner and more sustainable method for L-tyrosine production, which is currently extracted from plant tissues with low yields [68].

Metabolic pathways are pivotal in regulating microbial activity during silage fermentation. KEGG pathway analysis revealed that L-serine is involved in 'glycine, serine, and threonine metabolism' and 'cyanoamino acid metabolism'. Tryptophan, an essential amino acid, promotes animal growth and improves small intestine morphology [69]. In this study, the 'tryptophan metabolism' pathway was significantly affected in the LCA, and tyrosine is a product of the tryptophan biosynthesis pathway. Tyrosine levels were upregulated in the LCA compared with those in the CON. These findings suggest that inoculation with *L. parafarraginis*, alone or in combination with citric acid improved the fermentation quality of hybrid *Pennisetum* silage and positively influenced its essential amino acid composition. Modulating key metabolic pathways related to organic acids, amino acids, and lipids highlights the crucial role of *L. parafarraginis* inoculants in optimising fermentation dynamics and enhancing silage quality.

Correlation of metabolites with silage indicators and microbial communities

The concentration changes of various metabolites reflect microbial metabolic activity during the ensiling process. The accumulation patterns of these metabolic products are closely linked to nutritional parameters, including DM, CP, WSC, fibre content, and fermentation quality indicators such as pH and LA concentration. Utilising metabolomics to explore the relationships between microbial metabolism and silage quality offers deeper insights into how microbial functions impact both the nutritional value and fermentation quality of silage. This provides critical evidence for optimising silage formulations and fermentation management practices.

Our findings suggest that the accumulation of 4-pentenoic acid, 2-ethyl-2-hydroxybutyric acid, and ethyl vanillin may be associated with the breakdown of organic matter and the formation of undesirable fermentation products during silage fermentation. The concentrations of 4-pentenoic acid and 2-ethyl-2-hydroxybutyric acid appeared to be linked with the metabolic activities of specific microbes, including *C. fermentans*, *Raoultella planticola*, *Enterobacteriaceae*, *Clostridium*, and *Enterococcus*. These microbes are likely responsible for the suboptimal silage quality. Consequently, in terms of silage quality indicators, the CON and LCAs were observed to be inferior to the LP.

Additionally, the results indicated that unclassified genera of *Lactiplantibacillus* promoted LA production and helped preserve norleucine. Metabolites, such as luteolin-6-C-glucoside, norleucine, and p-tolualdehyde, were associated with the degradation of ADF and NDF. These metabolites may be linked to the activity of unclassified genera of *Lactiplantibacillus* and *Lactiplantibacillus parafarraginis*.

Conclusion

In summary, inoculation with *L. parafarraginis* significantly improved the fermentation quality of hybrid *Pennisetum* silage. This treatment also reduced the abundance of undesirable microorganisms and enhanced the silage's organic acid profile, suggesting its potential for improvements in its flavour. When combined with citric acid, *L. parafarraginis* further enhanced amino acid metabolism, particularly by increasing L-tyrosine concentrations and enriching tryptophan metabolic pathways, this combination offers additional benefits in terms of both metabolic improvement and overall silage quality. These findings highlight the promising role of *L. parafarraginis* and its combination with citric acid as an effective silage additive for producing high-quality, nutritious forage.

Supplementary Information

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Supplementary Material 1

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Author contributions
YJL: Data curation, Formal analysis, Writing-Original Draft, Writing-Review & Editing, Visualization. WQL: Investigation, Writing-Review & Editing, Visualization. YL: Investigation, Methodology, Data curation, Validation, Formal analysis. YZ: Investigation, Writing-Review & Editing, Visualization. JL: Investigation, Writing-Review & Editing. SQC: Writing-Review & Editing. JZ: Conceptualization, Resources, Methodology, Data curation, Writing-Review & Editing, Supervision, Project administration, Funding acquisition. FLY: Conceptualization, Methodology, Writing-Review & Editing, Supervision, Project administration.All authors reviewed the manuscript.

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Data availability
The raw sequence data were submitted to the National Center for Biotechnology Information under BioProject PRJNA1054343.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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