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RESEARCH

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Evaluation of the fermentation performance and functional properties of bacterial communities of amaranth silage supplemented with *Limosilactobacillus fermentum* and *Latilactobacillus graminis*

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Abstract

Background The objective was to determine the effects of different lactic acid bacteria (LAB) on amaranth (*Amaranthus hypochondriaus*) silage fermentation characteristics, changes in bacterial communities and their functional properties, and to investigate the preferential effects and mechanisms of action of *Limosilactobacillus fermentum* (*L. fermentum*) and *Latilactobacillus graminis* (*L. graminis*) on the bacterial communities of silage. LAB were screened and identified from native grasses and its naturally fermented silage, and their effects on fermentation performance and bacterial community of amaranth after 60 days of fermentation were investigated. BL1 and BL5 strains were identified as *L. fermentum* and *L. graminis*, respectively. They could grow normally at concentrations of 3.0% and 6.5% NaCl, tolerating pH and temperature ranges of 3.5–9.0 and 4.0–9.0, 30–45 ℃ and 10–45 ℃, respectively. The distilled water, *Lactiplantibacillus plantarum* (commercial inoculant), *L. fermentum*, and *L. graminis* were inoculated into amaranth at a total of 1×10^5 cfu/*g* fresh material and labeled AhSCK, AhSLP, AhSLF, and AhSLG, respectively, while the fresh amaranth was labeled YLS.

Results As expected, the water soluble carbohydrates of AhSCK was lower than AhSLG ($p < 0.05$). The highest number of LAB was found in AhSLF ($p < 0.05$). The lactic acid (LA) content of AhSLP was significantly higher ($p < 0.05$) than other silage. Nevertheless, acetic acid (AA) content of AhSLF was significantly different from other silage ($p < 0.05$). All the amaranth silage was well preserved with low pH (4.16–4.51) and acceptable levels of butyric acid (0.00–0.86%FM). At the species level, *Pantoea agglomerans*, *Pseudomonas oryzihabitans*, and *Cenchrus americanus* served as the dominant strains in fresh amaranth, with relative abundances lower than 1% after ensiling.. *Lentilactobacillus buchneri*, *Levilactobacillus brevis*, and *L. fermentum* were the dominant strains of AhSCK, AhSLP and AhSLF. Nevertheless, the predominant strains of AhSLG was *Xanthomonas oryzae*. Both fresh material and silage, L. fermentum, L. plantarum, Levilactobacillus brevis, Pantoea agglomerans, Pseudomonas oryzihabitans, Cenchrus americanus, Pseudomonas coleopterorum, Priestia aryabhattai, and Exiguobacterium sibiricum were significantly different in relative abundance ($p < 0.01$).Functional prediction analysis showed that in pyruvate metabolism, glycolysis/gluconeogenesis and amino sugar and nucleotide sugar metabolism were enriched after ensiling. AhSLF showed the most obvious trend of increasing abundance of pentose phosphate pathway, biosynthesis of secondary metabolites and biosynthesis of antibiotics, while the abundance of metabolic pathways decreased significantly ($p < 0.05$).

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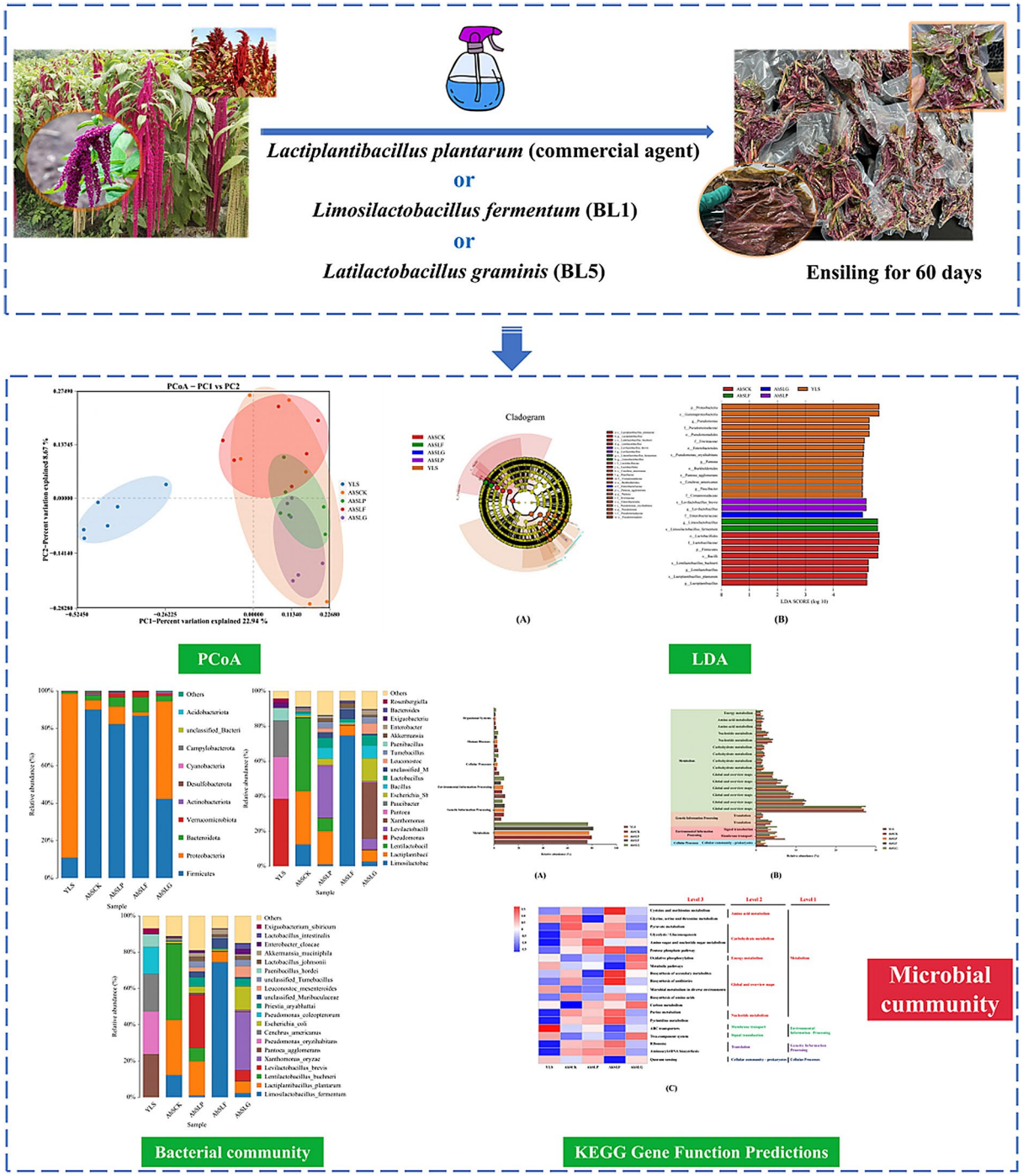
Conclusions *L. fermentum* can be recommended as an additive to improve the quality of amaranth silage, as it regulates the epiphytic microbiota in silage, improving the fermentation quality and metabolic capacity of the bacterial community.

Keywords Lactic acid bacteria, Fermentation quality, Bacterial community, Amaranth, Silage

Graphical Abstract



Background

Recently, the rapid development of China's livestock industry has caused problems such as feed shortages, climatic conditions in semi-arid and arid regions and water shortages that reduce the availability of feed. Consequently, the investigation of new, nutrient-rich and tolerant forage resources, including amaranth (Amaranthus hypochondriaus), has been initiated. Amaranth is an annual plant, which is suitable for poor soils and areas with infertile rainfall and high temperatures [1, 2]. It is a forage resource with ruminant feed potential [3]. Amaranth has a high growth rate and yield. It has good adaptability and plenty of protein, starch and fat [4, 5]. In the case of amaranth, the thickness of the stalk and moisture content may affect its drying rate, thus limiting its utilization as a hay forage [6, 7]. Ensiling has been applied successfully to preserve fresh amaranth [8, 9]. Amaranth silage preserves the freshness and juiciness, especially during the rainy seasons when it is difficult to produce hay.

The fermentation process of silage can be influenced by temperature, moisture, nutrient content and sugar content of the raw material, microbiome in the raw material and some human factors [10, 11]. In particular, the ecological diversity of microorganisms (epiphytic lactic acid bacteria (LAB)) is closely related to silage preservation [12]. When the fermentation substrate of the forage is insufficient to support LAB fermentation, the fermentation characteristics of the forage are generally enhanced by exogenous addition of silage additives [13]. Numerous additives are widely utilised for their characteristics of improving silage quality with small nutrient losses. Of these, LAB is appreciated for its properties of improving the quality of fermentation and extending the ensiling time of raw materials [10, 14]. LAB inoculants rapidly inhibit complex microorganisms in raw materials. Moreover, new silage inoculants can be isolated and identified from various materials [15]. However, amaranth has a low water-soluble carbohydrate (WSC) content and high moisture content, and natural fermentation may affect the final fermentation of silage. Therefore, the fermentation characteristics of amaranth were improved by adding LAB, which were classified as homofermentative and heterofermentative LAB. It also has been shown in previous studies that homofermentative LAB can use glucose to accelerate the fermentation process of lactic acid (LA) [16]. LA is produced via the Embden Meyerhof pathway (EMP) by strains such as *L. plantarum*, *Pediococcus acidilactici*, and *Pediococcus pentosaceus,* while heterotypic fermentative LAB are also widely used for the production of LA, acetic acid (AA), and ethanol, such as certain strains of *Lactobacillus buchneri*, *Limosilactobacillus fermentum* (*L. fermentum*) [17–22].

The diversity of strains implies that there are some variation in their effectiveness on silage, leading to the impossibility of a generic type of LAB production to modulate the fermentation characteristics of different forage silages [23, 24]. The fermentation process of silage can be considered as an ecosystem in which complex and dynamic interactions among microorganisms occur that can drive community structure and functional properties [12, 25]. In addition, there are important priority effects in microbial communities that have been shown to affect microbial community assemblages in various habitats [26–28]. Nevertheless, there are fewer studies on how priority effects affect the structural and functional properties of bacterial communities in amaranth silage inoculated with different LAB strains. Consequently, we hypothesised that *L. fermentum* and *Latilactobacillus graminis* (*L. graminis*) would have a positive effect on the fermentation characteristics, bacterial community structure and 16S rRNA gene-predicted metabolic characteristics of amaranth silage, and investigated the preferential effects and mechanisms of action on silage bacterial communities.

Methods

Lactic acid bacteria strains

Based on the method of Wang et al. [29], a total of 2 LAB strains were isolated from native grass and naturally fermented silage samples. Native grasses were harvested in July 2019 and 2020 (milk stage) in meadows, typical and desert steppe of the Inner Mongolian plateau in China. Samples (10 *g*) of the fresh and fermented material were aseptically mixed with 90 mL of sterile distilled water. Serial dilutions were spread to the de Man, Rugose, Sharpe (MRS) agar (Difco Laboratories, Detroit, MI, USA), and the plates were incubated at 35 ℃ for 2 days under anaerobic conditions for the isolation of LAB. Thereafter, the presumptive homo and heterofermentative LAB strains were purified third by streaking on MRS agar plates and stored at − 80 ℃ in MRS containing 25% glycerol [12].

Physiological and morphological tests

The assays for Gram staining, peroxidase activity and glucose gas production were referred to previous studies [30, 31]. Reference was made to Cai et al. [32] for temperature and acid resistance (pH adjustment with hydrochloric acid or NaOH) tests. Salt tolerance of the strains was determined with reference to the method of Wang et al. [33, 34], and its growth was measured and evaluated using optical density (OD) values. API 50 CH (BioMérieux, Marcy l' Etoile, Lyons, France) for the determination of carbohydrate assimilation [34].

Lactic acid bacteria identification by 16S rRNA sequencing

To extract the DNA of the screened strains, Bacterial DNA kit was used (Tiangen Biotech Co., Ltd., Beijing, China). The primers 27F (5′-AGAGTTTGATCCTGG CTCAG-3′) and 1492R (5′-TACGGCTACCTTGTT ACGACT-3′) were used in the polymerase chain reaction (PCR) (Cai et al. 1999) [33]. The 16S rRNA sequences were identified using BLAST analysis on GenBank [35].

Experimental design and silage preparation

The test material was red-fruited amaranth, and it was planted on June 9, 2021. The test site was located at the forage trial base of Inner Mongolia Agricultural University (111°430 E, 40°480 N). Three 1-m^2 plots were selected for harvesting (mature stage) on August 10. The collected red fruit amaranth was placed on a clean plastic sheet and dried until the moisture content reached about 70%. The raw material was cut into lengths of approximately 2–3 cm using a hand-held guillotine (Mode-8, 200; Minghong Business, Shandong, China). Strains BL1 and BL5, and commercial LAB additive (*L. plantarum* JYLP-002 was supplied by Shandong Zhongke Jiayi Biological Engineering Company) were used as inoculants. All LAB strains were supplemented separately at 1×10^5 cfu/*g* fresh material (FM). The control group (YLS) silage was sprayed with an equivalent deionized water. The amaranth (500 *g*) were packed in polyethylene plastic bags (size: 300 mm×400 mm; Shenyang Huasheng Plastic Packaging Products Co., Ltd., Shenyang, China), which were then vacuum sealed (DZ400/2D vacuum sealer; Wenzhou Dafeng Machinery Co., Ltd. Wenzhou, China) and ensiled at ambient temperature (24–30 ℃) for 60 days. Chemical and fermentation characteristics, microbial populations, and microbial community of the control (AhSCK), *L. plantarum* (AhSLP), strain BL1 (AhSLF), and strain BL5 (AhSLG) silage were then measured.

Chemical and fermentation characteristics analysis

Each amaranth silage sample had six replicates. Dry matter (DM) content was measured following the method of Zhang et al. [36]. The crude protein (CP) content was measured according to the method of Patrica [37], utilizing on a Kjeldahl nitrogen tester (Gehart Vapodest 50 s, Germany). Determination of neutral detergent fiber (NDF) and acid detergent fiber (ADF) content was performed according to the method of Van et al. [38] by using the Ankom A2000i fiber analyzer (Ankom Technology, Macedon, NY, USA). The WSC content was measured following the method of Chen et al. [39].

A sample of silage (10 *g*) was mixed with 90 g deionized water following the description of Cai [40] and stored in a refrigerator at 4 ℃ for 24 h. The leachate was filtered through four layers of gauze and filter paper and the pH, ammonia nitrogen (NH_3-N), and organic acids of the leachate were measured. pH was measured using a glass electrode pH meter (Leici pH S-3C, Shanghai, China). The content of LA, AA, propionic acid (PA) and butyric acid (BA) in silage was determined by high performance liquid chromatography (HPLC; model: Waters e2695, Milford, USA) [26]. The method of Broderick and Kang [41] was used to determine ammoniacal nitrogen (NH_3-N) concentrations. Microbial populations (LAB, yeasts, mold, anerobic bacteria, and coliform bacteria) in the FM were assessed as described in a previous report [42].

Microbial community analysis

The bacterial community composition of amaranth fermented for 60 days was analyzed by 16S rRNA gene sequencing. Based on the investigation of Liu et al. [43], total DNA was extracted from fresh and silage samples of amaranth. The procedures of metagenomic DNA extraction and PCR amplification of the bacterial 16S rRNA gene were performed according to Guo et al. [44]. Briefly, DNA was amplified with primers 27F (5′-AGRGTTTGATYNTGGCTCAG-3′) and 1492R (5′-TASGGHTACCTTGTTASGACTT-3′). PCR conditions were an initial denaturation at 98 ℃ for 2 min, 30 cycles of denaturation at 98 ℃ for 30 s, annealing at 50 ℃ for 30 s, and elongation at 72 ℃ for 60 s, followed by a final extension at 72 ℃ for 5 min. The PCR products were purified for sequencing and analysis. Each treatment was performed in six copies. 16S rRNA gene sequence were stored in NCBI with BioProject accession number PRJNA911007.

NGS sequencing was performed by Biomarker Technologies (Beijing, China) on a Pacbio_SMRT platform (Pacbio Sequel II, CA, USA). Coverage of alpha-diversity indicators Chao1 and Good was calculated using QIIME v1.9.1 [45]. Principal coordinate analysis (PCoA) was performed using the R program (version 3.2.5) on the basis of beta-diversity unweighted or weighted unifrac distances. The SILVA (version 128) 16S rRNA database was classified by Operational Taxonomic Units (OTUs) using the Ribosomal Database Project (RDP) classifier (version 2.2) with a minimum confidence of 0.7, and then classified by phylum, genus, and species. Mothur (version v.1.30) software was used to evaluate the alpha-diversity indices (ACE, Chao 1, Simpson, and Shannon) of the samples. LEfSe (Linear Discriminant Analysis (LDA) effect size) was able to find biomarkers that are statistically different between groups [46]. It was performed using a free online platform (https://international.biocloud.net).

Phylogenetic Investigation of Communities by Reconstruction of Unobserved States II (PICRUSt2) software was used to predict microbial functions from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Statistical analysis

One-way analyses of variance (ANOVA) based on SAS' General Linear Model (GLM) program (version 9.3; SAS Institute Inc., Cary, NC, United States) were conducted on fermentation and nutritional characteristics and microbial counts of fresh and silage amaranth. Effects were considered significant when $p < 0.05$. The graphs of microbial community data were created using the BMK Cloud online platform and GraphPad prism 8.

Results

The selection of isolated lactic acid bacteria based on morphological and physiological tests

Table 1 shows the selection of isolated LAB based on morphological and physiological tests. Two isolated LAB strains were Gram-positive and catalase-negative. And both isolated strains were bacillus-like and gram-positive and peroxide-negative. The optimum growth temperature of strain BL1 was 30 – 45 ℃, and the pH could be reproduced normally in the range of 3.5 – 9.0. And strain BL5 was able to grow normally from 10 – 45 ℃, and the growth was well at pH 4.0 – 9.0. The growth of strain BL1 and BL5 was normal at the concentrations of 3.0% and 6.5% NaCl.

Both strains possessed different fermentation patterns based on the results of gas detection on glucose, with strain BL1 being a hetero-fermented LAB and strain BL5 being a homo-fermented LAB, while different ferment carbohydrates are indicated in Table 2. GenBank database was used to analyze the similarity by BLAST in this study. Strain BL1 showed high similarity to *L. fermentum* (99.93%) and strain BL5 showed high similarity to *L. graminis* (99.65%). The nucleotide sequences of strains BL1 and BL5 were registered to Genbank under accession numbers OP984707 and OP984708, respectively (Table 3).

Chemical and microbial composition of pre-ensiled amaranth

Table 4 shows the chemical composition and microbial population of the fresh amaranth. The DM content of the raw material was 32.03% of FW. CP, WSC, NDF, and ADF were all DM-based with 7.99%, 1.35%, 47.39% and 36.70%, respectively. The numbers of LAB, mold, yeast and *Escherichia coli* attached to the raw material were 3.90, 0.90, 5.26, 4.03, and 4.65 log10 cfu/*g* FM, correspondingly.

Chemical and fermentation characteristics, and microbial populations after 60 days of ensiling

The chemical composition, fermentation characteristics and microbial population of silage for 60 days are shown in Table 5. Compared to AhSCK, the additive-treated group showed a reduction in DM content and an increase in CP content ($p > 0.05$). NDF content was lower than AhSCK in all treatments except AhSLF ($p > 0.05$), but ADF content was significantly lower in AhSLF than AhSCK ($p < 0.05$). While the WSC content of AhSLG was significantly higher than AhSCK ($p < 0.05$), it was not significantly different from AhSLP and AhSLF ($p > 0.05$). The number of LAB was significantly increased compared to AhSCK ($p < 0.05$), with AhSLF having the highest number of LAB. The number of yeast and aerobic bacteria was reduced in the additive groups. After ensiling, no molds and *Escherichia coli* were detected in all treatments. All the amaranth silage was well preserved with low pH (4.16 – 4.51) and acceptable levels of BA (0.00 – 0.86%FM). And LA content of the additive treatments was significantly higher than AhSCK ($p < 0.05$). The AA content of AhSLF was significantly different from other silage, PA and NH_3-N contents were not significantly different between treatments ($p > 0.05$). In particular, higher BA content was detected in AhSCK and AhSLG ($p < 0.05$).

Table 1 The selection of isolated lactic acid bacteria on the base of the morphological and physiological tests

Items	BL1	BL5
Shape	Rod	Rod
Gram stain	+	+
Gas for glucose	–	–
Catalase	–	–
Fermentation type	He	Ho
Growth at temperature (℃)		
5	–	w
10	w	+
30	+	+
45	+	+
Growth at pH		
3.0	w	–
3.5	+	–
4.0	+	+
4.5	+	+
9.0	+	+
Growth in NaCl (%)		
3.0	+	+
6.5	+	+

Ho homo-fermentation, *He* hetero-fermentation, *w* weak, + positive, – negative

Bacterial community of high-moisture amaranth silage

This study also analyzed the bacterial diversity in silage (Table 6). The sequencing coverage values for all samples demonstrated that the sequencing depth was sufficient to effectively characterize the bacterial community (coverage values > 99%). The various indices of alpha diversity in silage showed differences compared to the raw material. The OTUs, ACE and Chao1 were significantly ($p < 0.05$) increased for silage compared to YLS. The highest OTUs and Chao1 indexes were found for AhSLG and the highest ACE index for AhSLP among different silage. There was a significant difference in the Simpson index of AhSCK and AhSLF compared to YLS ($p < 0.05$). Both AhSLP and AhSLG had significantly higher Shannon indices than AhSCK and AhSLF ($p < 0.05$), with no significant difference with YLS ($p > 0.05$).

For the determination of whether there were differences in bacterial community structure in fresh amaranth and silage, PCoA was performed based on unweighted uniFrac distances (Fig. 1). Figure 1 showed a clear separation of bacterial communities in fresh material and silage. In addition, AhSCK, AhSLP, AhSLF and AhSLG were separated.

The relative abundance of bacterial communities in fresh material and 60-day silage is shown in Fig. 2. The dominant phylum of YLS were Proteobacteria and Firmicutes (Fig. 2A). After ensiling, the relative abundance of Firmicutes increased, the relative abundance of Proteobacteria decreased, but remained the dominant phylum for treatments with different inoculants. As shown in Fig. 2B, there was a highly significant difference in the relative abundance of Firmicutes and Proteobacteria between fresh materials and silage at the phylum level ($p < 0.01$). The relative abundance of Firmicutes of AhSLP, AhSLF and AhSLG was lower than AhSCK. Among them, the relative abundance of AhSLG was quite low (42.21%), and the relative abundance of Proteobacteria was as high as 52.30%.

Relative abundances of bacteria at the genus level are presented in Fig. 2C. The dominant genus of YLS were *Pseudomonas*, *Pantoea*, *Paucibacter*. For AhSCK, the dominant genus were *Limosilactobacillus*, *Lactiplantibacillus*, *Lentilactobacillus*. The dominant genus of AhSLP were *Lactiplantibacillus*, *Levilactobacillus*. The predominant genus of AhSLF were *Limosilactobacillus*, *Lactiplantibacillus*. For AhSLG, the dominant genus were *Xanthomonas*, *Escherichia* and *Shigella*. As indicated in Fig. 2D, there were significant or highly significant differences ($p < 0.05$ or $p < 0.01$) in the relative abundance of *Limosilactobacillus*, *Lactiplantibacillus*, *Pseudomonas*, *Levilactobacillus*, *Pantoea*, *Paucibacter*, *Bacillus*, *Paenibacillus*, *Enterobacter*, and *Exiguobacterium* for both fresh materials and silage at the genus level.

Relative abundances of bacteria at the species level are presented in Fig. 2E. The predominnant strains of YLS

Table 2 The characteristics of isolated lactic acid bacteria on the base of carbohydrate fermentation

Items	BL1	BL5
L-Arabinose	+	+
Ribose	+	+
D-Xylose	+	–
D-Galactose	+	+
D-Glucose	+	+
D-Fructose	+	+
D-Mannose	+	+
D-Mannitol	w	–
D-Sorbitol	–	–
Methyl-αD-mannopyranoside	–	–
N-Acetyl glucosamine	–	+
Amygdalin	–	w
Arbutin	–	–
Esculin	–	+
Salicin	–	w
Cellobiose	+	+
Maltose	+	–
Lactose	+	–
Melibiose	+	–
Sucrose	+	–
Trehalose	+	+
Melezitose	–	–
Raffinose	+	–
Gentiobiose	–	w
D-Tagatose	–	–
D-Arabitol	–	–
Gluconate	w	–

w weak, + positive, – negative

Table 3 The results of isolated lactic acid bacteria on the base of 16S rRNA gene sequences

Strain	Accession number	16S rRNA gene sequencing data(closest relative)	Similarity (%)
BL1	NR_113335.1	*Limosilactobacillus fermentum* NBRC 15885	99.93
BL5	NR_042438.1	*Latilactobacillus graminis* G90 (1)	99.65

Table 4 Chemical composition and microbial population of amaranth prior to ensiling

Items	Amaranth
Dry matter (%FW)	32.03
Crude protein (%DM)	7.99
Neutral detergent fiber (%DM)	47.39
Acid detergent fiber (%DM)	36.70
Water-soluble carbohydrates (%DM)	1.35
Lactic acid bacteria (log10 cfu/*g* FM)	3.90
Mold (log10 cfu/*g* FM)	0.90
Yeast (log10 cfu/g FM)	5.26
Aerobic bacteria (log10 cfu/*g* FM)	4.03
Coliform bacteria (log10 cfu/*g* FM)	4.65

FM fresh matter, *DM* dry matter, *CP* crude protein, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *WSC* water-soluble carbohydrate

were *Pantoea agglomerans*, *Pseudomonas oryzihabitans*, *Cenchrus americanus* and *Pseudomonas coleopterorum*. The dominnant strains of AhSCK were *L. plantarum* and *Lentilactobacillus buchneri*. The dominant strains of AhSLP were *Levilactobacillus brevis* and *L. plantarum.* The dominant strains of AhSLF was *L. fermentum*. The predominnant strains of AhSLG were *Xanthomonas oryzae* and *L. plantarum*. As shown in Fig. 2F, both fresh material and silage, *L. fermentum*, *L. plantarum*, *Levilactobacillus brevis*, *Pantoea agglomerans*, *Pseudomonas oryzihabitans*, *Cenchrus americanus*, *Pseudomonas coleopterorum*, *Priestia aryabhattai*, *Paenibacillus hordei*, *Enterobacter cloacae*, and *Exiguobacterium sibiricum* were significantly or highly significantly different in relative abundance ($p < 0.05$ or $p < 0.01$).

Table 5 Chemical compositions, fermentation characteristics, and microbial populations on 60 days of ensiling

Items	AhSCK	AhSLP	AhSLF	AhSLG	SEM	*p*-value
DM (%FM)	31.46 ± 0.72a	31.37 ± 0.51a	30.76 ± 0.62a	31.29 ± 1.00a	0.1562	0.8148
CP (%DM)	7.92 ± 0.85a	8.59 ± 0.75a	8.40 ± 1.09a	8.27 ± 0.91a	0.1269	0.8731
NDF (%DM)	46.44 ± 1.94a	46.17 ± 0.74a	46.61 ± 1.42a	44.32 ± 0.33a	0.5285	0.2707
ADF (%DM)	39.23 ± 1.29a	38.52 ± 1.59ab	36.36 ± 0.78b	36.73 ± 1.59ab	0.6928	0.1003
WSC (%DM)	0.16 ± 0.03b	0.25 ± 0.06ab	0.23 ± 0.07ab	0.33 ± 0.04a	0.0351	0.0412
LAB (log10 cfu/g FM)	4.36 ± 0.33c	5.84 ± 0.38b	7.37 ± 0.19a	4.68 ± 0.32c	0.6810	< 0.0001
Mold (log10 cfu/g FM)	ND	ND	ND	ND	ND	ND
Yeast (log10 cfu/g FM)	5.19 ± 0.36a	2.93 ± 2.54ab	1.44 ± 0.12b	3.37 ± 0.51ab	0.7706	0.0614
Aerobic bacteria (log10 cfu/g FM)	5.80 ± 0.14a	4.96 ± 0.69a	4.66 ± 0.50a	4.85 ± 0.84a	0.2512	0.1949
Coliform bacteria (log10 cfu/g FM)	ND	ND	ND	ND	ND	ND
pH	4.51 ± 0.04a	4.20 ± 0.01c	4.16 ± 0.03bc	4.38 ± 0.09b	0.0515	0.0074
LA (%FM)	0.85 ± 0.03c	1.46 ± 0.09a	1.16 ± 0.15b	1.08 ± 0.05b	0.1253	0.0005
AA (%FM)	0.21 ± 0.03b	0.19 ± 0.04b	0.76 ± 0.29a	0.20 ± 0.01b	0.1396	0.0106
PA (%FM)	0.23 ± 0.14a	0.18 ± 0.06a	0.17 ± 0.03a	0.21 ± 0.03a	0.0154	0.3185
BA (%FM)	0.31 ± 0.07b	0.00c	0.00c	0.86 ± 0.12a	0.2028	< 0.0001
NH_3-N (%TN)	1.17 ± 0.08a	1.04 ± 0.22a	1.01 ± 0.07a	0.94 ± 0.13a	0.0469	0.4076

FM fresh matter, *DM* dry matter, *CP* crude protein, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *WSC* water-soluble carbohydrate, *LAB* lactic acid bacteria, *LA* lactic acid, *AA* acetic acid, *PA* propionic acid, *BA* butyric acid

Different lowercase letters indicate significant differences among different treatments ($p < 0.05$); same letter indicate not significant ($p > 0.05$)

Table 6 Alpha-diversity of bacterial diversity of amaranth silage

Items	YLS	AhSCK	AhSLP	AhSLF	AhSLG	SEM	*p*-value
OTUs	55.8333c	94.8333b	109.5000ab	79.1667bc	129.6667a	12.63	0.0006
ACE	64.8553c	224.9584a	130.7388b	137.8942b	166.4252b	26.01	0.0002
Chao1	62.7195c	152.4080ab	130.4236ab	124.7025b	163.6675a	17.52	< 0.0001
Simpson	0.7747a	0.3876b	0.7666a	0.4012b	0.6499ab	0.09	0.0088
Shannon	2.5582ab	1.6501b	3.2548a	1.4932b	2.9462a	0.35	0.0173
Coverage	0.99915a	0.9961b	0.9972b	0.9970b	0.9961b	0.00	0.0023

Different lowercase letters indicate significant differences among different treatments ($p < 0.05$); same letter indicate not significant ($p > 0.05$)

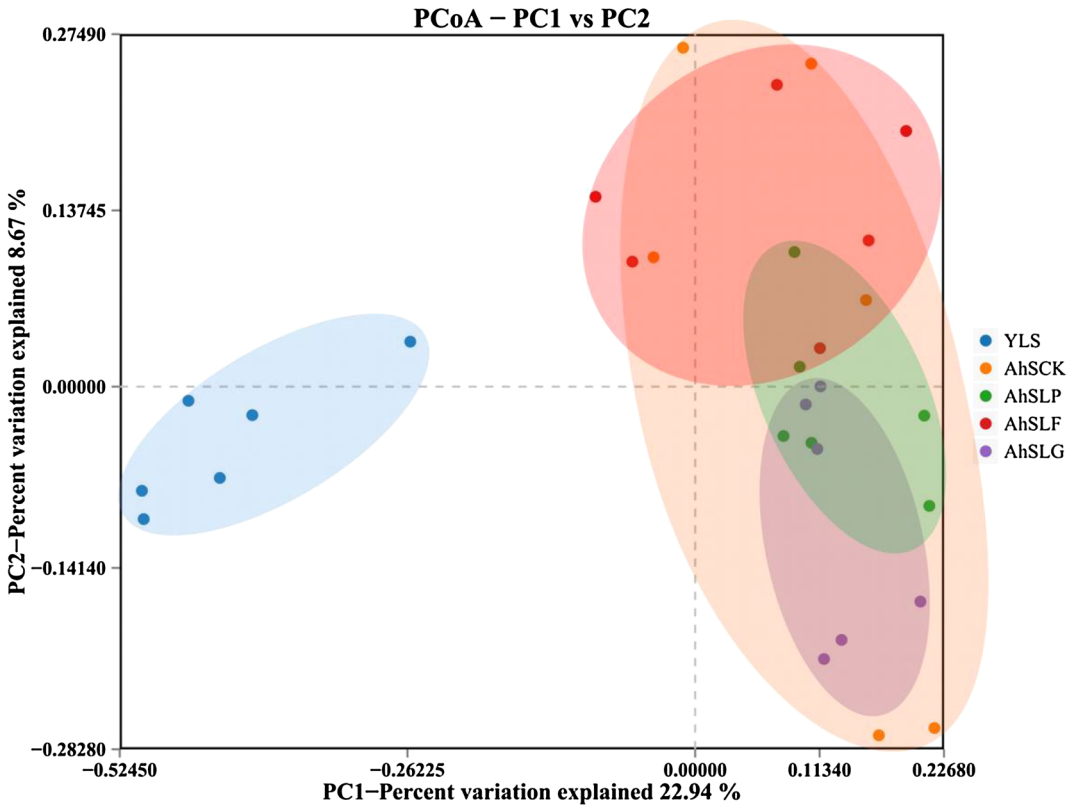


Fig. 1 Principal coordinate analysis (PCoA) of the bacterial community of fresh material and amaranth on 60 days of ensiling. YLS, fresh amaranth; AhSCK, control group; AhSLP, commercial inoculant group; AhSLF, strain BL1 group; AhSLG, strain BL5 group

The LEfSe method was used to evaluate differences in microbial communities between fresh material and silage and to explore specific bacterial species in each group [LDA score, > 5.0]. In YLS, 14 bacteria were significantly enriched, with Proteobacteria (LDA score, 5.94) having the highest LDA score (Fig. 3). There were six bacteria significantly enriched in AhSCK, with Firmicutes (LDA score, 5.95) having the highest LDA score. *Levilactobacillus* and *Levilactobacillus brevis* were significantly enriched in AhSLP, with both LDA scores of 5.46. Within AhSLF, *Limosilactobacillus* and *L. fermentum* were significantly enriched with an LDA score of 5.87. For AhSLG, only *Enterobacteriaceae* were significantly enriched with an LDA score of 5.25.

KEGG gene function predictions

PICRUSt predicts the bacterial metabolic function in accordance with the KEGG pathway. In total, there were six different metabolic pathways shown in YLS and silage (Fig. 4A). The main predicted genes were assigned to metabolism, accounting for about 70% in the YLS and silage followed by the genetic information processing, environmental information processing and cellular process. The top 20 metabolic functions in level 2 are shown as Fig. 4B, where most of these metabolic pathways (15 pathways) were assigned to metabolism. Figure 4C showed the top 20 metabolic pathways at the third pathway level, carbohydrate metabolism and global and overview maps were the critical metabolic pathways at level

(See figure on next page.)

Fig. 2 The bacterial community of fresh material and amaranth on 60 days of ensiling. **A** The bacterial community was shown at the phylum level. **B** The extended error bar plot displaying the significant differences among groups at the phylum level. **C** The bacterial community was shown at the genus level. **D** The extended error bar plot displaying significant differences among groups at the genus level. **E** The bacterial community was shown at the genus level. **F** The extended error bar plot displaying significant differences among groups at the genus level. *Shows that the significant difference was at $p < 0.05$ level. **Shows that the significant difference was at $p < 0.01$ level. YLS, fresh amaranth; AhSCK, control group; AhSLP, commercial inoculant group; AhSLF, strain BL1 group; AhSLG, strain BL5 group

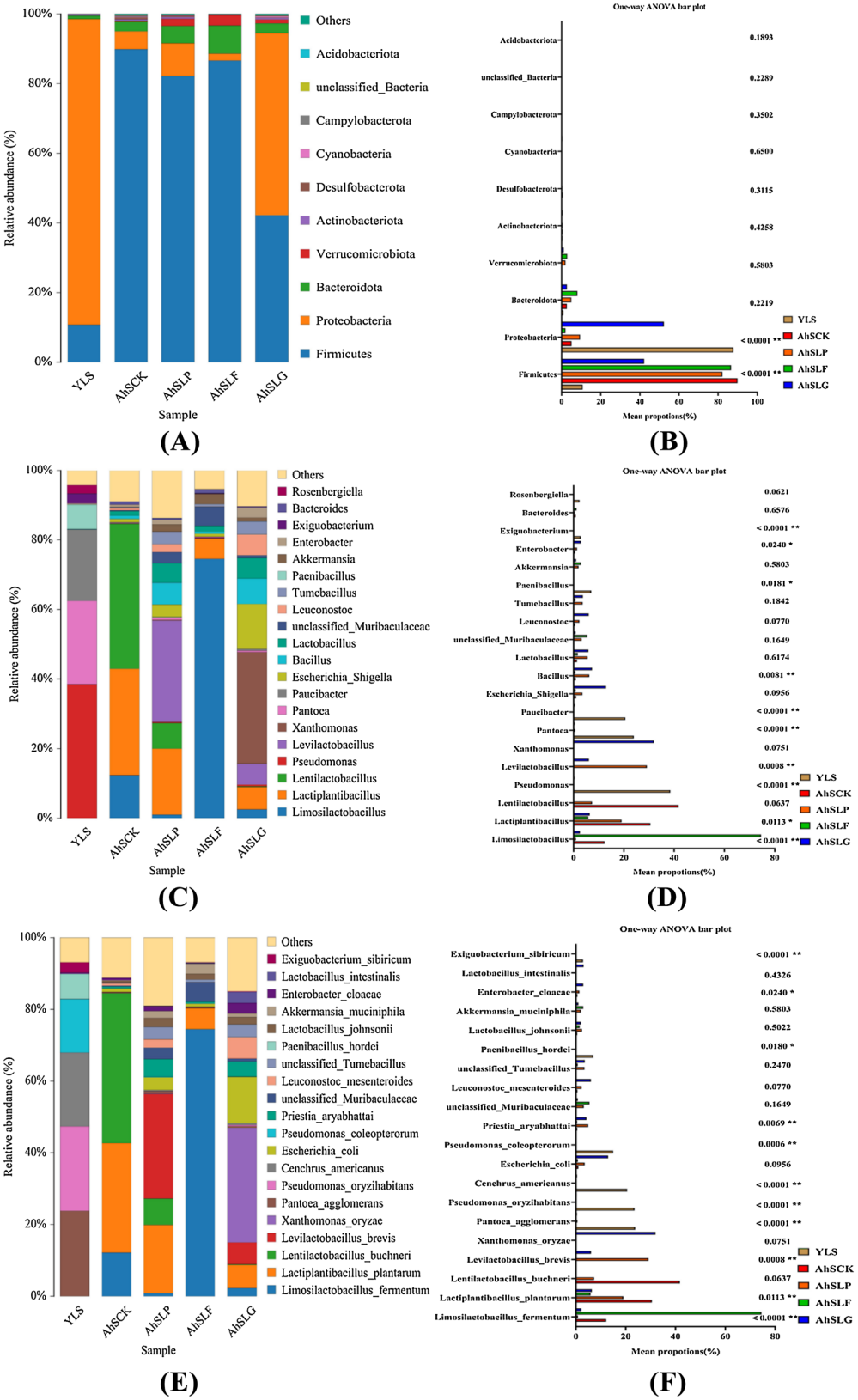


Fig. 2 (See legend on previous page.)

3. More interestingly, pyruvate metabolism, glycolysis/gluconeogenesis, and amino sugar and nucleotide sugar metabolism were enriched after ensiling. It is worth mentioning that the abundance of AhSLP for amino sugar and nucleotide sugar metabolism were significantly increased compared to YLS.

Among the pentose phosphate pathway, the trend of increasing abundance of AhSLF was the most pronounced. Overall, the performance of AhSLG in carbohydrate metabolism was poor compared to other treatments. Biosynthesis of antibiotics and Biosynthesis of amino acids were enriched after anaerobic fermentation process. However, the abundance of microbial metabolism in diverse environments decreased during ensiling. The metabolic pathways of AhSLF were significantly reduced and the biosynthesis of secondary metabolites and biosynthesis of antibiotics were significantly increased compared to fresh samples and silage. Importantly, the abundance of carbon metabolism in the silage without LAB addition was significantly lower than in the YLS and other treatments. Some metabolic pathways of amino acid metabolism, energy metabolism, nucleotide metabolism, and translation remained stable after fermentation.

Disscusion

LAB has been found and identified in forage crops, silage, and dairy products [31].In addition, LAB is the dominant microorganism in forage and silage [32]. Nonetheless, it can be a challenge to identify species among dichotomous species by morphological, physiological and biochemical tests [47]. The 16S rRNA sequence analysis identifies organisms by genus and species [35].

Strain BL1 was able to grow normally at pH 3.5–9.0 and weakly at pH 3.0 in our study. Strain BL5 was able to grow normally at pH 4.0–9.0. It indicated that strain BL1 had a high tolerance to acidity, and it was able to grow in a low pH condition. This conclusion was confirmed earlier in previous studies where *L. fermentum* showed intentional tolerance to low pH [48, 49]. It was found that both strains differed in the ability to grow in acidic environments, as well as in the temperature of the growth environments. Strain BL5 grew well at 10–45 ℃ and was able to grow weakly at 5 ℃. Nevertheless, strain BL1 was able to grow normally at 30–45 ℃ and weakly at 10 ℃, which can be attributed to long-term evolution and natural selection in a unique environment typical of

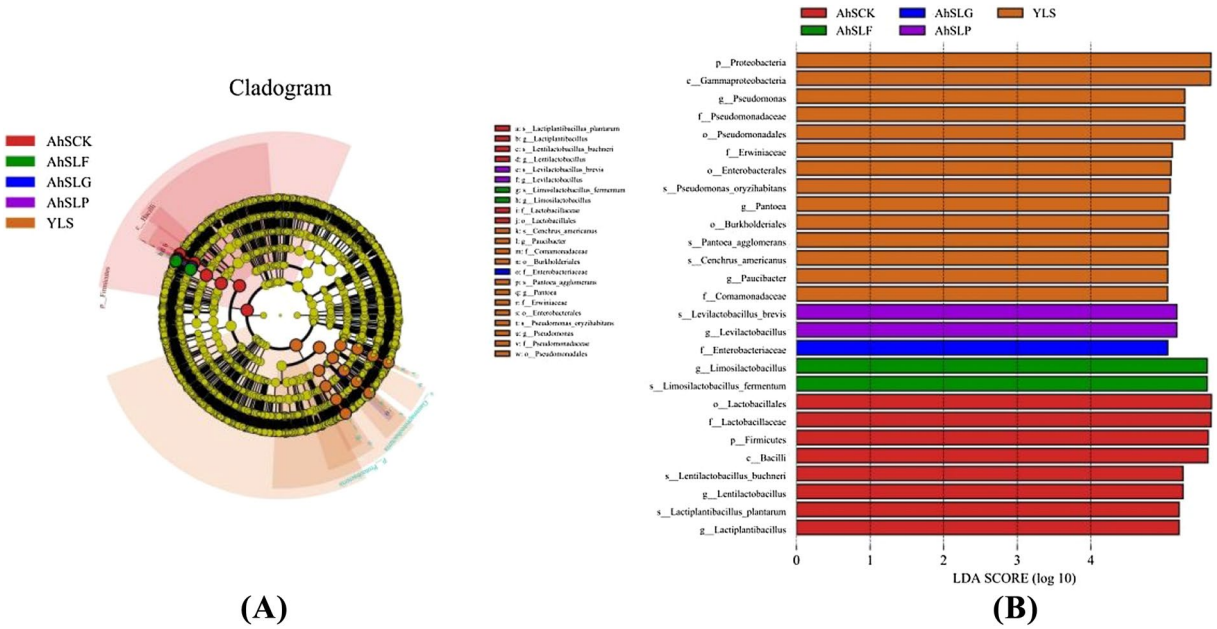


Fig. 3 The linear discrimination analysis (LDA) coupled on the bacterial community of fresh material and amaranth on 60 days of ensiling, with effect size (LEfSe) analysis. The significant difference in species was estimated by an LDA score greater at default score = 5. The length of the histogram shows the LDA score of differences in these groups. The circles radiating from the inside to the outside of the evolutionary branching diagram represent the taxonomic levels from phylum to species; each small circle at a different taxonomic level represents a taxon at that level, and the size of the diameter of the small circle is proportional to the size of the relative abundance; the colouring principle is that the species with no significant differences are uniformly coloured in yellow, and the other differences are coloured in accordance with the subgroup of the species with the highest abundance. Different colours indicate different subgroups, and nodes of different colours indicate microbiota that play an important role in the subgroup represented by the colour. YLS, fresh amaranth; AhSCK, control group; AhSLP, commercial inoculant group; AhSLF, strain BL1 group; AhSLG, strain BL5 group

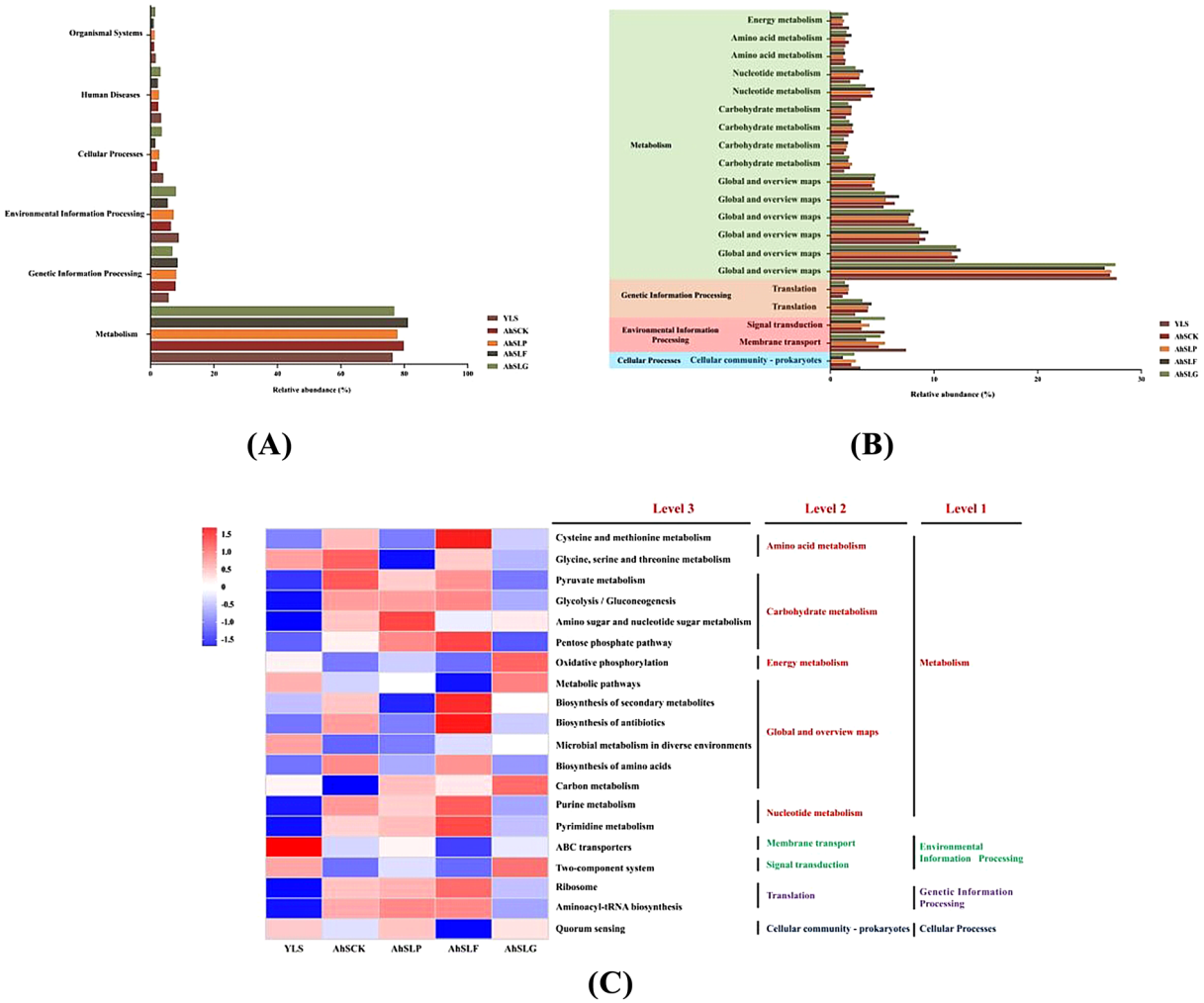


Fig. 4 Functional predictions for silage microbiota with significantly different KEGG pathways ($p < 0.05$) among the three groups of silage (YLS, AhSCK, AhSLP, AhSLF, and AhSLG). KEGG pathways at Level 1 (**A**), Level 2 (**B**), and Level 3 (**C**) are represented. YLS, fresh amaranth; AhSCK, control group; AhSLP, commercial inoculant group; AhSLF, strain BL1 group; AhSLG, strain BL5 group. *$p < 0.05$; **$p < 0.01$

grasslands [50]. In addition, strain BL1 was able to ferment more products compared to strain BL5.

Amaranth is a high protein forage grass. The CP and WSC contents of fresh amaranth were 9.19 and 0.52% DM, respectively, which were lower than those previously reported by Abbasi et al. [51]. The low levels of CP and WSC in this study may be related to the climate of the experimental area as well as photosynthesis, etc. [52, 53]. Fertilizer application, harvest season, sowing density and irrigation are key factors affecting the nutrient content of forage grasses [54]. The necessary conditions for obtaining high quality silage and high DM recovery are known to be a DM content of 300–350 *g*/kg and WSC content > 50 *g*/kg DM of raw material [29]. In this study, the DM content of fresh amaranth was in the range of DM content of high quality silage. While the WSC content of the fresh amaranth failed to satisfy the criteria for high-quality silage. And the amount of LAB attached to fresh amaranth was lower than 10^5 log10 cfu/*g* FM [55], the number of other harmful microorganisms attached was low. In previous studies, fresh material of different forage species (e.g. *Leymus chinensis*, paper mulberry, rice straw and whole-plant quinoa) had higher WSC content than amaranth, but the number of attached molds, yeast, aerobic bacteria, and *Escherichia coli* was similarly higher than fresh amaranth. However, the fermentation quality of the silage was good in all cases under poorer raw material conditions and by different means of silage [56–61], which is consistent with the results obtained in our study. The main explanation of this phenomenon may be due to the fact that when the amaranth enters the anaerobic fermentation stage, the reproductive metabolic activity of the anaerobic microorganisms attached to the surface gradually ceases. Meanwhile,

these microorganisms utilise the sugars of amaranth for warmth and metabolic activities. However, the low sugar content of amaranth results in a relatively small amount of available sugar. The available sugar was used by LAB to proliferate and inhibited the reproductive metabolic activities of other aerobic microorganisms, which contributed to the proliferation of LAB and the sustained decrease in pH. It is worth mentioning that fresh amaranth has a low number of harmful micro-organisms (such as molds, yeast, aerobic bacteria, and *Escherichia coli*) attached to it. In addition, NDF and ADF contents were comparable to the fresh maize and Italian ryegrass [34, 44].

The abundance, taxonomy and characteristics of the attached microorganisms (especially LAB) are crucial for ensiling [32]. The addition of different types of LAB increased the content of fermentation substrates and promoted the growth of LAB to different degrees, and the LA which they produced decreased the pH and inhibited the proliferation of harmful bacteria (yeast and aerobic bacteria). Interestingly, the highest amount of LAB was attached to AhSLF, but LA content generated was lower than AhSLP ($p < 0.05$). This may be due to the fact that *L. fermentum* acts as a heterotypic fermenting LAB, producing LA, AA and CO_2 through the pentose phosphate pathway, thus creating an acidic environment for silage [62]. This was also confirmed by the high AA content of AhSLF. BA was detected in AhSCK and AhSLG, especially the addition of *L. graminis*, which did not inhibit the production of spoilage acid and had a higher BA content than control group ($p < 0.05$). This may be explained by the high abundance of *Xanthomonas oryzae* which is high enough to be one of the dominant strains of AhSLG for silage fermentation (Fig. 2). pH is one of the key indicators to evaluate the good quality of silage [3]. It has been shown that spoilage bacteria can be inhibited by a lower acidic environment, and silage was considered well fermented when pH reached 4.20 [32, 63]. In this study, the pH of AhSLP and AhSLF were lower than 4.20, indicating that both treatments attained a good level of fermentation quality. The higher pH and lower CP content of AhSCK in comparison to the additive treatment may be due to the high accumulation of alkaline nitrogenous substances (e.g. ammonia and amines). The NH_3-N content of silage with additives was lower than the control, while the CP content was higher than the control. Most of the NH_3-N in silage is the result of protein degradation, which coincides with why silage without additives had low CP and high NH_3-N content [64].

We performed high-throughput sequencing techniques to evaluate bacterial communities and structure, and to predict changes in metabolic function. Both Chao1 and Shannon indices have characteristics that reflect bacterial abundance and species diversity [65]. In this study, a significant difference in bacterial diversity was observed between YLS and different treatments of silage in terms of changes in Shannon's index, which is consistent with previous reports, as undesirable microorganisms were inhibited by pH and LAB gradually dominated the entire fermentation process [66, 67], hence reduced α-diversity was observed. The coverage of fresh amaranth and silage exceeded 0.99, which indicated that sequencing could accurately screen the bacterial community with high confidence [68]. YLS and all treatments were well separated (Fig. 1), which suggested that the different additives had a definite effect on the bacterial community, which would be similar to the confirmed reports that additives increased the variability of the bacterial community and could explain the differences in silage quality [69].

Proteobacteria was the dominant phylum in YLS, accounting for about 90% of all bacterial species. In amaranth silage, the dominant phylum shifted from Proteobacteria to Firmicutes (Fig. 2), which was consistent with the results of previous studies [70]. At the genus level, there were significant differences between YLS and silage. In general, well-fermented silage were dominated by *Lactobacillus*, *Lactococcus*, *Pedicoccus*, *Weissella*, and *Leuconostoc* of the Firmicutes, which had the ability to decrease the initial pH of the silage and accelerate the anaerobic fermentation process. Pseudomonas, Pantoea and Paucibacter are Gram-negative bacteria harmful to silage fermentation [71], and they accounted for most of the epiphytic bacteria in YLS. They can compete with LAB for nutrients and cause deterioration of food and fermented feed. After ensiling, Pseudomonas, Pantoea and Paucibacter are reduced or completely suppressed. Some variation also existed in the strains of bacteria epiphytic in silage containing different LAB. The dominant species of AhSCK were *L. plantarum* and *Lentilactobacillus buchneri*, which confirmed that epiphytic harmful bacteria of fresh materials were rapidly inhibited under anaerobic environment and low pH stress. There are differences in the effect of different types of LAB on the silage fermentation. The dominant species of AhSLP was *Levilactobacillus brevis*. The dominant strains of AhSLG was *Xanthomonas oryzae*, a phytopathogenic bacterium capable of causing multiple diseases in globally important crops [72]. It successfully attached to the host plant by adhering to the plant surface, invading the cellular interstices of the host tissue, thereby obtaining nutrients and counteracting the plant defense response. It is noteworthy that *L. graminis* was not seen among the AhSLG-attached isolates, the reason for this being that *L. graminis* is in the early stages of fermentation, and is not present in sufficient numbers or abundance to be detected in the late stages of silage fermentation

[72]. In addition, the abundance of pathogenic bacteria and *Escherichia coli* accounted for a high percentage of the epiphytic bacteria. These strains competed with LAB for substrate and adversely affected the fermentation of silage, as corroborated by the high BA content of AhSLG. The dominant species of AhSLF was *L. fermentum*. Antimicrobial activity of L. fermentum is mainly due to its ability to produce organic acids (mainly LA and AA) and antimicrobial peptides [73]. The potential probiotic properties of *L. fermentum* strains were verified by in vitro digestion techniques. It has also been shown that *L. fermentum* is capable of fermenting dietary fiber and releasing short-chain fatty acids (including PA, BA, AA, and LA) in the intestine [74, 75]. This is sufficient to show that silage supplemented with *L. fermentum* had the most epiphytic LAB, the lowest pH and relatively high LA and AA content. The culture supernatant of *L. fermentum* at low pH showed greater inhibition of *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa*, demonstrated a significant correlation between the inhibition activity of this strain and the low pH caused by organic acids [76], it showed similar results to the present study.

Ensiling is dominated by microbial activity. It degrades substrates or converts metabolites through complex metabolic pathways. The functional characteristics of bacterial communities in raw materials and silage were predicted using PICRUSt software, based on the KEGG databases, and the metabolic pathways of their epiphytic bacteria were analyzed. In KEGG analysis, metabolism was the main metabolic pathway, indicating that bacteria can convert substrates into various metabolites through bacterial activity in the metabolic pathway. Carbohydrate metabolism and global and overview maps were the most important metabolic categories for raw materials and silage. Pyruvate metabolism, glycolysis/gluconeogenesis, amino acid sugar, and nucleotide sugar metabolism were essential components of carbohydrate metabolism. In the present study, the proportion of carbohydrate metabolic pathways was higher in silage than in raw materials, reflecting the community dynamics and metabolic activity of the main LAB. In particular, the propionic acid metabolism, pentose phosphate pathway and glycolytic abundance of AhSLG were lower than other treatments, which suggested that the *L. graminis* was not as metabolically competent as homozygous fermentative LAB [62], resulting in high pH and low production of LA and AA in *L. graminis*-containing silage, which consequently failed to inhibit the growth and reproduction of epiphytic harmful microorganisms. The abundance of pentose phosphate pathway was higher in AhSLF than in other silage. This is due to the fact that *L. fermentum* is a parthenogenic hetero-fermentation LAB that metabolizes pentose through the pentose phosphate pathway to produce LA and AA. The high LA and AA content detected in AhSLF and the high abundance of epiphytic *L. fermentum* and *L. plantarum* also corroborate this phenomenon.

Metabolic pathways, biosynthesis of secondary metabolites, biosynthesis of antibiotics, microbial metabolism in diverse environments, biosynthesis of amino acids, and carbon metabolism were the essential parts of global and overview maps. The synthesis of secondary metabolites varies from strain to strain and also controls the synthesis of antibiotics. However, until now, there is no agreement on the physiological functions of secondary metabolites and antibiotics in silage. The abundance of microbial metabolism in different environments was higher in the fresh material and decreased after the fermentation process. It shows that the fermentation process of silage effectively reduced some undesirable microorganisms attached to the environment, which is consistent with the data of microbial diversity (Fig. 2). The high metabolic abundance of AhSLG indicates that the microbial cellular activity at the early stage of silage fermentation in response to changes in the growth environment is rapidly changing metabolic responses. It also can further prove that *L. graminis* is more active in the early stage of fermentation. Amino acid synthesis occurs primarily through transamination, where the amino group of an amino acid is transferred to a keto acid to form a new amino acid. Microorganisms can consume some amino acids in excess to obtain some amino acids in lesser amounts through transamination. The metabolic abundance of amino acid synthesis was higher for AhSCK and AhSLF than other treatments, which also had relatively high amino acid metabolic abundance. Amino acid metabolism is necessary to promote primary metabolism and plant protein synthesis in plants [77]. The level of metabolic abundance may reflect the ability of the initial microbial population in the silage to synthesize amino acids. In our study, the abundance of cysteine and methionine, glycine, serine and threonine metabolism was higher in AhSCK and AhSLF than in other treatments. This may be related to the high abundance of epiphytic *L. fermentum*, but it needs further verification. Thus, the dynamics of amino acid metabolism observed in fresh materials and silage may reflect the metabolism of dominant populations throughout the ensiling process.

Conclusions

The bacterial community of fresh amaranth was found to be dominated by *Pantoea agglomerans*, *Pseudomonas oryzihabitans*, *Cenchrus americanus*. The bacterial community of amaranth with and without the addition of LAB underwent significant changes after fermentation.

Silage with the addition of strain BL1 (*L. fermentum*) and strain BL5 (*L.graminis*) had substantially different fermentation characteristics due to their fermentation pathways. It is noteworthy that in silage containing *L.graminis*, pathogenic bacteria, *Xanthomonas oryzae*, became the dominant strain in the silage process. And *L.graminis* failed to inhibit the growth and reproduction of harmful microorganisms. With the addition of *L. fermentum*, it dominates and thus reduces the pH. By comprehensive consideration, *L. fermentum* can be recommended as an additive to improve the quality of amaranth silage, as it regulates the epiphytic microbiota in silage, improving the fermentation quality andmetabolic capacity of the bacterial community.

Author contributions
MZ: conceptualization, investigation, visualization, methodology, formal analysis, writing—original draft. JB: conceptualization, investigation, formal analysis. ZW: supervision, writing—review & editing. SD: conceptualization, investigation, formal analysis. CG: conceptualization, investigation, formal analysis. DN: conceptualization, investigation, formal analysis. XY: conceptualization, investigation, formal analysis. GG: conceptualization, methodology, validation, investigation, writing—review & editing, funding acquisition.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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