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Exploring the bacterial community succession and metabolic profiles of *Lonicera japonica* Thunb. residues during anaerobic fermentation

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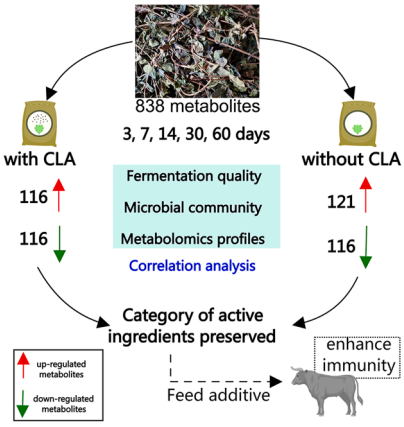
HIGHLIGHTS

- First reported the metabolomics of LJT residues during anaerobic fermentation.
- Anaerobic fermentation is a practical approach to utilize the LJT residues.
- Anaerobic fermentation couldn't cause a functional deficiency of metabolites.
- Compound additives improved fermentation quality and reduced undesirable bacteria.

GRAPHICAL ABSTRACT



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ABSTRACT

Discarding *Lonicera japonica* Thunb. (LJT) residues containing many active metabolites create tremendous waste. This study aimed to effectively use LJT residues by anaerobic fermentation. Fermentation significantly decreased the pH values and reduced the abundance of undesirable bacteria (potential pathogenic and biofilm-forming) while increasing *Lactobacillus* abundance. Compound additive use further improved fermentation quality (significantly increased the lactic acid (LA) content and decreased the pH values and ammonia nitrogen (a-N) content) and nutrient quality (significantly decreased the acid detergent fiber (ADF) content and increased the water-soluble carbohydrate (WSC) content) and optimized the microbial community (increased the *Lactobacillus* abundance). Fermentation also altered the flavonoids, alkaloids and phenols contents in the residues with minor

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effects on the functional metabolites amounts. The LJT residues metabolic profile was mainly attributed to its epiphytic bacteria, with a small contribution from the compound additive. Thus, compound additives may improve anaerobic LJT residue fermentation without functionally impairing the metabolites.

1. Introduction

Lonicera japonica Thunb. (LJT) is native to East Asia and has great medicinal value. In China, LJT flowers have long been used in traditional Chinese medicine to treat certain diseases, such as exogenous wind-heat and febrile diseases (Shang et al., 2011). Modern pharmacological studies have shown that the active ingredients of LJT possess broad pharmacological effects, such as anti-inflammatory activity (Park et al., 2005). The main components of LJT include organic acids and flavonoids, which are closely related to its pharmacological effects (Li et al., 2019). In addition to treating some diseases, Chinese herbal medicines have also been used in fermented feed applications to improve the fermentation quality and flavor and increase the beneficial metabolites, thereby having a favorable effect on livestock (Lin et al., 2020). Because of the medicinal value of LJT flowers, they are frequently used to treat diseases (Upadhyay and Mohan Rao, 2013), however, the LJT residues (i.e., stems and leaves) are directly discarded as a byproduct of drug production, resulting in a large waste of resources.

Anaerobic fermentation can inhibit the growth of most aerobic spoilage bacteria and has been applied for the utilization of herbal residues (Li et al., 2022c). Ardó et al. (2008) reported that LJT enhances the immune response and disease resistance of fish. As LJT residues also contain large amounts of active ingredients, these residues can be used as an anaerobic fermentation additive. Fermented LJT feed may have the potential to increase feed intake and improve animal immunity, which could bring substantial economic benefits to the breeding industry and allow the reuse of LJT residues. Li et al. (2022c) found that herbal residue addition could change fermentation quality and reduce the ammonia nitrogen (a-N) content by altering the microbiota. This provides a reference for better utilization of LJT residues. Ruminants in the breeding industry have a greater demand for fermented feed. Fermented feed provides livestock with nutritious and delicious food in the winter when fresh fodder is unavailable (Borreani et al., 2018). Traditional fermented feed can provide essential nutrition for animals, but it may not promote immune function in livestock. Therefore, LJT residues may extend the related functions since they contain substantial amounts of active ingredients (Shang et al., 2011). However, it is necessary to determine whether the active ingredients of LJT residues will change significantly during fermentation or not.

Lactic acid bacteria (LAB) and enzymes are often used as additives to improve fermentation quality and nutritional value (Muck et al., 2018). For instance, *Lactobacillus plantarum* B90 combined with *Lactobacillus hilgardii* 60TS-2 can reduce dry matter (DM) loss and improve the aerobic stability of sugarcane tops (Wang et al., 2020). Cellulase can degrade fibers and increase the water-soluble carbohydrate (WSC) content, so it is often applied with LAB (Wang et al., 2022). Fermentation trials using compound additives have also been conducted and produced ideal results both in the laboratory and on livestock farms (Wang et al., 2020).

Therefore, this study first aimed to evaluate the fermentation quality of LJT residues. Then, 16S rDNA full-length sequencing technology and wide-target metabolomics were used together to explore the dynamic changes in the microbial community and metabolites of the LJT residues during fermentation with or without a compound additive. This study provides a scientific theoretical basis for applying LJT residues as a feed additive, reducing its waste.

2. Materials and methods

2.1. Materials and silage preparation

The LJT residues were provided by the Institute of Biology Co., Ltd., Henan Academy of Science. The samples were adjusted to approximately 40 % DM and then chopped into 1–2 cm pieces with a crop chopper (ZS-2, Zhongsheng Agricultural Machinery Company, Tangshan, China). This study was carried out at IMCAS, Beijing (40.00° N, 116.38° E), indoors at room temperature. The LC group was treated with compound additive, which was composed of *L. plantarum* B90 (CGMCC No. 13318), *Lactobacillus fermentum* 17SD-2 (CGMCC No. 15448), and *Lactobacillus farciminis* GMX4 (CGMCC No. 19434), each at 10^6 CFU/g fresh sample (FS), and cellulase (1 ‰ FS), and the CK group was treated with an equal volume of distilled water. The experiment included a total of 30 sealed bags (2 treatments × 3 replicates × 5 time points), which were stored at room temperature (21–30 °C). The LC group included the samples LC1-LC3 (LCD3), LC4-LC6 (LCD7), LC7-LC9 (LCD14), LC10-LC12 (LCD30), and LC13-LC15 (LCD60), while the CK group included the samples CK1-CK3 (CKD3), CK4-CK6 (CKD7), CK7-CK9 (CKD14), CK10-CK12 (CKD30) and CK13-CK15 (CKD60). LCD3 and CKD3, LCD7 and CKD7, LCD14 and CKD14, LCD30 and CKD30, and LCD60 and CKD60 are the fermentation samples unpacked at 3, 7, 14, 30 and 60 days (D), respectively (see Supplementary material).

2.2. Sampling and culture-based microbial analyses

The detection of culturable microorganisms (CMs) was performed using the methodology described by Sun et al. (2021). Briefly, 10 g samples were resuspended in 90 mL of sterile phosphate-buffered saline (PBS) and used to measure the CMs after each bag was unpacked. The gradient dilution method was used to enumerate CMs. LB, MRS, and PDA media were used to enumerate aerobes, LAB, and yeasts, respectively. The colonies were identified and converted into the corresponding number of colonies proportionally, and the number of colonies per gram of sample was calculated.

2.3. Fermentation quality analysis

Fermentation quality was analyzed according to the previous method described by Sun et al. (2021). Briefly, 10 g of each sample were resuspended in 90 mL of sterile water and incubated overnight at 4 °C. The supernatant was centrifuged at 5000 rpm for 20 min at 4 °C and decontaminated by filtration using a 0.22 μm filter membrane. Then, the pH values, contents of organic acids (i.e., lactic acid (LA), acetic acid (AA), propionic acid (PA), butyric acid (BA)) and the a-N content in the sterile supernatant were determined. A pH meter (pH 213, HANNA, Italy) was used to determine the pH values. High-performance liquid chromatography (HPLC, 1200, Agilent, California, USA) instrument was used to determine the contents of organic acids. The mobile phase consisted of 0.004 mol/L H_2SO_4 and elution was carried out at a flow rate of 0.6 mL/min. The colorimetric ninhydrin and phenol-hypochlorite methods were used to analyze the a-N levels (Broderick and Kang, 1980).

2.4. Chemical composition analysis

Samples of approximately 150 g were used to analyze the chemical composition including DM, crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) and WSC. The samples were treated at

Table 1
The pH values, chemical composition and cultivable microbial population of fresh and fermented LJT residues.

	FS	CKD3	CKD7	CKD14	CKD30	CKD60	LCD3	LCD7	LCD14	LCD30	LCD60	F value
pH values	6.54 ± 0a	4.51 ± 0.08c	4.66 ± 0.01b	4.24 ± 0.03e	4.24 ± 0.04e	4.36 ± 0.15d	4.08 ± 0.02f	4.33 ± 0.02de	3.94 ± 0 g	3.95 ± 0.01 g	3.95 ± 0.03 g	546.67
DM (%)	37 ± 0a	37.3 ± 0.03a	34.47 ± 2.31bcd	35.4 ± 0.02b	34.8 ± 0.10bcd	35.37 ± 0.06b	35.3 ± 0.02b	35.1 ± 0.01bc	33.4 ± 0d	33.5 ± 0.02d	33.73 ± 0.06 cd	10.14
CP (DM %)	7.47 ± 0.15ab	7.27 ± 0.06b	7.8 ± 0.61a	7.3 ± 0.17ab	7.37 ± 0.06ab	7.8 ± 0.26a	7.4 ± 0.2ab	7.3 ± 0.17ab	7.4 ± 0.1 ab	7.4 ± 0.1ab	7.53 ± 0.12ab	1.89
NDF (DM %)	74.96 ± 0.55c	76.9 ± 0.66abc	80.5 ± 5.89a	77.2 ± 0.53abc	76.23 ± 0.15bc	78.87 ± 0.25ab	75 ± 0.96c	74.93 ± 0.51c	74.27 ± 0.99c	74.17 ± 1.10c	74.83 ± 0.15c	3.48
ADF (DM %)	58.43 ± 0.35d	62.03 ± 0.06bc	63.93 ± 4.65ab	61.97 ± 0.25bc	62.37 ± 0.15bc	65.8 ± 0.87a	60.07 ± 0.90 cd	60.77 ± 0.32 cd	60.13 ± 0.29 cd	61.07 ± 0.49bcd	59.53 ± 0.42 cd	5.99
WSC (DM %)	12.81 ± 0.91a	1 ± 0.03e	1.03 ± 0.03e	1.02 ± 0.03e	1.09 ± 0.01e	1.2 ± 0.21de	1.64 ± 0.06c	1.6 ± 0.06 cd	1.73 ± 0.057c	1.82 ± 0.04c	2.45 ± 0.65b	41.655
	Cultivable microbial population (lg CFU/g FS)											
LAB	2.54 ± 0.12f	7.6 ± 0.10b	7.57 ± 0.07b	7.5 ± 0.07b	6.38 ± 0.07c	5.52 ± 0.11d	8.36 ± 0.37a	7.72 ± 0.05b	7.34 ± 0.16b	6.2 ± 0.11c	4.32 ± 0.21e	233.55
Yeast	4.51 ± 0.19bc	5.37 ± 0.08a	0 ± 0d	0 ± 0d	4.13 ± 0.50bc	3.97 ± 0.33c	4.65 ± 0.44b	0 ± 0d	0 ± 0d	4.38 ± 0.39bc	4.03 ± 0.61c	176.58
Aerobes	6.73 ± 0.17a	4.89 ± 0.02bc	4.82 ± 0.08bc	4.97 ± 0.04b	4.77 ± 0.09c	4.82 ± 0.11bc	4.99 ± 0.03b	4.83 ± 0.12bc	4.98 ± 0.14b	4.88 ± 0.05bc	4.89 ± 0.06bc	108.18

DM, dry matter; FS, fresh sample; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; WSC, water-soluble carbohydrates; LAB, lactic acid bacteria; CFU, colony-forming units. The degree of freedom of these data was 32. Means ± SDs within the same row with different letters present significant differences ($P < 0.05$).

65 °C for 48 h and then dried at 105 °C until they reached a constant weight. The DM content was calculated according to a previous report (Sun et al., 2021). Then, the dried samples were ground for chemical composition analysis. The CP content was determined by the AOAC official method 988.05 with $CuSO_4/TiO_2$ mixed catalyst and Kjeldahl method according to Helrich (1990). The WSC content was detected by phenol–sulfuric acid method according to Rover et al. (2013). NDF and ADF were determined by the filter bag technique according to Van Soest et al. (1991).

2.5. Sequencing-based microbial analysis

The extraction of DNA from the samples was performed according to the method of Wang et al. (2020). Briefly, 10 g samples were shaken with sterile PBS for 10 min, then each mixture was filtered through four layers of cheesecloth, and the filtrate was centrifuged at 10,000 rpm for 10 min at 4 °C. The deposit was used for DNA extraction by the MolPure Soil DNA Kit (Yeasen, Beijing, China) according to the manufacturer's instructions. Total DNA was used for 16S rDNA sequencing. The universal primer pairs 27F (5′-AGRGTTYGATYMTGGCTCAG-3′) and 1492R (5′-RGYTACCTTGTTACGACTT-3′) were used to amplify the whole sequence of 16S rDNA sequence (Callahan et al., 2019). The products of three repeat amplifications were mixed and used for sequencing with the PacBio Sequel II System (Pacifc Biosciences, CA, USA) by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China). The raw data from sequencing were subjected to quality control, and optimized data were used for OTU analysis. The similarity among the microbial communities in different samples was determined by nonmetric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity. Linear discriminant analysis (LDA) effect sizes (LEfSe) was performed to identify bacterial taxa that were significantly enriched among the different groups (Segata et al., 2011). The co-occurrence networks were used to investigate the internal community relationships among the samples. The metagenomic function was predicted by PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Douglas et al., 2020).

2.6. Metabolomics analysis

To determine changes in metabolite levels, wide-target metabolomics was performed with the assistance of Shanghai Biotree Biotech Co., Ltd. Lyophilized samples were crushed with a grinder at 60 Hz for 2 min. A total of 1000 μL of a methanol/water mixture (v:v = 3:1) was used to extract the metabolites. The samples were vortexed the samples for 30 s and then sonicated in an ice bath for 15 min, followed by shaking overnight at 4 °C. Then, the samples were centrifuged at 12,000 rpm for 15 min at 4 °C. The resulting supernatants were passed through a 0.22 μm filter membrane and stored at −80 °C until ultrahigh-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS, AB Sciex QTOF 5600^+) analysis was performed. Quality control samples were prepared by mixing equal amounts of supernatant from all samples. For analysis, a Waters ACQUITY UPLC HSS T3 column (100 × 2.1 mm, 1.8 μm) was used, mobile phase A consisted of 0.1 % formic acid, and acetonitrile was used as mobile phase B. The column temperature was adjusted to 40 °C, the autoinjector temperature was adjusted to 4 °C and the injection volume was 2 μL. The high-resolution mass spectrometry data were transformed into mzXML format using ProteoWizard and processed by MAPS software (version 1.0). The pretreatment results generated a data matrix that included retention time, mass-to-charge ratio (m/z) values, and peak intensities. The internal MS2 database was applied for metabolite identification.

2.7. Statistical analyses

Data processing and graphing were performed using GraphPad Prism 7 and OriginPro 2021 (9.8.0.200) software. Data are presented as Means ± SDs. IBM SPSS Statistics (R23.0.0.0) was used to analyze the degrees of freedom, F values and P values. Significant differences between the two groups were analyzed using Student's t-test, and significant differences among multiple groups were analyzed using two-way ANOVA (with the Duncan test). Correlation analysis was performed using the package in R 4.0.2 software. Significance was declared at $P < 0.05$.

3. Results and discussion

Anaerobic fermentation has already been used to prevent plant byproduct waste (Wang et al., 2020), providing a reference to tackle the issue of LJT residue waste. However, little is known about the dynamics of the microbial community and the active ingredients of LJT residues after fermentation. Li et al. (2022c) studied 36 kinds of herbal residues in the anaerobic fermentation of alfalfa, and they proposed that the increased bioactive ingredients may play a critical role in improving the fermentation quality. Therefore, it was hypothesized fermentation would change the active ingredient content and modify the

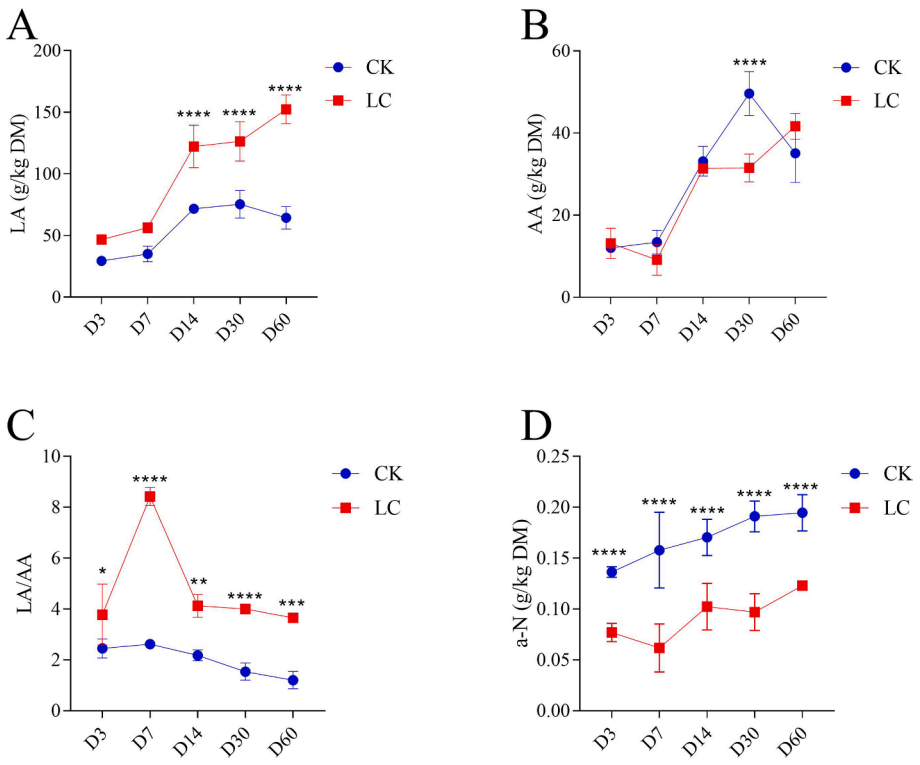

Fig. 1. Fermentation quality of *Lonicera japonica* Thunb. (LJT). LA content (A), AA content (B), the ratio of LA/AA (C) and a-N content (D) of LJT. LA: lactic acid; AA: acetic acid; a-N: ammonia nitrogen. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

pharmacological value of LJT residues.

3.1. Characteristics of fresh LJT residues

The chemical composition and microbial population of the fresh LJT residues are shown in Table 1. The DM was 37 %, and CP, NDF, ADF, and WSC accounted for 7.47 %, 74.96 %, 58.43 %, and 12.81 % of the DM, respectively. The epiphytic LAB, yeast and aerobe abundances were 2.54, 4.51 and 6.73 lg CFU/g FM, respectively. Anaerobic fermentation by microbial decomposition of WSC can produce organic acids, thereby lowering the pH values and inhibiting the proliferation of deteriorating bacteria (Muck et al., 2018). A high WSC content may lead to excess microbial fermentation and affect the active ingredients of the LJT residues. In this study, even with enough WSC as a substrate, it was also difficult to achieve good fermentation when the LAB abundance was insufficient (<5 lg CFU/g FM) (Sun et al., 2021). Therefore, based on the low LAB and high fiber contents, LAB and cellulase addition were needed to speed up the fermentation process and improve the fermentation quality.

3.2. Dynamic culturable microbial population, chemical composition, and fermentation parameters of LJT residue fermentation

The dynamic culturable microbial population, chemical composition, and fermentation parameters are shown in Table 1. The LAB amount increased rapidly during the initial phase (D3), at which this value was significantly higher in the LC samples than in the CK samples, followed by a gradual decrease over time. A similar trend was reported by Ni et al. (2017), in which the LAB amount increased during the initial stage and gradually decreased as fermentation progressed. However, the amount of LAB in the CK group (5.52 lg CFU/g FM) was significantly ($P < 0.05$) higher than that in the LC group (4.32 lg CFU/g FM) at D60, which may be due to the proliferation of the LAB that had been added to the LC samples, resulting in the utilization of nutrients and the production of large amounts of LA that inhibited LAB growth. Nair et al. (2019) reported that the use of silage inoculants containing yeast has the potential to positively affect the nutrient digestibility of beef cattle. Yeast used as a direct-fed microbial (DFM) can increase the level of CP by 38–41 % (Muck et al., 2018). In this study, yeast was not detected at D7 and D14 but was detected again at D30 and D60, which was an inexplicable phenomenon. However, the fermentation quality was not affected in this study, indicating at a later stage, these yeasts have the potential to be used as DFMs. The aerobes decreased by an order of magnitude at D3 compared with the FS and they remained stable until D60. These results indicate that the higher levels of LAB in the LC samples contribute to the rapid reduction in pH value. The DM decreased over time, but neither the fermentation process nor the compound additive affected the CP contents. These slight differences in nutrient composition (DM, CP, NDF, and ADF) indicated that fermentation had only a small impact on the nutritional value of LJT. The LJT CP level was similar to that of whole plant corn silage (Jiao et al., 2021), and as there were no significant differences in the proportions of CP during the LJT fermentation process, it was determined that this process does not cause protein loss. The NDF content of LJT did not display a continuous decrease with the fermentation time, which was consistent with the results of whole crop wheat silage (Xia et al., 2018). The compound additive reduced the NDF at D7 and ADF at D60 and increased the WSC content, probably due to the action of cellulase, as reported by Su et al. (2019). These results are also in accordance with the report of Xia et al. (2022), and indicated that the fermented quality in the LC group was superior to that in the CK group.

The pH value is considered a critical measure to monitor the fermentation process, and a pH value below 4.2 is beneficial for long-term storage and aerobic stability (Li et al., 2022b). The pH values of the CK and LC samples dropped from 6.54 to 4.51 and 4.08 at D3, respectively. The pH values increased slightly at D7 before continuing to decrease and stabilized at a relatively low level (CK: 4.36, LC: 3.95) (Table 1). The pH values were reduced sharply in the LC samples compared with the CK samples, indicating that the compound additive facilitated the conversion of WSC into organic acids. The pH value of the

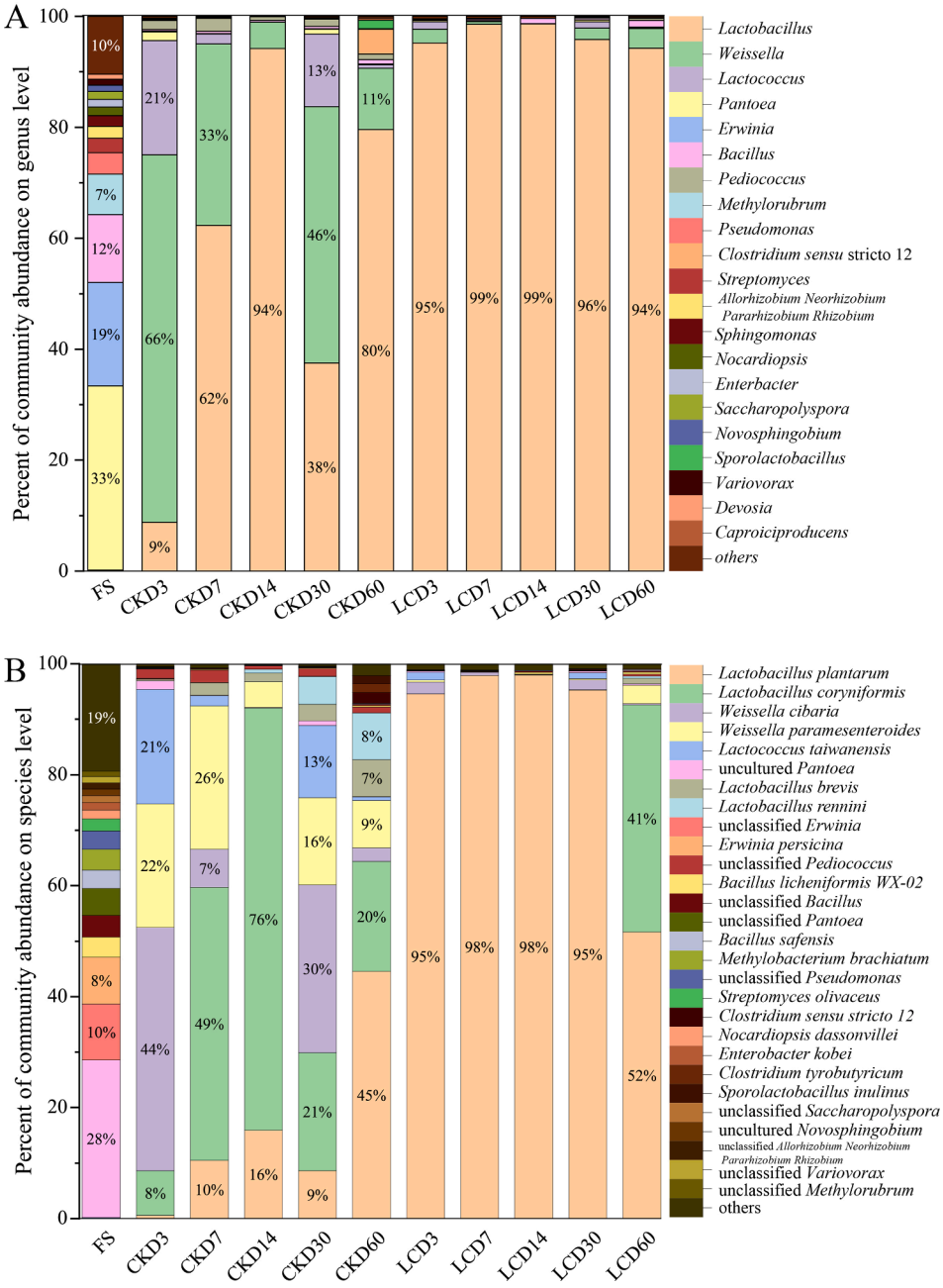

Fig. 2. Bacterial community barplot analysis of LJT. Percent of community abundance at the genus level (A). Percent of community abundance at the species level (B).

CK group was lower than that of the tea leaves (pH value = 4.8) reported by Lin et al. (2021). These tea leaves contained many bioactive ingredients but a low WSC content (4.33 % DM), highlighting that the high WSC content in the CK group contributed to the pH reduction. The drop in pH value was mainly due to the accumulation of organic acids, especially LA (Kung Jr et al., 2018). The LA and AA contents in the LC and CK samples increased continuously, and LA accounted for over 70 % of the total acid. The LA in the LC samples was always significantly higher than that in the CK samples (Fig. 1A-C), indicating that the fermentation quality of the LC group would be better than that of the CK group. In addition to the organic acids (LA and AA) produced by LAB, there might be several other organic acids in the LJT residues that may directly decrease the pH value (Li et al., 2022a). The content of a-N is an important index of protein degradation. In this study, the compound additive significantly reduced the a-N content ($P < 0.05$, Fig. 1D), which may help to reduce protein loss. Similar to Li et al. (2022c), the low level of a-N content in the LC samples might be due to the rapid reduction in pH, thus inhibiting the proteolytic bacteria, such as *Enterobacter*. To elucidate the essence of the changes in fermentation quality, the microbiota and metabolites of LJT residues were further studied.

3.3. Dynamics of the microbial community of LJT residue fermentation

Fermentation quality is associated with the microbiota (Muck et al., 2018); therefore, the dynamic changes in the microbial community of the LJT residues during fermentation were further investigated. NMDS analysis showed that all fermented samples were distant from the FS and relatively close to each other (see Supplementary material), highlighting that the microbial community composition of the fermented samples was significantly different from that of the FS. The variability between

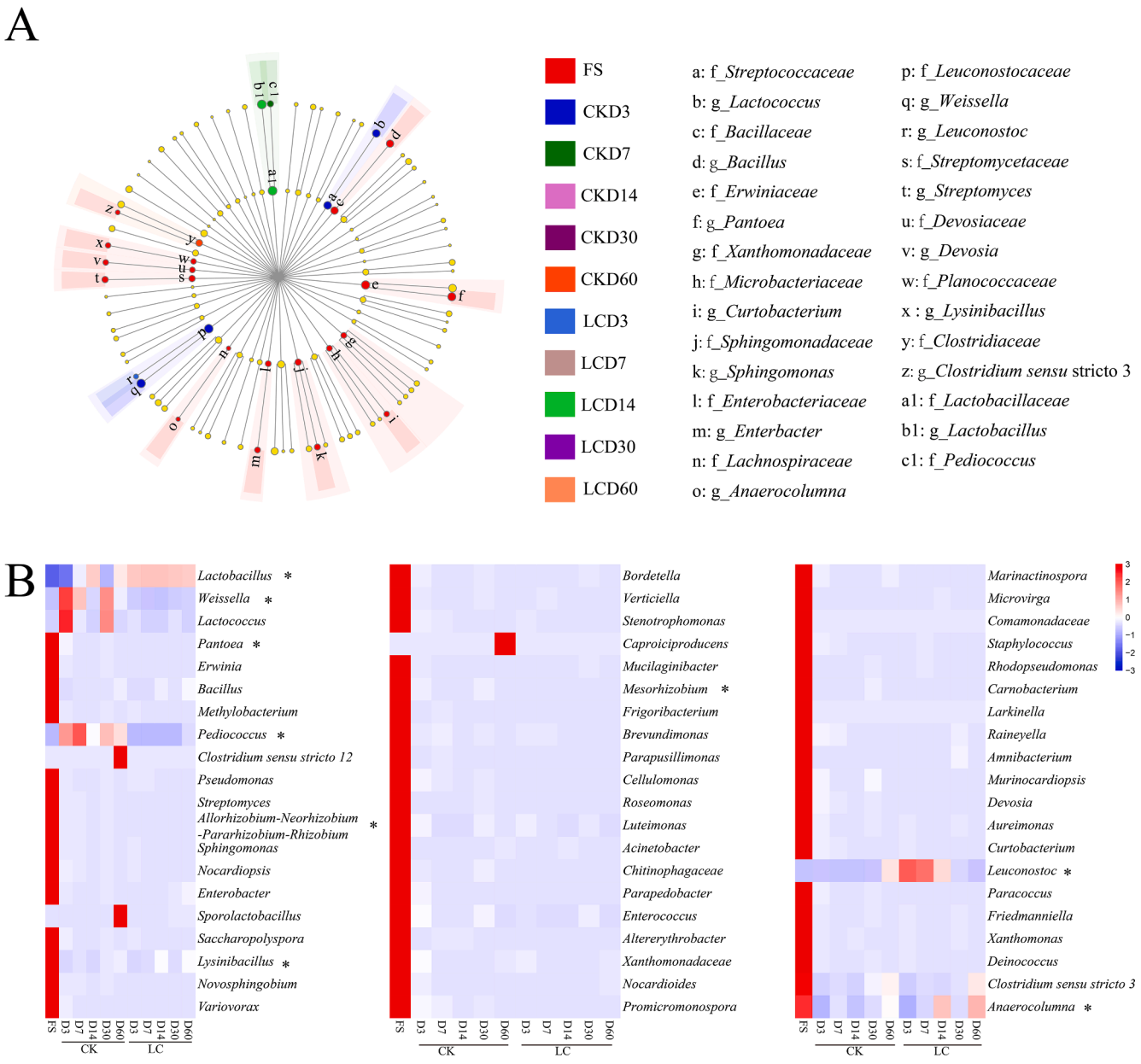

Fig. 3. Species variation analysis of LJT silages. LEfSe multilevel species difference discriminant analysis (A). Analysis of species variation analysis among groups (B). *, Variable genera with significant contributions to component differences derived from random forest analysis.

the LC and CK samples was greater in the early fermentation stages; however, this variation diminished gradually with increased fermentation time. This result indicated that the fermentation process changed both the alpha and beta diversity, which was consistent with Sun et al. (2021) and Xia et al. (2022). The primary explanation is that favorable microorganisms such as LAB can produce antibacterial compounds (including hydrogen peroxide, organic acids, and bacteriocins) under anaerobic conditions to inhibit or kill undesirable bacteria (Ibrahim et al., 2021). These bacteria usually affect fermentation quality by competing for WSC and degrading proteins, producing toxic compounds and ultimately leading to a reduction in fermentation quality (Oladosu et al., 2016). Therefore, it was necessary to further explore the dynamic changes in the microbial community.

The FS mainly included the genera of *Pantoea* (33 %), *Erwinia* (19 %), *Bacillus* (12 %), *Methylorubrum* (7 %), etc. (Fig. 2A). Low abundance of *Lactobacillus* in FS had been previously reported by Keshri et al. (2018). However, *Lactobacillus* became the dominant bacteria in the LC and CK samples (Fig. 2A). The presence of Gram-negative bacteria such as *Pseudomonas* and *Enterobacter* may decrease fermentation quality, the abundances of which decreased in the LC and the CK samples (Fig. 2A and Fig. 3). These bacteria are considered undesired bacteria since they compete with LAB for WSC, thus forming a-N and slowing acidification (Silva et al., 2016). This result indicated that anaerobic fermentation improved the microbial composition of the LJT residues. In addition, Wu et al. (2020) reported that *Clostridium sensu* stricto would result in poor fermentation quality since they can convert WSCs into BAs, in this study, the abundance of *Clostridium sensu* stricto in the FS group was significantly higher ($P < 0.05$) than that in the CK and LC samples (Fig. 3A and Table 2). The dynamics of certain specific genera were also analyzed, and most undesirable epiphytic bacteria were significantly reduced during the fermentation process (Fig. 3B). These results were consistent with that of Li et al. (2022c), who found that the microbiota of herbal residues was changed after fermentation.

The species compositions of the samples are shown in Fig. 2B. The FS mainly included uncultured *Pantoea* (28 %), unclassified *Erwinia* (10 %), and *Erwinia persicina* (8 %), of which *E. persicina* is a plant pathogen that

Table 2
The OTU abundance of specific genera.

	FS	CK				
		D3	D7	D14	D30	D60
a	7.67 ± 0.58 g	1104.33 ± 346.23 fg	8790.67 ± 3304.8de	17436 ± 3784.2abc	6328 ± 1824.11ef	11316 ± 3362.32cde
b	2 ± 1c	8302 ± 383.01a	4580 ± 1517.96b	876 ± 230.83c	8029.67 ± 3296.02a	1658.67 ± 986.13c
c	4543.33 ± 502.90a	196 ± 24.56b	13 ± 5.29d	3.67 ± 4.73d	138 ± 50.11bc	16 ± 10.44d
d	0 ± 0d	208.67 ± 65.9abc	330.67 ± 148.6a	119.67 ± 22.74bcd	238.33 ± 167.02ab	150 ± 117.9bcd
e	287.33 ± 12.7a	3.33 ± 1.53b	3.33 ± 0.58b	0 ± 0b	4.33 ± 1.53b	0 ± 0b
f	101.33 ± 14.29a	2.33 ± 1.53d	5.33 ± 0.58 cd	3.33 ± 1.53d	8.67 ± 5.51bcd	9.33 ± 3.51bcd
g	0.67 ± 1.15c	1.33 ± 1.53c	0.67 ± 1.15c	0 ± 0c	2 ± 2c	20.33 ± 19.55bc
h	35 ± 2a	1.33 ± 1.53de	2 ± 1de	1.67 ± 2.08de	6 ± 4.58bcd	8 ± 3.61bc
i	35.33 ± 8.33a	1.33 ± 1.15b	0.33 ± 0.58b	0.33 ± 0.58b	1 ± 1b	0 ± 0b
j	2557 ± 272.63a	23.67 ± 1.53b	2.67 ± 2.08d	0 ± 0d	14.33 ± 2.52c	2 ± 1d
k	188.67 ± 26.08a	5.67 ± 1.53b	0.67 ± 1.15b	0.33 ± 0.58b	4.33 ± 2.08b	1 ± 1.73b
l	1000.67 ± 54.5a	3.67 ± 2.52b	0.33 ± 0.58b	0.33 ± 0.58b	1.67 ± 1.15b	0.33 ± 0.58b
m	119.33 ± 14.05a	5 ± 4.36b	3 ± 1b	0.33 ± 0.58b	2 ± 1.73b	0.67 ± 0.58b
n	522.33 ± 26.63a	3.33 ± 2.08b	1.33 ± 1.15b	0 ± 0b	2.67 ± 2.52b	1 ± 1b
o	145.67 ± 27.79a	3 ± 1b	0 ± 0b	0 ± 0b	0.33 ± 0.58b	0.33 ± 0.58b
	LC					F value
	D3	D7	D14	D30	D60	
a	14027 ± 3241bcd	21810.67 ± 2584.62a	19297 ± 5340ab	15140 ± 1084bc	15909 ± 4211abc	16.206
b	347.67 ± 17.67c	124.67 ± 54.72c	12.33 ± 9.29c	330.33 ± 67.68c	592.33 ± 114.02c	23.51
c	39.67 ± 12.86 cd	17.33 ± 17.04d	7 ± 8.19d	47.33 ± 29.69 cd	38.67 ± 23.76 cd	234.93
d	2 ± 1d	2.67 ± 3.06d	0.67 ± 0.58d	4.33 ± 3.06d	56.67 ± 8.5 cd	6.57
e	0.67 ± 0.58b	0.67 ± 0.58b	0 ± 0b	0.33 ± 0.58b	0.33 ± 0.58b	1464.38
f	2.33 ± 2.08d	6 ± 4.58 cd	19.33 ± 4.51b	4.33 ± 3.21d	17.33 ± 9.02bc	70.14
g	44 ± 19.08ab	66.67 ± 40.92a	30 ± 22.27bc	5.67 ± 6.66c	1.33 ± 1.53c	5.58
h	0.67 ± 0.58e	4 ± 2cde	3.33 ± 3.21cde	3.33 ± 2.31cde	10.67 ± 3.06b	42.28
i	0 ± 0b	0 ± 0b	0 ± 0b	0 ± 0b	0 ± 0b	51
j	4 ± 0d	0.33 ± 0.58d	0 ± 0d	5 ± 6.93d	0 ± 0d	262
k	1.33 ± 0.58b	1.33 ± 0.58b	0.33 ± 0.58b	3.33 ± 4.16b	14 ± 5.29b	140.49
l	1 ± 1b	0 ± 0b	0.33 ± 0.58b	0.67 ± 0.58b	0 ± 0b	1006.16
m	1.67 ± 0.58b	0.33 ± 0.58b	0 ± 0b	1 ± 1b	1.67 ± 1.15b	186.05
n	2.67 ± 0.58b	9 ± 6.56b	0.67 ± 1.15b	0 ± 0b	0.33 ± 0.58b	1058.4
o	0.33 ± 0.58b	0 ± 0b	0 ± 0b	0 ± 0b	0 ± 0b	81.79

a, *Lactobacillus*; b, *Weissella*; c, *Pantoea*; d, *Pediococcus*; e, *Allorhizobium -Neorhizobium – Pararhizobium – Rhizobium*; f, *Lysinibacillus*; g, *Leuconostoc*; h, *Clostridium sensu* stricto 3; i, *Mesorhizobium*; j, *Erwinia*; k, *Enterobacter*; l, *Methylobacterium-Methylorubrum*; m, *Curtobacterium*; n, *Pseudomonas*; o, *Variovorax*. The degree of freedom of these data was 32. Means ± SDs within the same row with different letters present significant differences ($P < 0.05$).

causes the necrosis and wilting of alfalfa (Zhang and Nan, 2014). After fermentation started, these bacteria almost disappeared and were replaced by favorable bacteria. *Lactobacillus coryniformis* progressively increased from 8 % to 76 % from CKD3 to CKD14, while the opposite trend was observed for *Weissella cibaria* and *Weissella paramesenteroides*. It has been reported that *L. coryniformis* is often found in plant materials and they can directly produce bactericidal substances (Magnusson and Schnürer, 2001) or convert substrates into bacteriostatic substances (Tanaka et al., 2009). This may partially explain the undesirable bacteria in the CK samples also declined rapidly. The composition of the FS bacterial community varied significantly from that of the bulk material (alfalfa) (Su et al., 2019), but was quite similar to that of tea leaves (Lin et al., 2021). Moreover, the observed changes in the abundances of *Lactobacillus*, *Methylobacterium,* and *Sphingomonas* in fermented tea leaves reported by Lin et al. (2021) were consistent with those found after LJT residue fermentation. This may be due to the tea leaves also having many active ingredients similar to LJT. *L. plantarum* was the dominant species in the LC samples throughout the fermentation process. Its relative abundance was over 95 % until D30, but then decreased significantly to 52 % at D60 when 41 % L. coryniformis was observed. *L. plantarum* and *L. coryniformis* constituted the largest proportion of the CK and LC samples at D60, respectively. This may be due to the changes in nutrient composition and microenvironment during fermentation, such as pH, which led to different adaptations by the strains. Notably, equal levels of *L. plantarum*, *L. fermentum*, and *L. farciminis* were added to the LC samples, but *L. plantarum* was overwhelmingly dominant within 3 D in stark contrast to the rare detection of *L. fermentum* and *L. farciminis* by 16S rDNA sequencing. The possible reason could be that the latter two species cannot adapt to the nutritional characteristics of LJT residues, or they are more sensitive than *L. plantarum* to the active ingredients of LJT residues since LAB contain different phylogenetic and basic characteristics (Li et al., 2022c; Sun et al., 2014). Hence, the compound additive could be simplified to just *L. plantarum* plus cellulase, a combination that needs to be re-evaluated.

The composition of the bacterial community exhibited dynamic changes that could alter the functions of the bacterial community in fermented samples (Hisham et al., 2022). The co-occurrence network diagram demonstrated the coexistence of species in environmental samples (see Supplementary material), and the obtained results were in line with the microbial composition data. The functional phenotype prediction and contribution analysis of the bacterial community showed that the FS group contained a higher level ($P < 0.05$) of potential pathogenic bacteria (PPB; i.e., *Pantoea*, *Erwinia*, *Enterobacter*, *Pseudomonas,* and *Variovorax,* etc.) compared with the fermented samples (Table 2). The reason for the low levels of PPB in the fermented samples could be attributed to the organic acids produced by LAB coupled with the anaerobic environment. The treatment administered to the LC samples could minimize the level of these PPBs, which was associated with a rapid reduction in pH value by the compound additive. Moreover, these PPBs can also form biofilms, which enhance tolerance to different external stressful environments (Lebeaux et al., 2014). Gavrilova et al. (2019) isolated *L. plantarum* strain AG10 from clover silage, which inhibited biofilm formation and the spreading of foodborne pathogens, suggesting that this compound additive can reduce the risk of biofilm formation in livestock.

3.4. Metabolomics profiles of LJT residue fermentation profiles

The changes in active ingredients were investigated by wide-target metabolomics. A total of 838 metabolites (including flavonoids, alkaloids, phenols, amino acids and derivatives, nucleotides, and derivatives) were detected. Principal component analysis showed

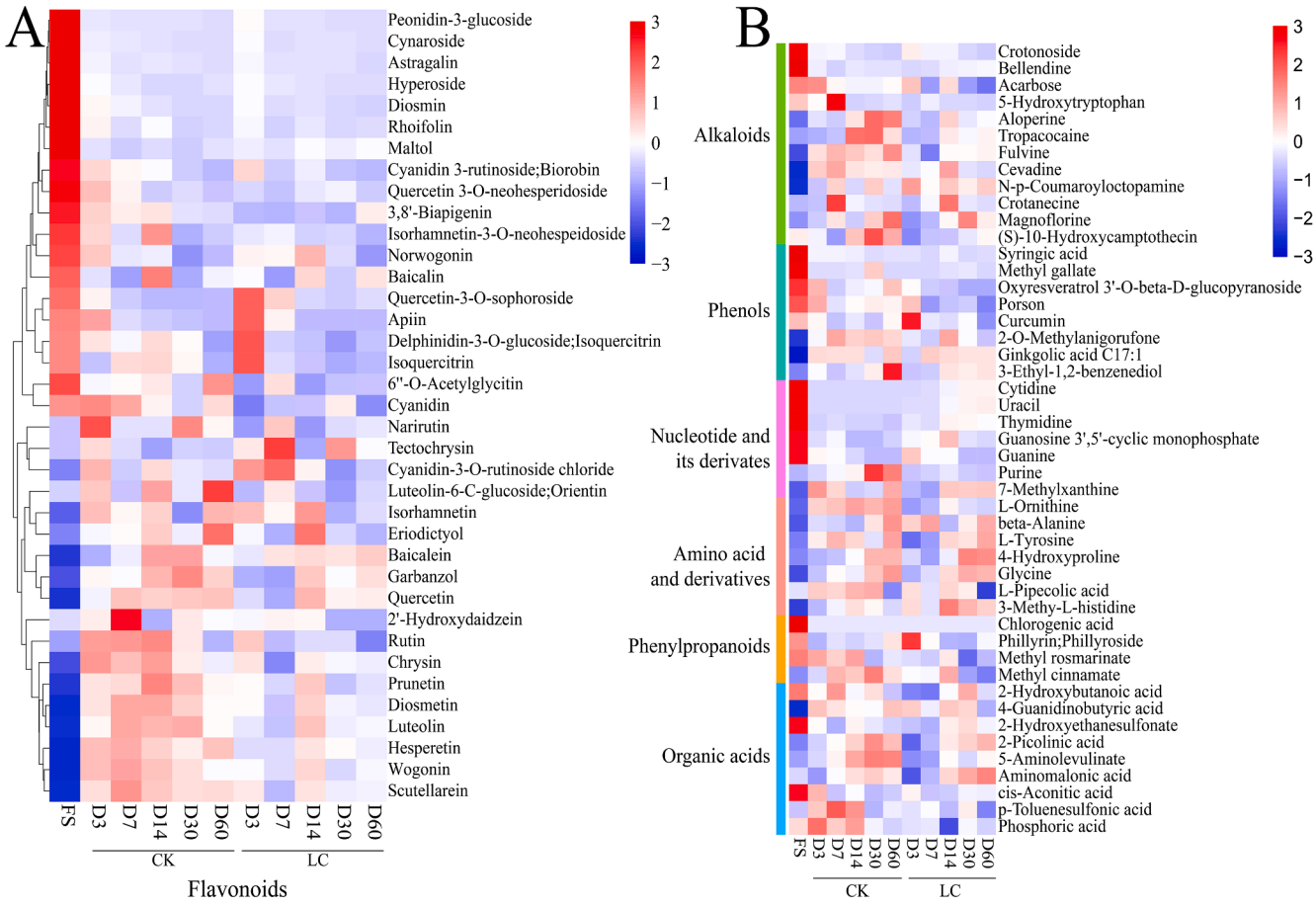

Fig. 4. Metabolite dynamics of LJT silage samples. Certain specific active ingredients included flavonoids (A) and alkaloids, phenols, amino acids and their derivatives, nucleotides and their derivatives (B) in each group in terms of their relative abundance.

considerable variation between the FS and fermented samples and a minor difference between the CK and LC samples. The differences became increasingly diverse from the previous period as the fermentation was prolonged. Compared with the FS group, the CKD60 group had 121 upregulated and 116 downregulated metabolites, while the LCD60 group had 116 upregulated and 116 downregulated metabolites (see Supplementary material). The percentages of metabolites with significant changes in the CKD60 and LCD60 groups compared to FS were 28.28 % (237/838) and 27.68 % (232/838), respectively. Therefore, the anaerobic fermentation process changed the secondary metabolic profile of the LJT residues. The significantly altered metabolites included flavonoids, alkaloids, phenols, etc. Flavonoids possess promising antioxidant activity and can treat various diseases (Williamson et al., 2018). The results here showed that fermentation caused a change in the metabolic profile with a similar number of up-and down-regulated metabolites. Overall, there was no significant difference in the number of differential metabolites between the CK and LC samples. However, fermentation of the LJT residue with or without compound additive did not change the degree of metabolism within the same category, and the metabolic pathways enriched by the differential metabolites were also similar, i.e., the pyrimidine metabolism, valine, leucine and isoleucine biosynthesis, flavone and flavonol biosynthesis and phenylalanine metabolism pathways (see Supplementary material). Although fermentation affected the metabolite profile of LJT residues, the categories of these functional compounds did not change greatly. Lin et al. (2022) fermented pruned tea branches and found that the fermentation process preserved the main bioactive components of the raw material, which is data that supports the findings of this study.

Nevertheless, there were also differences in metabolites between the CKD60 and LCD60 groups. There were 86 upregulated and 85 downregulated metabolites in the LCD60 group compared with the CKD60 group (see Supplementary material). Similarly, a certain quantity of differential metabolites existed between the CK and LC groups at each fermentation stage. Thus, the focus was turned to metabolites that changed significantly throughout the fermentation process compared to the FS group, including flavonoids (e.g., peonidin-3-glucoside, cynaroside, astragalin, hyperoside, scutellarein, wogonin, and luteolin), alkaloids (e.g., crotonoside and magnoflorine), phenols (e.g., syringic acid and curcumin), amino acids and derivatives (e.g., beta-alanine, glycine), nucleotides and derivatives (e.g., cytidine and 7-methylxanthine), etc. (Fig. 4). These significantly changed flavonoids have certain biological activities against diseases. For example, the contents of wogonin (3.85 fold), scutellarein (3.37 fold), hesperetin (23.34 fold), luteolin (2.35 fold) and quercetin (5.21 fold) were significantly upregulated ($P < 0.05$) in the CKD60 group, similar trends were shown in the LCD60 group (see Fig. 4 and Supplementary material). Each of these metabolites has some preventive and therapeutic effects against various cancers, as well as anti-inflammatory and antioxidant activities. In contrast, the contents of metabolites with similar effects (Erlund, 2004; Guo et al., 2019) were downregulated significantly ($P < 0.05$) in the CKD60 group, such as peonidin-3-glucoside (313.89 fold), cynaroside (203.9 fold), astragalin (39.7 fold), quercetin 3-O-neohesperidoside (22.9 fold), and quercetin 3-O-sophoroside (15.70 fold), similar trends observed in LCD60 group (see Fig. 4 and Supplementary material). Interestingly, as the contents of some anti-inflammatory metabolites declined (e.g., cynaroside, quercetin 3- O - neohesperidoside, and quercetin 3- O -sophoroside), the contents of metabolites with similar biological functions (e.g., luteolin and quercetin) (Odontuya et al., 2005) increased in the fermented

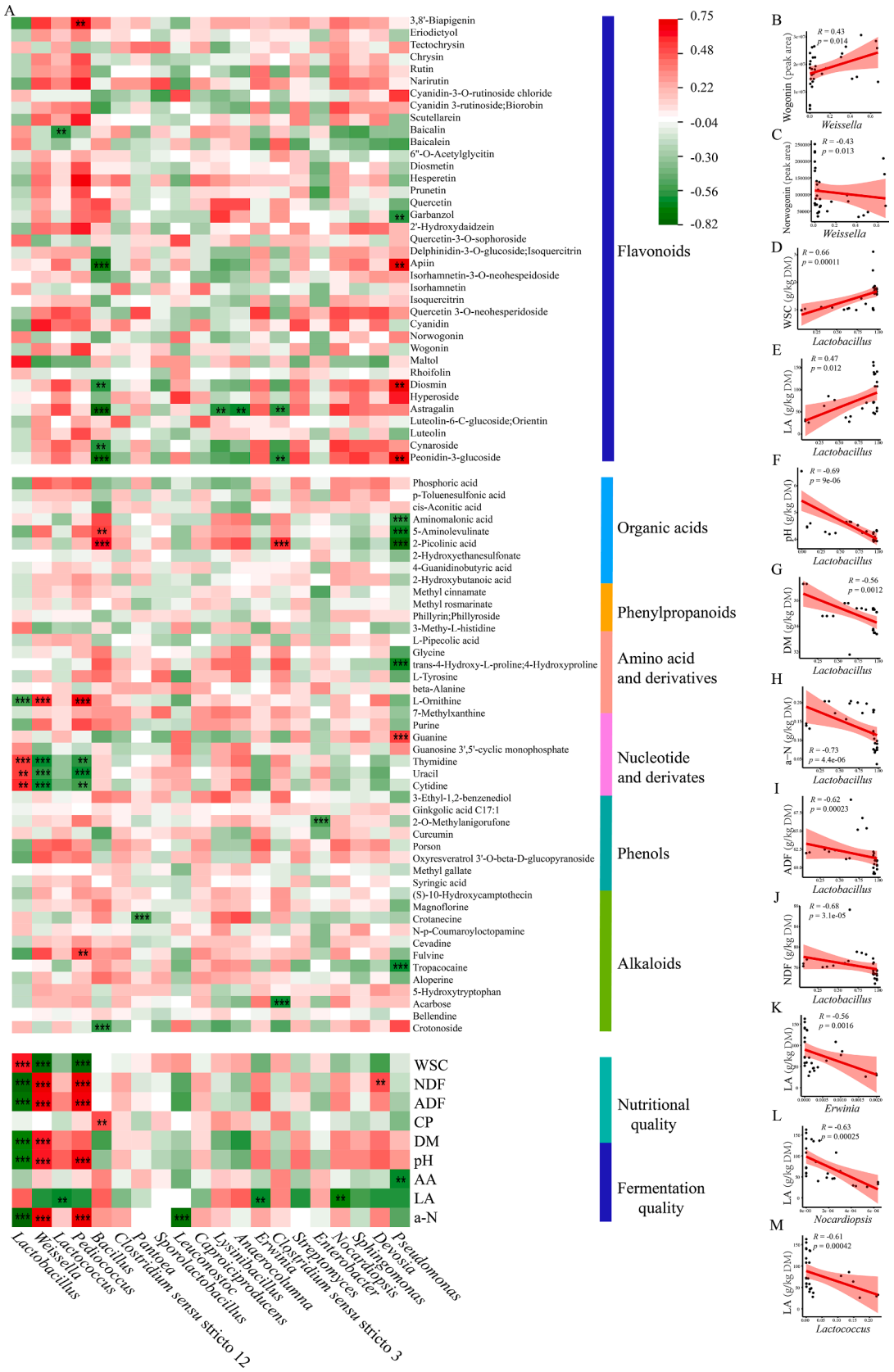

Fig. 5. Correlations between the main microorganisms and the LJT metabolites/fermentation quality (A). Specific correlation analysis of the bacteria in terms of Wogonin content (B), Norwogonin content (C), WSC (D), LA content (E, K, L and M), pH values (F), DM (G), a-N content (H), ADF content (I), NDF content (J). The X-axis represents the relative abundance of the genus. Correlations > 0.5 and < 0.05 were considered significant. **, $P < 0.01$; ***, $P < 0.001$.

samples. As these metabolites might transform into one another, it was speculated that some might have been converted into other forms by the effects of microbial.

It has been reported that quercetin promotes protein digestion and absorption, thus suggesting that the upregulation of quercetin content in the LC (4.50 fold) and CK (5.21 fold) groups may foster protein intake in livestock (Cheng et al., 2021). The levels of most amino acids and derivatives i.e., glycine (3.45 fold), 3-methyl-L-histidine (13.53 fold), 4-hydroxyproline (6.90 fold) and L-tyrosine (4.55 fold) were significantly upregulated ($P < 0.05$) in the LCD60 group compared with the FS (see Fig. 4 and Supplementary material). Proteins are digested by gastric and intestinal juices and villus cells into amino acids before being absorbed into the blood (Whitcomb and Lowe, 2007). The above results suggested that LJT residue fermentation could promote the degradation of proteins to amino acids that could be directly absorbed and utilized with no impact on the total CP content.

3.5. Correlation analysis of the microorganisms in terms of fermented quality parameters and metabolites

Changes in the microbial composition and metabolite profile occurred throughout the whole fermentation process. Thus, elucidating microbial correlations could facilitate controlling the quality of fermentation in an effective way. The Spearman correlation coefficient was calculated to further investigate the correlation between metabolite changes, fermentation parameters, and microbial succession. Overall, the abundances of the top 20 genera and the 83 important metabolites were analyzed, of which only 2.5 % (41/1660) had a significant correlation (Fig. 5A). In particular, Weissella was positively correlated with wogonin content ($R = 0.43$, $P < 0.05$), but negatively correlated with norwogonin content ($R = 0.43$, $P < 0.05$) (Fig. 5B-5C). This indicated that the differences in metabolites may be related to the changes in the bacterial community during fermentation. *Lactobacillus* showed significant positive correlations ($P < 0.05$) with WSC and LA (Fig. 5D-5E) and significant negative correlations ($P < 0.001$) with pH, DM, a-N, ADF, and NDF (Fig. 5F-5 J) levels, which is in agreement with Xia et al. (2022). The opposite correlations were observed for *Weissella*, *Lactococcus*, *Pediococcus*, and *Devosia* (see Fig. 5A and Supplementary material). These data were supported by Li et al. (2020), who also found that Lactobacillus increased LA content and decreased pH value, whereas Weissella produce the opposite result. In addition, *Erwinia*, *Nocardiopsis* and *Lactococcus* (Fig. 5K-5M) showed significant negative correlations ($P < 0.01$) with LA. This result indicated that the changes in the bacterial community are closely related to the fermentation quality. *Lactobacillus* displayed significant correlations with few metabolites, but there were also many strong correlations between some intrinsic epiphytic bacteria (e.g., *Bacillus* and *Pseudomonas*) and the metabolites. These data suggested that most of the alterations to the metabolic profile of LJT residues might be due to the effects of its intrinsic epiphytic bacteria, with a small proportion attributed to the exogenous addition of the compound additive.

4. Conclusion

In summary, the high WSC content of the LJT residues enabled a rapid reduction in pH value. The fermentation process reduced both bacterial diversity and undesirable bacteria, resulting in domination by *Lactobacillus*. The compound additive improved fermentation quality by increasing LA production and reducing a-N production. The fermentation process and the compound additive did not cause a functional loss in the active ingredients in the LJT residues. Based on their abundant active ingredients, LJT residues have the potential to be used as a feed additive to improve animal immunity, and further feeding experiments will be performed for validation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.128264.

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