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Effect isolated lactic acid bacteria inoculation on the quality, bacterial composition and metabolic characterization of *Caragana korshinskii* silage

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Abstract

Feed shortage has hindered the development of the world's livestock industry, and the rational use of non-conventional forages can help resolve this issue. To improve the fermentation quality of *Caragana korshinskii* silage, this study isolated lactic acid bacteria (LAB) from fresh *Caragana korshinskii* and silage samples, as alternatives to traditional commercial LAB inoculants. Utilizing Single Molecule, Real-Time (SMRT) sequencing technology and metabolomics analysis, the microbial community structure and metabolome were examined after 60 days of fermentation to elucidate the mechanism by which isolated LAB affect the fermentation quality of *Caragana korshinskii* silage. After 60 days of ensiling, the dominant microbial population in the silage shifted from harmful bacteria such as *Erwinia*, *Pantoea*, and *Enterobacter* to LAB. Compared to commercial LAB, the isolated *Lactiplantibacillus plantarum* significantly reduced the microbial diversity of *Caragana korshinskii* silage. The species composition of LAB became simpler with *Lentilactobacillus parakefiri* dominating and achieving a higher relative abundance. By down-regulating Butanoate metabolism and the Pentose phosphate pathway and up-regulating the Ascorbate and aldarate metabolism pathway, the isolated *Lactiplantibacillus plantarum* significantly lowered the pH of the silage, increased the content of water-soluble carbohydrates (WSC) and lactic acid (LA), reduced the neutral detergent fiber content (NDF), and improved in vitro neutral detergent fiber digestibility (IVNDFD), thereby enhancing the fermentation quality of *Caragana korshinskii* silage. On the other hand, the isolated *Limosilactobacillus fermentum* had the opposite effect, with its ensiling outcome being less effective than that of commercial LAB. The results of the study indicate that the addition of the isolated *Lactiplantibacillus plantarum* improved the quality of *Caragana korshinskii* silage by regulating metabolic pathways and the composition of microbes within the ensiled feed.

Keywords *Caragana korshinskii*, Lactic acid bacteria, Microbial community, Metabolome
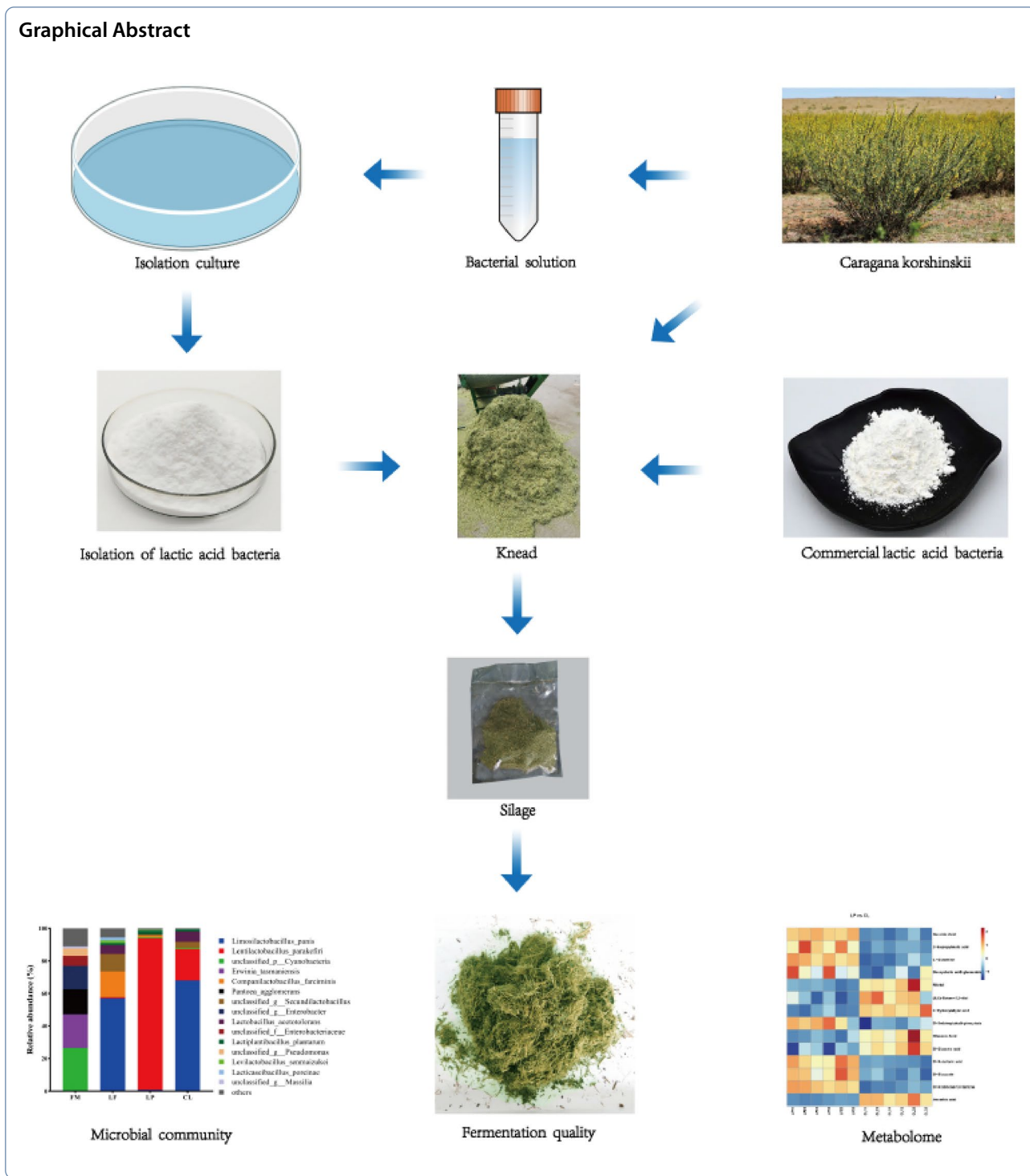
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Graphical Abstract

Background

With the decrease in arable land and rise in demand for animal products such as meat, eggs, and dairy [1], traditional forages and grain feeds can no longer meet the food requirements of ruminants. Thus, there is an urgent need to find new and unconventional feed resources to alleviate feed shortages. *Caragana korshinskii*, as an ecological energy crop, is widely cultivated in the arid and semi-arid regions of Asia and Europe. However, the excessive growth of *Caragana korshinskii* can hinder the regeneration of fresh branches and reduce the diversity of surrounding vegetation [2]. Therefore, it is pruned every

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3–5 years. It is estimated that China harvests over 4 million tons of *Caragana korshinskii* branches and leaves annually [3]. In recent years, numerous researchers have discovered the great potential of *Caragana korshinskii* as silage feed [4, 5]. However, due to its nature as a legume, its lower fermentation substrate leads to unsatisfactory silage results [6].

Silage is made by fermenting soluble carbohydrates with LAB, producing organic acids such as LA, thereby lowering the pH value to inhibit the growth of harmful microorganisms, thus preserving the nutritional value of the feed [7]. The quantity of LAB naturally present on plants is a crucial factor for anaerobic fermentation, but the number of these bacteria is usually low, less than 5 Log_{10} cfu/g fresh material (FM), which is not enough for high-quality fermentation. Therefore, the addition of exogenous LAB becomes an effective way to ensure high-quality fermentation. However, whether the added LAB can have a positive interaction with the raw materials is key to achieving high-quality fermentation [8]. Previous studies have shown that LAB isolated from the natural grass itself used for the plant's silage production can match or even surpass the effectiveness of commercially available LAB strains [9]. Therefore, isolating and utilizing the LAB naturally present on plants for the production of silage feed is necessary, but currently, research on isolating LAB from *Caragana korshinskii* and applying them to silage feed is quite rare.

Silage involves a dynamic process of microbial community succession and changes in metabolic products [10]. In recent years, third-generation PacBio SMRT sequencing and metabolomics have brought new insights into the research of silage feed [11]. The SMRT sequencing technology can identify microbial species at the species level, thus more comprehensively reflecting the microbial community structure of silage samples. Metabolomics can study the metabolic products during the silage process as well as differential metabolic pathways, and delve more deeply into the exploration of fermentation mechanisms. However, the metabolome in *Caragana korshinskii* silage feed is currently unclear, making it significantly important to investigate the regulatory mechanisms of *Caragana korshinskii* silage fermentation through SMRT sequencing technology and metabolomics studies.

In this study, we isolated two strains of bacteria with rapid acid production and strong growth capabilities (*Limosilactobacillus fermentum* and *Lactiplantibacillus plantarum*) from *Caragana korshinskii* raw materials and *Caragana korshinskii* silage feed to be used as additives in the production of *Caragana korshinskii* silage feed. Furthermore, we utilized SMRT sequencing technology and metabolomics to analyze the microbial community structure, metabolic products, and metabolic pathways

during the *Caragana korshinskii* silage process, to clarify the mechanism by which the isolated LAB affect the fermentation quality of *Caragana korshinskii* silage feed.

Materials and methods

Lactic acid bacteria strains

A total of 56 strains of LAB were isolated from 39 samples of *Caragana korshinskii* raw material and *Caragana korshinskii* fermented silage according to the method of Cai et al. [12]. The raw materials were obtained from the horqin sandy land of Xing'an league, Inner Mongolia Autonomous Region. Five grams of each sample were taken and mixed with 45 mL of sterile distilled water. The diluted solution was evenly spread on MRS agar and incubated under anaerobic conditions at 37 °C for 48 h to isolate LAB. The suspected LAB colonies were streaked on MRS agar (Difco Laboratories, Detroit, USA) and each LAB colony was purified five times. The purified LAB colonies were preserved in liquid MRS containing 15% glycerol at a temperature of − 80 °C. Growth curve assays and acid production curve tests were conducted on 56 isolated strains, resulting in the selection of 2 strains with rapid acid production and strong growth capabilities for subsequent experiments. Peroxidase activity, Gram staining and glucose gas production were tested according to Duan et al. [13]. The strain was tested for acid and salt tolerance using MRS broth, and 49 different compounds were identified for carbohydrate assimilation and fermentation using API 50 CH (bioMe´rieux, Inc., Marcy l'Etoile, France) [14]. Genomic DNA of the screening strains was extracted using a bacterial DNA kit (Sangon Bioengineering (Shanghai) Co., Ltd., Shanghai, China), and the isolated DNA was subjected to polymerase chain reaction (PCR) using primers 27F (5′-AGTTTGATC-MTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTT ACGACTT-3′). The 16SrDNA sequences were then compared on the ribosomal database http://rdp.cme.msu.edu/index.jsp.

Silage preparation

The *Caragana korshinskii* raw material used in this study was taken from the horqin sandy land of Xing'an League, Inner Mongolia Autonomous Region (44°77′E, 121°86′N), which was flat-cropped in August 2019 and harvested at the fruiting stage in August 2022, the third year of its growth. The harvested material was kneaded (fineness below 1.5 mm and length between 2 and 5 cm) using a *Caragana korshinskii* kneading machine (The kneading machine, model FS800-3, was purchased from Jilin Jiyuan Caragana Machinery Equipment Co., Ltd.), and the kneaded material was divided into three equal parts, and distilled water was added to adjust the moisture content to 60%. Silage additives were self-screened

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Lactiplantibacillus plantarum (abbreviated as LP), *Limosilactobacillus fermentum* (abbreviated as LF) and a commercial LAB strain (*Lactiplantibacillus plantarum*, abbreviated as CL). The self-screened strains were lyophilized using a vacuum freeze dryer FDU-2200 (Hangzhou Jiawei Innovation Technology Co., Ltd.) according to the method of Chen et al. [15], and the material was vacuum sealed in polyethylene bags after inoculation of *Caragana korshinskii* at a dose of 10^5 cfu/g FM. Five replicates of 500 g each were set up for each treatment and silage from the different treatments was analyzed after 60 days of storage at room temperature (25 ℃).

Analysis of chemical composition, fermentation parameters, and in vitro digestibility
Five gram sample from each bag of raw material and silage was weighed, mixed with 45 mL of sterilized distilled water, filtered through 4 layers of gauze and filter paper, and then tapped for 2 min with a Aseptic Homogenizer JC-JZ-08 (Qingdao Jucheng Century Environmental Protection Technology Co.) to obtain the organic acid solution. The content of LA (lactic acid), AA (acetic acid), PA (propionic acid) and BA (butyric acid) were determined by high performance liquid chromatography (HPLC) according to the method of Cai et al. [16]. The NH_3-N (ammonia nitrogen) content was determined by phenol-sodium hypochlorite colorimetric method. The pH was determined using a pH meter (Shanghai Yidi Electronic Science Instrument Co., Ltd.). Each bag of silage samples was first dried at 105 ℃ for 0.5 h to inhibit enzyme activity in the plant material, then dried at 65 ℃ for 48 h before the DM (dry matter) content was measured. The CP (crude protein) content was determined by Dumas nitrogen determination, the NDF (neutral detergent fiber), ADF (acid detergent fiber) and ADL (acid detergent lignin) content were determined by the method of Van Soest et al. [17]. The WSC content was determined by anthrone colorimetry [18]. Determination and calculation of cellulose and hemicellulose contents was performed according to Li et al. [19]. The assessment of LAB count in fresh samples was performed according to the method of Guo et al. [20]. Determination of IVDMD (in vitro dry matter digestibility) and IVNDFD content was performed according to the method of Ferreira et al. [21].

Bacterial community analysis
Fresh *Caragana korshinskii* and silage stored for 60 days were selected for bacterial community analysis. Sample DNA was extracted using the Soil Rapid DNA SPIN kit (MP Biomedicals, Solon, USA), and a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, USA) was utilized for DNA concentration and purity

assessment. Polymerase chain reaction (PCR) amplification was performed by Majorbio Technology Co. (Shanghai, China), and the obtained PCR products were further purified with AMPure ® PB beads (Pacifc Biosciences, CA, United States) to prepare the SMRTbell library. The specific primers were 27F (AGRGTTTGATYNTGGCTCAG) and 1492R (TASGGHTACCTTGTTASGACTT) primers. The purified libraries were sequenced on a Pacbio Sequel II system (Pacifc Biosciences, CA, United States) using SMRT sequencing technology. The raw data were subjected to pair-end double-ended sequence splicing using Flash (version v1.2.11), and the operational taxonomic units (OTUs) were clustered using the Uparse algorithm with 97% threshold identity, and each OTU was classified using the sequence classification annotation of the RDP Classifier (version 2.13) with a confidence level of 70%. Bioinformatics analysis of plant samples was performed on the Majorbio cloud platform. Kruskal–Wallis multiple comparisons ($p < 0.05$) were used to detect bacterial community structure and analyze bacterial community structure. QIIME2 was used for α-diversity and β-diversity analysis. Community composition maps and the linear discriminant analysis effect size (LEfSe) analysis maps were plotted at https://www.omicstudio.cn/tool.

Metabolite analysis
A 50 mg silage sample was placed in a 2 mL centrifuge tube containing grinding beads (6 mm diameter) and 400 µL of an extract mixture of methanol and water (4:1 volume ratio) containing 0.02 mg/mL of L-2-chloro chicory alanine as an internal standard was added. The samples were ground in a frozen tissue grinder at − 10 ℃ for 6 min at a frequency of 50 Hz and extracted by sonication at 5 ℃ for 30 min at 40 kHz. After extraction, the samples were refrigerated at − 20 ℃ for 30 min, followed by centrifugation at 13,000 rpm and 4 ℃ for 15 min. The supernatant was transferred to an injection vial containing an inserted tube to be analyzed. One QC sample was prepared for every six samples, and 20 µL of the supernatant was mixed as the QC sample. Ultra-high performance liquid chromatography-Fourier transform mass spectrometry (UPLC-FTMS) detection was performed using a Thermo Fisher UHPLC -Q Exactive HF-X analytical system with an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm i.d., 1.8 µm; Waters, USA), mobile phase A was 95% water + 5% acetonitrile (containing 0.1% formic acid) and mobile phase B was 47.5% acetonitrile + 47.5% isopropanol + 5% water (containing 0.1% formic acid). The injection volume was 3 µL, and the column temperature was set at 40 ℃. The samples were ionized with an electrospray source, and the mass

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spectrometry data were acquired in positive and negative ion modes, respectively.

The data were analyzed by partial least squares discriminant analysis (PLS-DA) using R package ropls (version 1.6.2), and differential metabolites were detected using variable importance projection (VIP) of the first principal component. The screening criteria for differential metabolites were a *p*-value of less than 0.05 and a VIP value of greater than 1. Special attention was paid to differences in carbohydrate metabolic pathways, and the identified differential metabolites were visualized.

Statistical analysis

Using SAS 9.3 software (SAS Institute, Inc., Cary, NC, USA), the Duncan's multiple range test was conducted to analyze the significant differences among groups in fermentation parameters, chemical composition, and bacterial alpha diversity. A probability below 5% was considered statistically significant.

Results
Lactic acid bacteria characteristics

Table 1 lists the morphological and physiological characteristics of the two bacterial strains isolated in this study and a commercial LAB. All three strains are Gram-positive, catalase-negative, and rod-shaped. Strain L1 is glucose-positive, whereas strains L2 and CL are glucose-negative, indicating that strain L1 is a hetero-fermenter, while strain L2 and CL are homo-fermenter. Moreover, when cultured in liquid MRS

medium, all three strains could grow normally under anaerobic conditions at pH values ranging from 3.5 to 7.0, with strain L1 showing weak growth at a pH value of 3.0. All three strains were capable of growing normally at NaCl concentrations of 3.0% and 6.5%.

The carbohydrate fermentation characteristics of the three strains are shown in Table 2. Differences in the fermentation characteristics of ᴅ-Xylose, ᴅ-Fructose, Mannitol, Sorbitol, Methyl-α-ᴅ-mannopyranoside, ᴅ-Lactose, ᴅ-Melezitose, ᴅ-Raffinose, Starch, ᴅ-Toulon sugar, ᴅ-Tagatose, ᴅ-Arbaitol, 2-Keto-potassium

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

	L1	L2	CL
Shape	Rod	Rod	Rod
Gram stain	+	+	+
Gas for glucose	+	−	−
Catalase	−	−	−
Fermentation type	Hetero	Homo	Homo
Growth at pH			
3.0	w	−	−
3.5	+	+	+
4.0	+	+	+
4.5	+	+	+
6.0	+	+	+
7.0	+	+	+
Growth in NaCl (%)			
3.0	+	+	+
6.5	+	+	+

+, positive; −, negative; w, weakly positive. Homo, homofermentative; Hetero, heterofermentative

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

	L1	L2	CL
ʟ-Arabinose	+	+	+
ᴅ-Ribose	+	+	+
ᴅ-Xylose	+	−	−
ᴅ-Galactose	+	+	+
ᴅ-Glucose	+	+	+
ᴅ-Fructose	W	+	+
ᴅ-Mannose	+	+	+
ʟ-Rhamnose	W	W	W
Mannitol	−	+	+
Sorbitol	−	+	+
Methyl-α-ᴅ-mannopyranoside	−	+	+
N-acetyl-glucosamine	+	+	+
Laetrile	+	+	+
Arbutin	+	+	+
Esculin and ferric citrate	+	+	+
Salicin	+	+	+
ᴅ-Cellobiose	+	+	+
ᴅ-Maltose	+	+	+
ᴅ-Lactose	W	+	+
ᴅ-Melibiose	+	+	+
ᴅ-Sucrose	+	+	+
ᴅ-Trehalose	+	+	+
ᴅ-Melezitose	−	+	+
ᴅ-Raffinose	+	+	−
Starch	−	W	W
ᴅ-Aentiobiose	+	+	+
ᴅ-Toulon sugar	−	+	+
ᴅ-Tagatose	+	−	−
ᴅ-Arbaitol	−	W	W
Potassium gluconate	W	W	W
2-Keto-potassium gluconate	W	−	−
5-Keto-potassium gluconate	W	−	−

All strains gave negative results for Glycerol, Erythritol, ᴅ-Arabinose, ʟ-Xylose, ᴅ-Ribitol, Methyl-β-ᴅ-pyranoside, ʟ-Sorbose, Dulcitol, Inositol, Methyl-α-ᴅ-glucopyranoside, Inulin, Glycogen, Xylitol, ᴅ-Lyxose, ᴅ-Fucose, ʟ-Fucose, and ʟ-Arbaitol. +, positive; −, negative; w, weakly positive

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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 (%)
L1	NR_113335.1	*Limosilactobacillus fermentum* NBRC 15885	99.86%
L2	NR_115605.1	*Lactiplantibacillus plantarum* JCM 1149	99.93%

Table 4 Characteristics of pre-ensiled *Caragana korshinskii*

Items	Sample
DM (g/kg FM)	524.41 ± 4.22
CP (g/kg DM)	112.9 ± 0.65
WSC (g/kg DM)	29.85 ± 3.68
NDF (g/kg DM)	726.67 ± 22.88
ADF (g/kg DM)	574.53 ± 15.59
ADL (g/kg DM)	217.63 ± 10.38
Cellulose (g/kg DM)	354.43 ± 5.98
Hemicellulose (g/kg DM)	152.14 ± 12.49
IVDMD (% DM)	17.75 ± 0.71
IVNDFD (% DM)	12.09 ± 1.01
pH value	5.51 ± 0.05
Lactic acid bacteria (Log$_{10}$ cfu/g FM)	4.35 ± 0.38

FM fresh material, *DM* dry matter, *CP* crude protein, *WSC* water soluble carbohydrates, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *ADL* acid detergent lignin, *IVDMD* in vitro dry matter digestibility, *IVNDFD* in vitro neutral detergent fiber digestibility

Table 5 Fermentation quality, chemical composition, and in vitro digestibility of silage

Item	Silage			SEM	*P*-value
	LF	LP	CL		
Fermentation quality					
pH value	4.13^a	3.70^b	3.71^b	0.23	0.00
LA (g/kg DM)	6.12^c	19.17^a	11.62^b	6.08	0.00
AA (g/kg DM)	4.49^a	2.21^b	1.29^c	1.47	0.00
PA (g/kg DM)	1.92	2.49	1.94	0.39	0.12
BA (g/kg DM)	1.77	ND	0.11	–	–
NH$_3$-N (g/kg DM)	0.25	ND	ND	–	–
Chemical composition					
DM (g/kg FM)	385.71	383.82	382.08	2.88	0.33
CP (g/kg DM)	104.62	104.56	104.42	0.35	0.99
WSC (g/kg DM)	10.44^c	17.14^a	13.89^b	3.00	0.00
NDF (g/kg DM)	714.60^a	638.28^b	687.66^a	36.21	0.00
ADF (g/kg DM)	553.27	527.43	553.07	17.74	0.11
ADL (g/kg DM)	210.81	217.14	214.55	8.10	0.96
Cellulose (g/kg DM)	311.83^a	279.97^b	311.84^a	6.77	0.06
Hemicellulose (g/kg DM)	161.33^a	110.85^c	134.59^b	7.70	0.00
In vitro digestibility					
IVDMD (% DM)	19.86^b	23.63^a	23.11^a	0.01	0.00
IVNDFD (% DM)	13.40^c	17.36^a	15.45^b	0.01	0.00

FM fresh material, *DM* dry matter, *CP* crude protein, *WSC* water soluble carbohydrates, *NDF* neutral detergent fiber, *ADF* acid detergent fiber, *ADL* acid detergent lignin, *IVDMD* in vitro dry matter digestibility, *IVNDFD* in vitro neutral detergent fiber digestibility, *LA* lactic acid, *AA* acetic acid, *NH$_3$-N* ammonia nitrogen. Different lowercase letters indicate significant differences between treatments ($p < 0.05$); *ND* no detected, *SEM* standard error of the mean

gluconate and 5-Keto-potassium gluconate were found among these three strains.

The 16S rRNA sequencing results were analyzed by BLAST. Strain L1 was highly similar to *Limosilactobacillus fermentum* and L2 was highly similar to *Lactiplantibacillus plantarum* (Table 3). The nucleotide sequences of strains L1 and L2 were registered in GenBank under accession numbers PP627243 and PP627245, respectively.

Characteristics of pre-ensiled *Caragana korshinskii*

The nutrient composition, in vitro digestibility, attached LAB count and pH value of *Caragana korshinskii* before ensiling are shown in Table 4. The content of DM was 524.41 g/kg FM, and the contents of CP, NDF, ADF, WSC, ADL, Cellulose and Hemicellulose were 112.90, 726.67, 574.53, 29.85, 217.63, 354.43 and 152.14 g/kg DM, respectively. The IVDMD and IVNDFD of fresh *Caragana korshinskii* were 17.75% and 12.09%. The LAB count attached to the raw material is 4.35 Log$_{10}$ cfu/g FM. The pH value of fresh *Caragana korshinskii* was 5.51.

Fermentation quality, chemical composition, and in vitro digestibility of silage

The fermentation quality, chemical composition, and in vitro digestibility of *Caragana korshinskii* silage after 60 days of ensiling are listed in Table 5. Overall,

the effects of different strain treatments on pH value, LA, AA, PA and NH$_3$-N, WSC, NDF, hemicellulose, IVDMD and IVNDFD of *Caragana korshinskii* silage were significant ($p < 0.05$). The pH value of the LP and CL groups was significantly lower than those of the LF group ($p < 0.05$), and the LA content was also significantly affected by the additives, with the LP group having the highest LA content. As expected, the AA content was significantly higher in the LF group than in the other two groups ($p < 0.05$). The PA content was not affected by the treatments, and the highest BA content was found in the LF group, followed by the CL group, and no BA was detected in the LP group. The NH$_3$-N was only detected in LF group compared to others

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groups. The effects of different additive treatments on DM, CP, ADF and ADL content were not significant ($p > 0.05$). The WSC content of LP group was significantly higher than the other two groups ($p < 0.05$), in contrast, the NDF and cellulose content was significantly lower than the other two groups ($p < 0.05$), and the difference between the CL and LF groups was not significant ($p > 0.05$). The LP group had the lowest hemicellulose content among the three groups. Compared to the CL group, isolated lactic acid bacteria with failed to improve IVDMD, however, the LP group improved IVNDFD.

**Effect of additives on microbial communities**

As shown in Fig. 1, we performed SMRT sequencing of the full-length 16S rRNA gene of fresh and silage samples of *Caragana korshinskii* and Alpha diversity analysis of bacterial communities. When the sequencing data were large enough, the sparse curves were nearly smooth for all groups (Fig. 1A). The Shannon index of silage (Fig. 1B) was significantly lower ($p < 0.05$) in the LP and CL treatment groups compared to FM, with the LP group having the lowest Shannon index. The PCoA plots using the weighted UniFrac distance metric showed that the silage samples were significantly separated from the FM (R = 0.8858, $p = 0.001$) (Fig. 2), and the LP group samples were significantly separated from the CL and LF groups samples, but there was no substantial difference among samples of CL and LF groups.

The microbial communities at phylum, genus and species levels in *Caragana korshinskii* silage are shown in Fig. 3. At the bacterial phylum level, the predominant attached bacteria in the fresh samples were Proteobacteria and Cyanobacteria. The Firmicutes predominated in all treatment groups after silage, with relative proportions exceeding 95%. At the genus level, silage from each treatment group presented different microbial abundances. The main bacteria attached to the fresh samples were unclassified_p__Cyanobacteria, *Erwinia*, *Pantoea* and *Enterobacter*. The LAB were the dominant bacteria in all treatment groups after silage, with *Limosilactobacillus* as the dominant genus in the LF and CL groups, and *Lentilactobacillus* as the dominant genus in the LP group. At the species level, the predominantly attached bacteria in the fresh samples were unclassified_p__Cyanobacteria (26.4%), *Erwinia_tasmaniensis* (20.7%), *Pantoea_agglomerans* (15.4%), and unclassified_g__Enterobacter (14.3%), but their abundances decreased substantially after silage
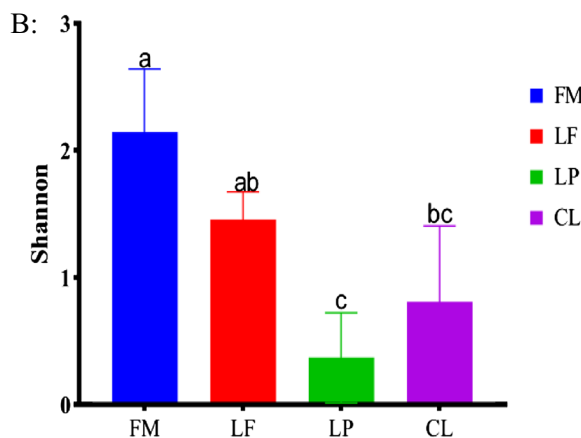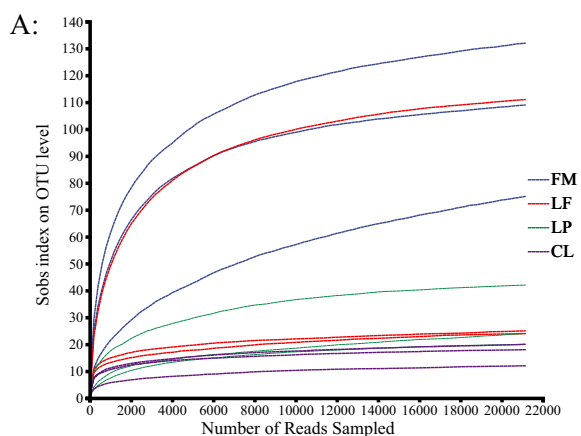
Fig. 1 A Rarefaction curves for OTUs number in different treatments of *Caragana korshinskii*. **B** Community diversity of *Caragana korshinskii* silage under different treatment conditions. *FM* fresh material, *LF Limosilactobacillus fermentum*, *LP Lactiplantibacillus plantarum*, *CL* commercial lactic acid bacteria. Different lowercase letters indicate significant differences between treatments ($p < 0.05$)
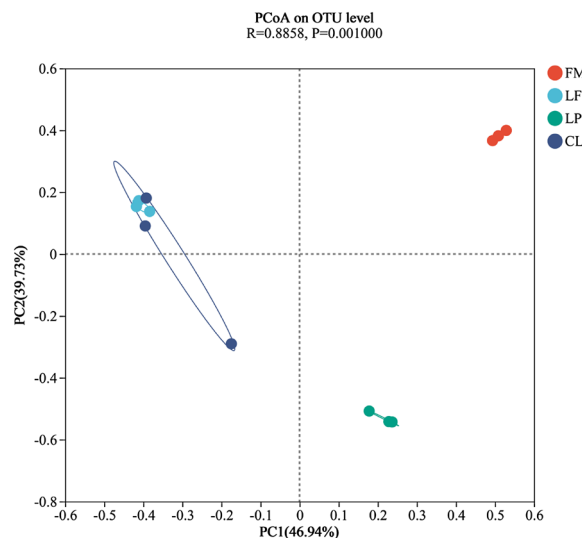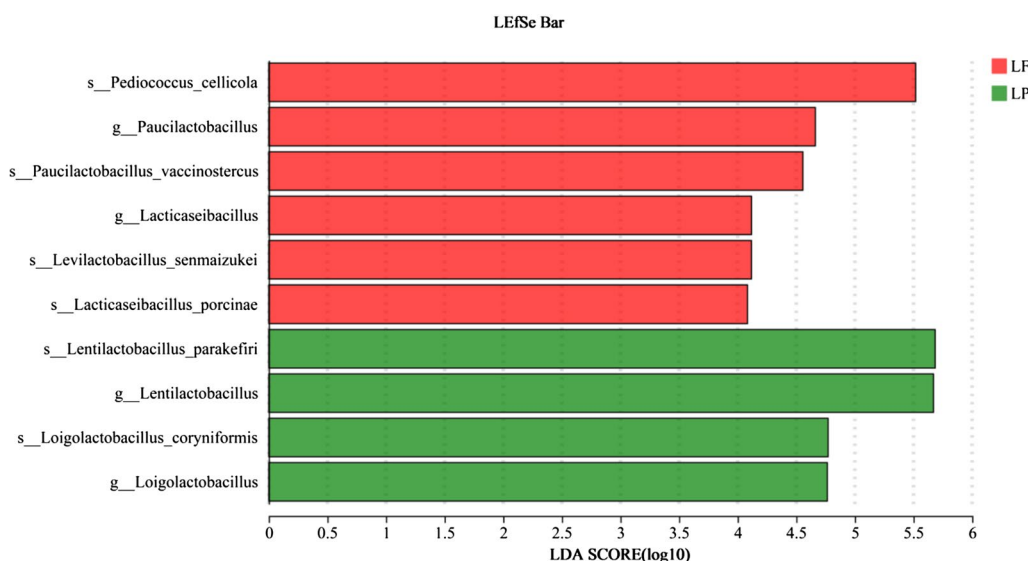

Fig. 2 Principal coordinate analysis (PCoA) of the bacterial community of fresh material and *Caragana korshinskii* on 60 days of ensiling. *FM* fresh material, *LF Limosilactobacillus fermentum*, *LP Lactiplantibacillus plantarum*, *CL* commercial lactic acid bacteria
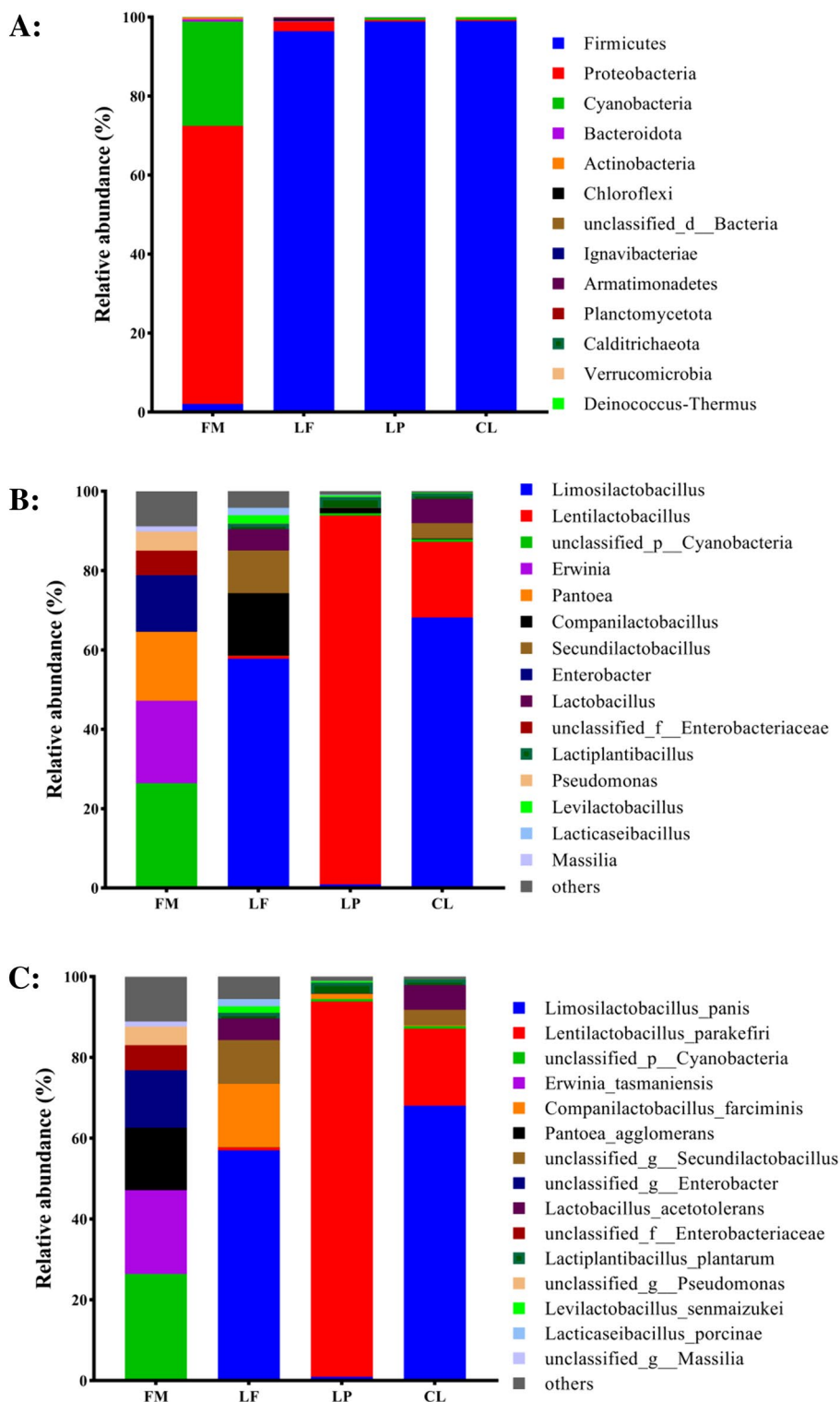
Fig. 3 Bacterial communities in fresh material and 60 days of *Caragana korshinskii* silage under different treatments. **A** The bacterial community was shown at the phylum level. **B** The bacterial community was shown at the genus level. **C** The bacterial community was shown at the species level. *FM* fresh material, *LF Limosilactobacillus fermentum*, *LP Lactiplantibacillus plantarum*, *CL* commercial lactic acid bacteria

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Fig. 4 The linear discrimination analysis (LDA) coupled on the bacterial community of fresh material and *Caragana korshinskii* 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 = 2.0. The length of the histogram shows the LDA score of differences in these groups. *FM* fresh material, *LF Limosilactobacillus fermentum*, *LP Lactiplantibacillus plantarum*, *CL* commercial lactic acid bacteria
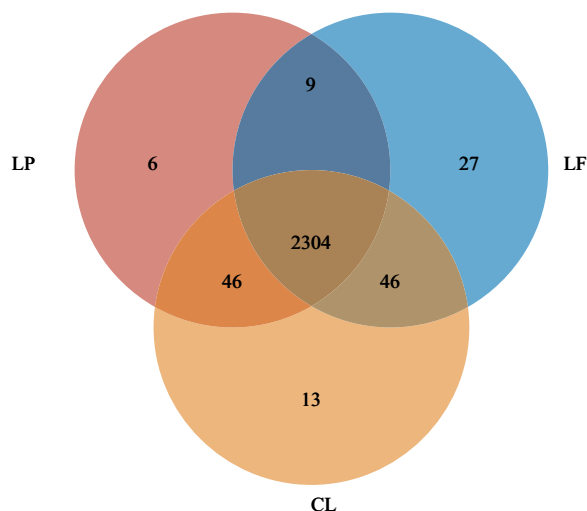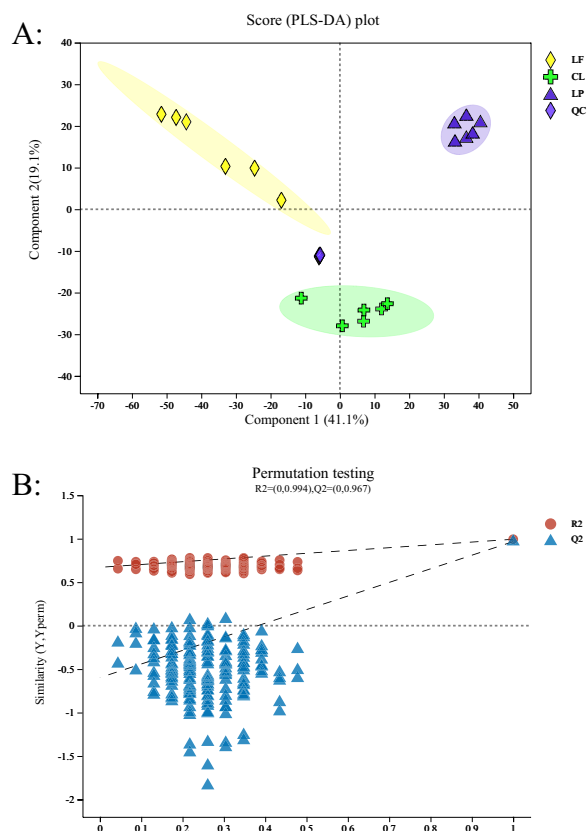

Fig. 5 Venn analysis of the metabolic products of *Caragana korshinskii* silage with the addition of lactic acid bacteria. *LF Limosilactobacillus fermentum*, *LP Lactiplantibacillus plantarum*, *CL* commercial lactic acid bacteria

(Fig. 3C). The predominant LAB in the LF group were *Limosilactobacillus_panis* (57.0%), *Companilactobacillus_farciminis* (15.7%), unclassified_g__Secundilactobacillus (10.8%), and *Lactobacillus_acetotolerans* (5.36%). The predominant LAB in the CL group were *Limosilactobacillus_panis* (68.0%), *Lentilactobacillus_parakefiri* (19.1%), *Lactobacillus_acetotolerans* (6.1%), and unclassified_g__Secundilactobacillus (3.9%). However, the *Lentilactobacillus_parakefiri* (92.9%) was most predominant LAB in the LP group.

The bar charts generated by LEfSe (Fig. 4) show the differences in taxa between treatments. The LF, LP and CL treatments had a significant effect on the microbial composition of silage at both genus and species level (LDA > 2). The LF treatment increased the relative abundance of *Pediococcus_cellicola* (LDA > 5.5), *Paucilactobacillus* (LDA > 4.5), *Paucilactobacillus_vaccinostercus* (LDA > 4.5), *Lacticaseibacillus* (LDA > 4), *Levilactobacillus_senmaizukei* (LDA > 4) and *Lacticaseibacillus_porcinae* (LDA > 4). The LP treatment increased the relative abundance of *Lentilactobacillus_parakefiri* (LDA > 5.5), *Lentilactobacillus* (LDA > 5.5), *Loigolactobacillus_coryniformis* (LDA > 4.5) and *Loigolactobacillus* (LDA > 4.5).
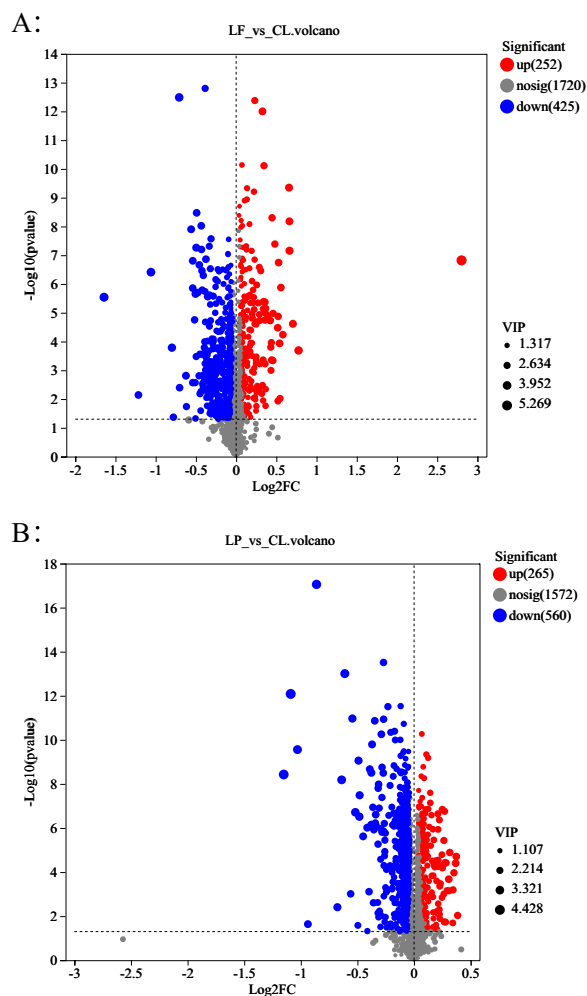
Differential metabolite analysis

A non-targeted metabolomics approach was utilized to study the effects of isolated LAB on the metabolic products of *Caragana korshinskii* silage. Among 18 samples, a total of 2491 metabolites were detected and 1250 metabolites were annotated. The LF, LP, and CL groups had a total of 2304 common metabolites, with 27 unique metabolites identified in the LF group, 6 unique metabolites in the LP group, and 13 unique metabolites in the CL group (Fig. 5). Partial least squares discriminant analysis (PLS-DA) was applied to differentiate metabolites within the samples, and significant discrimination was observed among the three groups after 60 days of ensiling (Fig. 6A). Permutation testing of the models indicated an R2 of 0.994 and a Q2 of 0.967 between the groups, demonstrating that these models are stable and reliable (Fig. 6B). We then screened for differential metabolites

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Fig. 6 Partial Least Squares-Discriminant Analysis (PLS-DA) of the metabolic characteristics of *Caragana korshinskii* silage with the addition of lactic acid bacteria additives. *LF Limosilactobacillus fermentum, LP Lactiplantibacillus plantarum, CL* commercial lactic acid bacteria

Fig. 7 Volcano plot analysis of the differential metabolites in *Caragana korshinskii* silage with the addition of lactic acid bacteria additives. *LF Limosilactobacillus fermentum, LP Lactiplantibacillus plantarum, CL* commercial lactic acid bacteria

under the following conditions: fold change ≥ 1, VIP ≥ 1, and p-value < 0.05. As shown in Fig. 7, the volcano charts demonstrate the differential metabolite composition of the LF and CL groups, and the LP and CL groups. We identified 674 and 823 differential metabolites between the LF and CL groups, and the LP and CL groups, respectively. Compared with CL, 253 metabolites were up-regulated and 421 metabolites were down-regulated in the LF group (Fig. 7A). The 266 metabolites were up-regulated and 557 metabolites were down-regulated in the LP group compared with the CL group (Fig. 7B).

Silage is a process in which LAB utilize soluble carbohydrates to produce organic acids, thereby inhibiting harmful microorganisms and preserving nutrients. In this study, we mainly wanted to verify the nutritional effects of isolation of LAB on the fermentation quality of *Caragana korshinskii* silage, so we focused on the differences in carbohydrate metabolism pathways among treatment groups. As shown in Fig. 8, the Differential Abundance Score (DA Score) demonstrates the overall changes of all differential metabolites in

the carbohydrate metabolic pathways between the LF and CL groups, and the LP and CL groups. Metabolite expression was up-regulated in pathways Propanoate metabolism, pyruvate metabolism, Butanoate metabolism, Pentose phosphate pathway, Citrate cycle and Glyoxylate and dicarboxylate metabolism and down-regulated in pathways Glycolysis/Gluconeogenesis, Amino sugar and nucleotide sugar metabolism, Ascorbate and aldarate metabolism, Pentose and glucuronate interconversions and Galactose metabolism in the LF group compared to the CL group. Metabolite expression was up-regulated in pathways Pyruvate metabolism, Ascorbate and aldarate metabolism, Citrate cycle, Glyoxylate and dicarboxylate metabolism, and Propanoate metabolism and down-regulated in pathways

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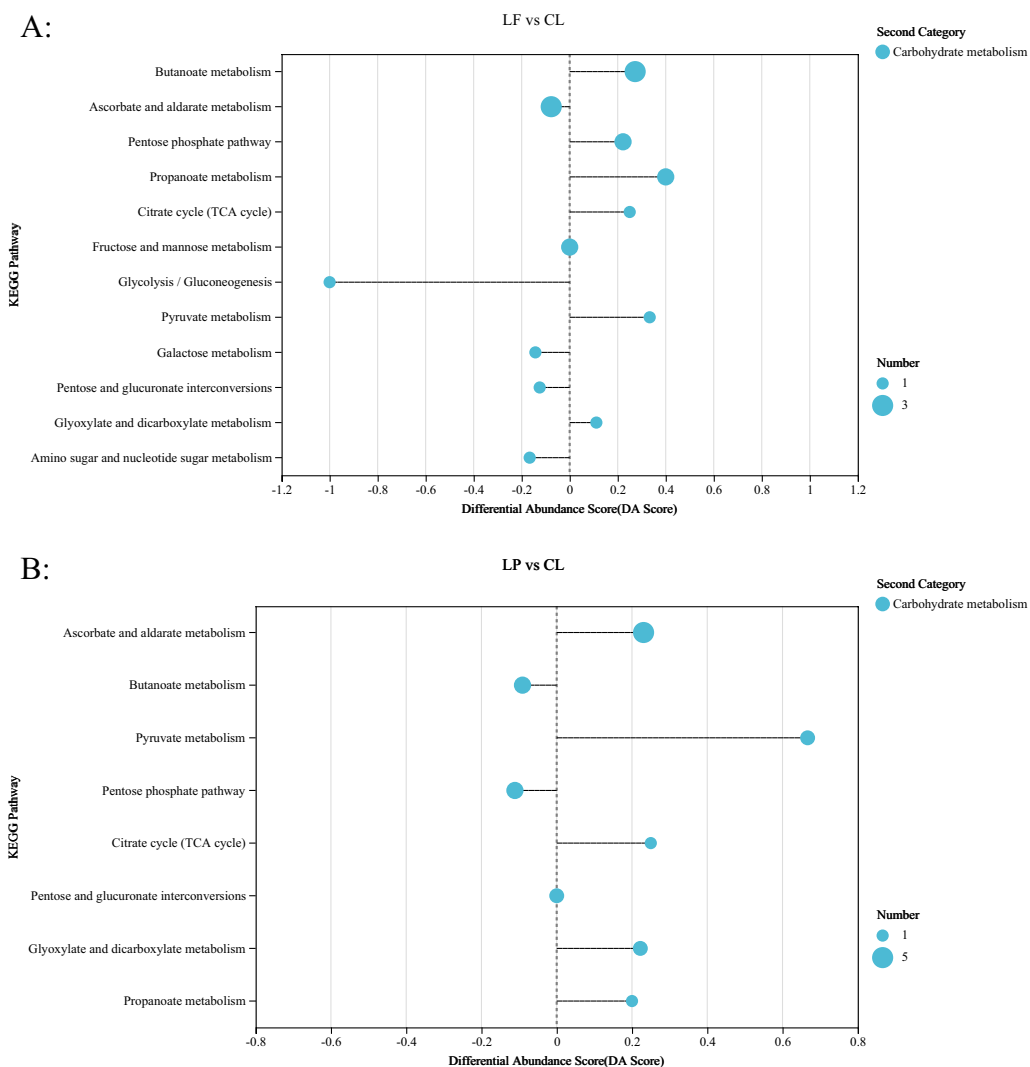
Fig. 8 Analysis of KEGG enrichment under the carbohydrate metabolic pathway. **A** LF vs CL; **B** LP vs CL. DA score represents the overall changes in all metabolites in the metabolic pathways; a score of 1 indicates up-regulated trend, while a score of − 1 indicates a down-regulated trend. Each bubble in the plot represents the number of associated metabolites

Pentose phosphate pathway and Butanoate metabolism in the LP group compared to the CL group. We next presented the differential metabolites in carbohydrate metabolism in a heat map (Fig. 9). Inoculation with LF significantly increased the levels of Gluconolactone, Gluconic Acid, Succinic Acid, 3-Hydroxybutanoic Acid, ʟ-rhamnofuranose, 2-Hydroxybutyric Acid, 3-Butyn-1-al and 2,3,4-Trihydroxybutyric acid and decreased the levels of ᴅ-Glucurono-6,3-lactone, Ascorbic acid, Cotinine glucuronide, ᴅ-Xylono-1,5-lactone, Salicin and ᴅ-Sorbitol compared with the CL group. Inoculation with LP significantly increased the content of ᴅ-Sedoheptulose 7-phosphate, Deoxycholic acid 3-glucuronide, ᴅ-Galactaric acid, ᴅ-Glucarate,

ᴅ-Arabinono-1,4-lactone, Succinic Acid, ʟ-Glutamine and 2-Isopropylmalic acid and decreased the content of Gluconic Acid, ᴅ-Gluconic acid, Ascorbic acid, (S,S)-Butane-2,3-diol, 3-Hydroxybutyric acid and Ribitol compared to inoculation with CL.

Discussion

The LAB play a key role in improving the health status of humans, animals and even plants, therefore, they are widely used in a variety of food and animal feed products with specific functions. Traditional identification methods of LAB mainly rely on the observation of their phenotypes and biochemical and physiological attributes to differentiate different species, but these methods cannot

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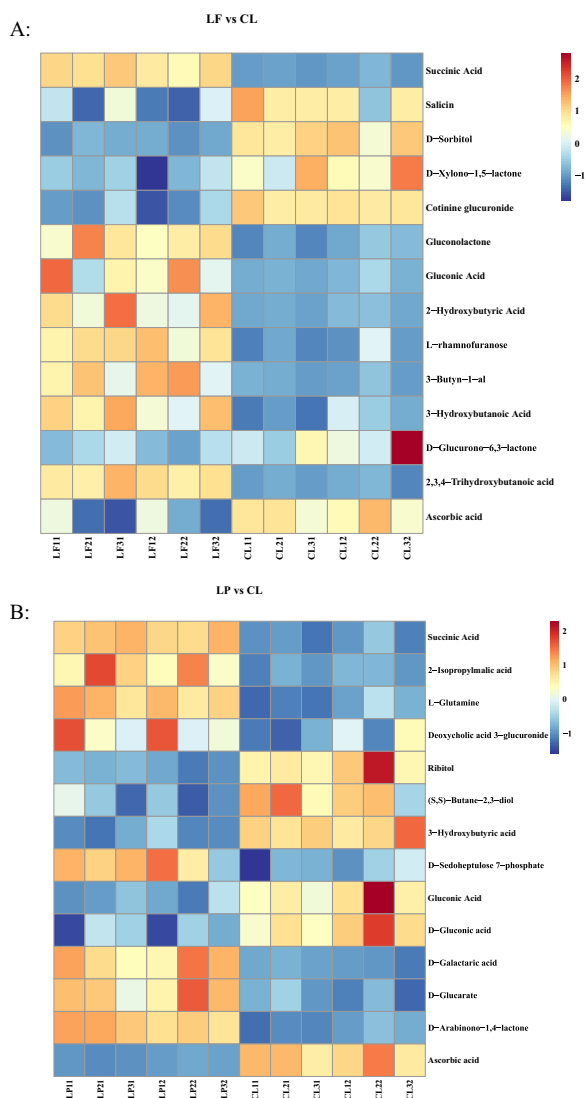
Fig. 9 Heat map of carbohydrate differential metabolite content of *Caragana korshinskii* silage under different treatments. Each colored cell corresponds to a value of different categories of metabolites. Red color indicates high content, while blue color indicates low content

completely ensure the standardized identification of strains. With the development of sequencing technology, 16S rDNA sequence analysis has become a widely used technique to accurately identify organisms at the species level [22], which significantly improves the screening efficiency and accuracy of LAB.

In our study, both strains of LAB selected by 16S rDNA sequence analysis could grow at pH 3.5–7.0. In comparison, *Limosilactobacillus fermentum* was still able to grow weakly at a pH value of 3.0, indicating its stronger tolerance to acidic environments, consistent with Bao's report [9]. Additionally, these three strains exhibited different

behaviors in experiments involving fermentation with various carbon sources. *Lactiplantibacillus plantarum* was capable of utilizing a wider variety of carbohydrates. Although the isolated *Lactiplantibacillus plantarum* and the commercial *Lactiplantibacillus plantarum* are the same species, but it was found that the isolated strain could utilize D-Raffinose, whereas the commercial strain could not. This might be the result of long-term evolution and selection in the unique environment of the Horqin sand. However, these characteristics only represent the traits of the strains themselves. When used as additives in silage fermentation, different plant materials can influence the fermentation outcome. Therefore, we conducted more in-depth testing and screening of the selected LAB strains in combination with the practical conditions of silage feed.

As population grows and arable land decreases, competition for food sources between humans and animals becomes more intense, resulting in conventional forage crops being unable to meet ruminant demand. Therefore, to alleviate the problem of feed shortage, woody forage has gained new attention in the field of animal feeding research in recent years [23]. *Caragana korshinskii* has been planted on a large scale in northern China due to its windbreak and sand fixation properties, and its high protein content, high nutritional value, and sizable biomass have made it a focus of great attention for livestock researchers recently [5, 24]. However, its high lignocellulose content and low WSC content make it difficult to ferment successfully. Previous studies have found that the minimum WSC concentration and the number of attached LAB for successful fermentation of legume forages are about 70.0 g/kg DM and 5.0 Log_{10} cfu/g FM, and that inoculation of silage with LAB ensures good fermentation quality and improves bioconversion efficiency [25]. In the present study, the WSC concentration of fresh *Caragana korshinskii* was 29.85 g/kg DM and the number of attached LAB was 4.35 Log_{10} cfu/g FM, which could not meet the silage fermentation requirements. Therefore, isolated and commercial LAB were used as silage additives in this experiment to improve its fermentation quality.

The rate of pH decline in silage is a major factor affecting fermentation quality [26]. Our previous study found that the pH of *Caragana korshinskii* decreased rapidly to below 4.2 after 3 days of silage [27], which greatly reduced CP loss. Protein hydrolysis of legumes during silage fermentation is a common problem and is mainly caused by phytolactic enzymes [10]. The loss of CP content in all three treatment groups of our study was less than 8%, which may be attributed to the inhibition of protease activity by the rapid decrease in pH. The LP and CL groups had significant lower pH value than the

LF group, which could be attributed to the fact that *Lactiplantibacillus plantarum* belongs to the homofermentative LAB strain, which has a strong lactic acid fermentation capacity in fermented feeds [28], and lactic acid is considered to be the most effective acid in reducing silage pH [29]. Although both LP and CL groups were supplemented with *Lactiplantibacillus plantarum*, the LA content was significantly higher in the LP group than in the CL group, which may be due to the fact that the efficacy of LAB silage inoculants is based on their ability to compete effectively relative to the epiphytic microbiota in the forage [30], and so native strains isolated from the *Caragana korshinskii* themselves had a competitive advantage and, therefore, produced more LA. *Limosilactobacillus fermentum* belong to the group of heterozygous fermenting LAB bacteria. This explains the higher AA content of the LF group. BA in silage is mainly produced by Clostridium, which is considered undesirable because it reduces ruminant intake [31], and no BA was detected in the LP group, suggesting that the metabolic activity of Clostridium was limited after fermentation. Many studies have found that inoculation of silage with LAB promotes lignocellulose degradation [32]. Mohamad's study also found that *Lactiplantibacillus plantarum* was able to produce cellulolytic and hemicellulolytic enzymes [33]. Therefore, we concluded that the NDF, cellulose and hemicellulose contents of the LP group were significantly lower than those of the other two groups, which may be due to the isolated *Lactiplantibacillus plantarum* producing cellulase-related enzymes that accelerate the degradation of lignocellulose, and also due to acid hydrolysis of structural carbohydrates during silage. It is well known that lignocellulose is more suitable for enzymatic hydrolysis after acid pretreatment [34], and part of the water-soluble cellulose is decomposed into WSC, which provides sufficient fermentation substrate for WSC-deficient *Caragana korshinskii* silage, consistent with the highest WSC content in the LP group. The IVDMD refers to the ratio of feeds degraded by microorganisms in the in vitro digestive process, which is a key measure of how efficiently the roughage is being utilized in the rumen fermentation process Indicator [35]. In our study, all three groups showed different increases in IVDMD after silage compared to fresh samples, with the LP group showing the highest increase in IVDMD, from 17.75% to 23.63%, which is likely due to the reduction of hemicellulose allowing rumen microbes to digest more efficiently components (e.g., nonfibrous carbohydrates), making them more dominant fermentation substrates. NDF in feed is a major factor influencing feed intake and milk production in dairy cows. Oba's study evaluated the relationship between NDFD and animal performance and found that each 1 unit increase in IVNDFD

increased milk production by 0.23 kg/d [36]. In this study, the LP group had a 43.6% increase in IVNDFD compared to raw materials, which is of major significance for the development and utilization of *Caragana korshinskii*. In combination with silage quality and in vitro digestion, *Caragana korshinskii* silage with the addition of isolated *Lactiplantibacillus plantarum* was the most effective.

When studying microbial diversity, rarefaction curves are commonly used to determine if enough sequencing data have been obtained to demonstrate the diversity of the sample. When the rarefaction curve flattens, it means that there is enough sequencing data [37], and the rarefaction curve in this experiment proves that these data are plausible. The replacement of harmful microorganisms by LAB as the dominant group after silage is the main reason for the decrease in microbial diversity. The Shannon index, commonly used to measure species diversity, indicates that the lower the index, the lower the species diversity. The LP group had the lowest Shannon index, which is due to the dominant bacterium in the LP group (*Lentilactobacillus parakefiri*, 92.9%) having a much higher relative abundance than in the CL group (*Limosilactobacillus panis*, 68%) and the LF group (*Limosilactobacillus panis*, 57%). Bai et al. suggest that when the abundance of dominant bacteria is high, the diversity of the microbial community decreases and the fermentation quality is better [38]. The highest LA content in the LP group corroborates this view. According to the PCoA analysis, there is a significant separation in the bacterial community of the LP group after ensiling, indicating that the LP group has a unique microbiome. Therefore, we conducted further analysis on the bacterial community.

The microbial communities naturally present on plants significantly influence the silage fermentation process [39]. In this study, bacteria belonging to the genera *Erwinia*, *Pantoea*, and *Enterobacter* were identified as the main epiphytic bacteria on *Caragana korshinskii* grass raw material; all these bacteria are members of the Enterobacteriaceae family. During the silage fermentation process, these enterobacteria compete with LAB for nutrients, which could reduce the quality of the silage feed. However, as the pH decreases during fermentation, the growth of these bacteria is inhibited, and they are eventually replaced by LAB. An intriguing point is that although the added *Limosilactobacillus fermentum* and *Lactiplantibacillus plantarum* belong to the native strains of *Caragana korshinskii* and they were able to grow normally in environments with pH as low as 3.5, they did not become the dominant bacterial flora in the silage samples after 60 days later in silage samples, they did not become the dominant bacterial flora. Instead, other LAB such as *Limosilactobacillus panis* and

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Lentilactobacillus parakefiri took over and became the dominant LAB. Powell's research suggests that acidity is not the sole contributor to antagonism among microbes; hydrogen peroxide, acetaldehyde, diacetyl, and bacteriocins also play a crucial role in this process [40]. Yildirim's research indicates that bacteriocins play a pivotal role in controlling the antagonistic interactions among LAB [41]. For example, in Shan's study, it was found that bacteriocin (Lactocin C-M2) produced by *Limosilactobacillus panis* effectively inhibits the growth and proliferation of *Pediococcus pentosaceus, Lactiplantibacillus plantarum, Levilactobacillus brevis, and Limosilactobacillus fermentum* [42]. This explains the significantly low abundance of *Limosilactobacillus fermentum* and *Lactiplantibacillus plantarum* in the LF and CL groups, while *Limosilactobacillus panis* dominates. Similarly, we speculate that the *Lentilactobacillus parakefiri* attached to the plant itself inhibit the added *Lactiplantibacillus plantarum* through the production of bacteriocins, and although studies on the bacteriocin production of *Lentilactobacillus parakefiri* have not yet been reported, Jannis's research has found that *Lentilactobacillus parakefiri* can produce bacteriostatic actives that inhibit the growth of a wide range of Gram-positive and negative bacteria [43]. In addition, the degradation of lignocellulose accompanies the silage process, and Giacon's study found that furan compounds, which are intermediate products of the lignocellulose degradation process, could stimulate the growth rate of heterofermentative LAB while inhibiting the growth and reproduction of homofermentative LAB [44]. This may explain the low relative abundance of *Lactiplantibacillus plantarum* added in the LP group and the final dominance of *Lentilactobacillus parakefiri*. Despite the low relative abundance of LAB added at the late silage stage, bao's study suggests that the added bacteria can play a key role in the early silage stage [9]. Isolated *Lactiplantibacillus plantarum* improved silage quality better than commercial LAB, probably because the effect of the inoculant dependent on the specific strain rather than the species.

The metabolomics analysis performed in this study using LC–MS was designed to investigate in depth the role of the addition of isolated LAB on the metabolites in *Caragana korshinskii* silage fermentation products. The metabolite differences among the three groups of samples were significant as shown by partial least squares discriminant analysis (PLS-DA) scatter plots, which indicated that the addition of isolated LAB significantly altered the metabolic composition of the silage process. Previous studies detected no more than 1000 metabolites in silage feed made from paper mulberry and hybrid *Pennisetum* [45, 46], whereas in this study, a total of 2491 different metabolites were identified across three *Caragana korshinskii* silage feed samples. This finding suggests that

the diversity and number of metabolites may be related to the different silage materials used. Du et al. demonstrated that the quality of silage is primarily determined by the hydrolysis of proteins and the glycolysis of sugars, a process largely due to the differences in microbial metabolic pathways for amino acids and carbohydrates [47]. Additionally, Du et al. further highlighted that the fermentation quality of paper mulberry silage is chiefly associated with the carbohydrate metabolism process driven by pyruvate and butyrate metabolism [48]. In our study of *Caragana korshinskii* silage, we noted that proteins were largely undegraded in the three silage samples, and we, therefore, shifted the focus of our study to differences in carbohydrate metabolic pathways. In terms of fermentation quality of the three groups, the silage with the addition of isolated *Lactiplantibacillus plantarum* was the best fermented and the one with the addition of *Limosilactobacillus fermentum* was the worst. Compared with the CL group, the LP group down-regulated Butanoate metabolism, Pentose phosphate pathway, and up-regulated Ascorbate and aldarate metabolism, while the LF group did the opposite. We suggest that these three carbohydrate metabolism pathways may be the key pathways affecting fermentation quality. 3-Butyn-1-al, 3-Hydroxybutanoic Acid and (S,S)-Butane-2,3-diol as precursors for the production of butyric acid we found to be down-regulated in the LP group and up-regulated in the LF group, which corresponded to higher butyric acid content in the LF group. Therefore, we hypothesized that lowering the Butanoate metabolism pathway might be beneficial for silage fermentation. The Pentose phosphate pathway and the Glycolysis pathway are two key pathways for the conversion of glucose to LA by LAB. Homofermentative lactic bacteria produces two molecules of LA from one molecule of glucose via the Glycolytic pathway, whereas heterofermentative lactic bacteria produce one molecule of LA from one molecule of glucose via the Pentose phosphate pathway. In the LF group, the enhancement of the Pentose phosphate pathway and attenuation of the Glycolysis pathway resulted in a significantly lower LA content than that of the LP and CL groups, which may be related to the different fermentation patterns of the isolated LAB. In the previous glycolysis test we found that only *Limosilactobacillus fermentum* could utilize D-Xylose, which is a precursor for the synthesis of 2,3,4-Trihydroxybutyric acid, which explains the highest 2,3,4-Trihydroxybutyric acid content in the LF group. Similarly, isolated *Lactiplantibacillus plantarum* could utilize D-Raffinose, while commercial *Lactiplantibacillus plantarum* could not, which corresponds to an indirect provision of more available carbohydrates for the silage process. Thus, in the present study, the isolated *Lactiplantibacillus plantarum* improved the

fermentation quality of *Caragana korshinskii* silage by down-regulating Butanoate metabolism, Pentose phosphate pathway and up-regulating Ascorbate and aldarate metabolism.

Conclusion

This study utilized SMRT sequencing technology and metabolomics techniques to analyze the microbial community and differential metabolic pathways in *Caragana korshinskii* silage with the addition of isolated LAB. Compared to commercial LAB, *Lactiplantibacillus plantarum* from the plants themselves proved to be more beneficial in enhancing the fermentation quality. This was achieved by reducing the complexity of the microbial community structure, simplifying the composition of LAB species in the silage, and increasing the relative abundance of the dominant *Lentilactobacillus parakefiri*. Moreover, by down-regulating the metabolic pathways of Butanoate metabolism and the Pentose phosphate pathway, the BA content was decreased, and LA content was increased, thereby improving the fermentation quality. Therefore, it is suggested to inoculate isolated *Lactiplantibacillus plantarum* into *Caragana korshinskii* silage to enhance fermentation quality.

Abbreviations

LAB	Lactic acid bacteria
CK	Control
LF	*Limosilactobacillus fermentum*
LP	*Lactiplantibacillus plantarum*
CL	Commercial lactic acid bacteria
FM	Fresh material
DM	Dry matter
WSC	Water-soluble carbohydrates
CP	Crude protein
NDF	Neutral detergent fiber content
ADF	Acid detergent fiber
ADL	Acid detergent lignin
LA	Lactic acid
AA	Acetic acid
PA	Propionic acid
BA	Butyric acid
NH_3-N	Ammonia nitrogen
IVDMD	In vitro dry matter digestibility
IVNDFD	In vitro neutral detergent fiber digestibility
OTUs	Number of operational taxonomic units
PCoA	Principal coordinate analysis
PLS-DA	Partial least squares-discriminate analysis

Author contributions
Conceptualization, methodology, data curation, writing-original draft preparation and writing-review and editing: B.C.B. Methodology: R.Q., J.B., and Y.C.L. Writing-original draft prepare ion, Writing-Review and editing, Investigation and resources: L.S. Writing-Review and editing: G.T.G. Project administration and funding acquisition: Y.S.J., Z.J.W. All authors have read and agreed to the published version of the manuscript.

Availability of data and materials
The raw sequence data were uploaded to the NCBI archive of sequence reads under study record number PRJNA1098938.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
There are no conflicts of interest in this work.

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