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SHORT COMMUNICATION

Identification and characterization of lactic acid bacteria isolated from tomato pomace

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Abstract The objective of this study was to isolate, identify, and investigate lactic acid bacteria (LAB) present in tomato pomace. In total, we isolated 108 LAB strains and subsequently identified 16 species (87.5 % homofermentative LAB). Among these, the isolation of *Lactobacillus harbinensis*, *L. manihotivorans*, *L. helveticus*, *L. camelliae*, *L. pontis*, *L. amylovorus*, *L. hilgardii*, *L. panis*, *L. vaginalis*, and *L. rapi* from vegetable samples is reported for the first time. This is the first report to describe the diversity and natural populations of LAB associated with tomato pomace. The physiological and biochemical properties of the isolates could provide a platform for the future design of LAB inoculants aimed at improving silage quality.

Keywords Characterization · Identification · Lactic acid bacteria · Tomato pomace · 16S rRNA gene

Tomato pomace has been evaluated for use in rations for poultry (Mansoori et al. 2008), dairy cows (Weiss et al. 1997), and small ruminants. The production of tomato pomace is a major industry in the city of Bayannuoer, Inner Mongolia province, as well as in other areas of China. Tomato pomace, a byproduct of the tomato juice industry, contains abundant nutrients, such as vitamins, minerals, vegetable fiber, and protein (Delvalle et al. 2006). Ventura et al. (2009) reported that the nutritional components of tomato pomace include dry matter (DM, 150–300 g kg^{-1} DM), neutral detergent fiber (550–650 g kg^{-1} DM), acid detergent fiber (400–500 g kg^{-1} DM), crude protein (150–200 g kg^{-1} DM), and lignin (250–300 g kg^{-1} DM). Tomato pomace has been included at concentrations of up to 200 g kg^{-1} DM (Fondevila et al. 1994) in complete rations for growing lambs or at 480 g kg^{-1} DM to make feed blocks (Ben Salem and Znaidi 2008). However, a major drawback of tomato pomace is that it is difficult to preserve. The use of artificial drying to preserve tomato pomace comes at the expanse of a loss of nutritive value. Silage is a common preserved feed in many countries, including China (Ruppert et al. 2003). The preservation of silage depends on the production of sufficient quantities of organic acids, mainly those produced by lactic acid bacteria (LAB), to inhibit the activity of undesirable microorganisms, such as clostridia and molds, under anaerobic conditions (Cai et al. 1997, 1999a). However, to our knowledge, no information is as yet available on the natural populations of LAB in tomato pomace. The aim of the study reported here was to isolate, identify, and characterize the LAB from tomato pomace.

Tomato pomace was collected from local commercial producers of tomato juice (Inner Mongolia, China). Tomato pomace is a residue that remains after the juice is extracted and consists of leftover flesh, seeds, and peels. The tomato pomace samples were collected in sterilized bags, kept in ice boxes, and transported to a microbiology laboratory where they were analyzed immediately. Serial dilutions were used to isolate the LAB using MRS agar (Huankai Microbial SCI. & Tech, Co., Ltd. Guangdong, China) at 37 °C for 48 h under anaerobic conditions (anaerobic jar; AnaeroPack, Anaero, Japan). The isolation process was carried out as follows. Colonies of different morphology (colony size, shape, and color) on MRS agar medium were picked from each sample; a total of

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108 strains were collected and purified twice by streaking on MRS agar. All of these strains were considered to be LAB based on Gram-stain appearance, the catalase test, and 16S rDNA sequencing. Gram-positive and catalase-negative bacteria were identified as LAB. The purified strains were stored at −80 °C in nutrient broth (Difco Laboratories, Franklin Lakes, NJ) and dimethyl sulfoxide (9:1 ratio) until further analysis (Cai et al. 1999b, c). Morphological characteristics and gas production from glucose were determined as described previously. Growth at different temperatures was detected in MRS agar after incubation at 5 and 10 °C for 10 days and at 45 and 50 °C for 7 days. Growth at pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 was observed in MRS broth after incubation at 37 °C for 7 days. Salt tolerance of the LAB isolates was tested in MRS agar containing 3.0 and 6.5 % NaCl. All isolates assigned to a particular LAB genus or species were identified on API 50 CH strips (Biomérieux, Marcy l'Etoile, France).

Molecular phylogeny analysis was conducted and the phylogenetic tree was constructed based on about 1,500 bases of the 16S rDNA sequences. Cells were grown at 37 °C for 8 h in MRS broth and used for DNA extraction and purification (Meroth et al. 2003). The concentration and purity of the DNA were determined using a spectrophotometric method at 260 and 280 nm (Liu et al. 2012). The 16S rRNA gene sequence coding region was amplified by PCR in a PCR thermal cycler (FlexCycler; Analytik Jena, Jena, Germany) with the prokaryotic 16S ribosomal DNA universal primers 27 F (5′-AGAGTTTGAT CCTGGCTCAG-3′) and 1492R (s) (5′-TACCTTGTTACGACTT-3′). Each PCR reaction consisted of a 50-μl volume containing 0.25 μl Takara Taq (5 U μl^{-1}), 1 μl 27 F (10 μM) and 1492R (s) (10 μM) primer, respectively, 5 μl of 10×PCR buffer (Mg^{2+} Free), 3 μl of $MgCl_2$ (25 mM), 4 μl of dNTP mixture, 2 μl of the template DNA, and ddH_2O up to 50 μl. The PCR thermal cycling conditions were one cycle at 94°C for 5 min, 30 cycles at 94 °C for 1 min, 53 °C for 1 min, and 72 °C for 2 min, followed by one cycle at 72 °C for 10 min. The purified PCR fragments were sent directly to Invitrogen Biotechnology Co., Ltd (Guangzhou, China) for 16S rDNA sequencing analysis in a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA). Sequence similarity searches were performed in the GenBank data library using the BLAST program, and the retrieved sequence information was then introduced into the CLUSTALW software program for assembly and alignment (Thompson et al. 1994). The 16S rRNA gene sequences of the isolated strains were compared with sequences from type LAB strains held in the National Center for Biotechnology Information (NCBI). Nucleotide substitution rates were calculated (Kimura and Ohta 1972), and the phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). *Bacillus subtilis* NCDO 1769 was used as an outgroup organism (Duan et al. 2008). The topologies of the tree were evaluated using bootstrap analysis of the sequence data with the molecular evolutionary genetics analysis (MEGA) 5 software (Tamura et al. 2007), based on 1,000 random resamplings and the Kimura two-parameter model (Eitan et al. 2006). The nucleotide sequences for the 16S rRNA gene described in this report have been deposited with GenBank under accession nos. KF418815, KF418816, KF418817, KF418818, KF418819, KF418820, KF418821, KF418822, KF418823, KF418824, KF418825, KF418826, KF418827, KF418828, KF418829, and KF418830 for the representative strains FQ002, FQ003, FQ005, FQ011, FQ015, FQ027, FQ034, FQ045, FQ060, FQ066, FQ072, FQ073, FQ076, FQ084, FQ097 and FQ103, respectively.

The characteristics of the LAB isolated in our study are shown in Tables 1 and 2. The isolated strains were identified as 16 species, all of which belonged to the genus *Lactobacillus*. These results showed that lactobacilli were the dominant LAB species in our samples of tomato pomace. Lactobacilli are generally preferred to lactic acid-producing cocci for silage fermentation because they play a more important role in the fermentation process and effectively promote lactic acid production for longer times compared to lactic acid-producing cocci. In this context, the isolated strains are likely to be good silage inoculants. FQ003, FQ005, FQ011, FQ015, FQ034, FQ072, and FQ066 grew well at 10 °C, suggesting that these strains would be valuable for use in the future design of appropriate inoculants for silage fermentation in cold areas. FQ103, FQ060, FQ045, FQ084, and FQ097 grew well at 45 °C, indicating that they are heat-resistant and could be added to silage stored at high temperatures. All of the isolates, with the exception of FQ015 and FQ060, grew well medium containing 3.0 % NaCl; FQ011 was also able to grow well at 6.5 % NaCl. All of the isolates, with the exception of FQ027, FQ060, and FQ076, grew well at pH levels ranging from 3.0 to 8.0, indicating that most of the isolated strains are resistant to acid and that they should grow well during the process of making silage. In addition, all isolates except FQ027 and FQ066 were homofermentative rods. Many studies (Cai et al. 1998, 1999a) have reported that the inoculation of forage with homofermentative lactobacilli such as *Lactobacillus casei* or *L. plantarum* has beneficial effects, such as promoting lactic acid fermentation and improving silage quality. In contrast, Cai et al. (1998) reported that inoculation of silage with heterofermentative *Leuconostoc* and *Weissella* strains did not improve silage quality and may have caused some fermentation loss. Based on these results, we suggest that the strains isolated in our study would be excellent inoculants for promoting silage fermentation.

The phylogenetic tree of isolated strains constructed from evolutionary distances using the neighbor-joining method are shown in Fig. 1. Based on these morphological characteristics and the results of the physiological and biochemical tests and phylogenetic analysis, strains FQ060, FQ034, FQ003, FQ073, FQ066, FQ076, FQ027, FQ045, FQ097, FQ084, FQ002, and

Table 1 Characteristics of representative strains[a] isolated from tomato pomace

Characteristic	FQ003	FQ002	FQ005	FQ011	FQ015	FQ027	FQ034	FQ072	FQ060	FQ066	FQ045	FQ103	FQ073	FQ084	FQ097	FQ076
Isolates (*n*)	17	6	36	11	3	3	7	4	4	3	2	2	2	2	3	3
Shape	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod	Rod
Gram stain	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Gas from glucose	−	−	−	−	−	+	−	−	−	+	−	−	−	−	−	−
Fermentation type[b]	Homo	Homo	Homo	Homo	Homo	Hetero	Homo	Homo	Homo	Hetero	Homo	Homo	Homo	Homo	Homo	Homo
Growth at temperature (°C):																
5.0	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10.0	+	W	+	+	+	−	+	+	−	+	−	−	−	−	−	−
45.0	−	−	−	−	−	−	−	−	+	−	+	+	−	+	+	W
50.0	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Growth in NaCl																
3.0 %	+	+	+	+	−	+	+	+	−	+	+	W	+	+	+	+
6.5 %	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−
Growth at pH:																
3.0	+	+	+	+	+	−	+	+	−	+	+	+	+	+	+	−
4.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
16S rRNA similarity[c] (%)	99.86	99.59	99.59	99.38	99.52	99.67	98.00	99.23	99.00	99.93	97.30	99.72	98.33	99.65	99.40	100

+, Positive; W, weakly positive; −, negative

[a] Based on the neighbor-joining (NJ) method or NJ designations

[b] Homo, Homofermentative; Hetero, heterofermentative

[c] 16S rDNA sequence similarity between isolate and each type strain was analyzed by BLAST search program

Table 2 Fermentation patterns (evaluated using API 50 CH strips; BioMérieux France) of lactic acid bacteria strains isolated from tomato pomace

Item	FQ003	FQ002	FQ005	FQ011	FQ015	FQ027	FQ034	FQ072	FQ060	FQ066	FQ045	FQ103	FQ073	FQ084	FQ097	FQ076
Glycerol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	W
Erythritol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Arabinose	W	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Arabinose	+	+	−	+	−	+	−	−	−	+	−	+	+	−	−	+
Ribose	−	+	+	+	−	+	−	−	−	+	−	−	+	−	−	+
D-Xylose	+	+	−	−	−	+	−	−	+	+	−	−	+	−	−	+
L-Xylose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Adonitol	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
β-Methyl-xylopyranoside	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+
Galactose	+	−	+	+	+	+	+	+	+	+	−	+	+	W	W	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	−	+	+	+	−	+
D-Fructose	+	+	+	+	+	−	+	+	+	+	−	+	+	−	−	+
D-Mannose	+	−	+	+	+	−	+	+	+	W	−	+	−	−	−	+
L-Sorbose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Rhamnose	−	−	−	−	−	−	W	+	−	−	−	−	−	−	−	−
Dulcitol	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Mannitol	−	−	+	+	+	−	−	−	−	W	−	+	−	W	−	+
Sorbitol	−	−	+	+	+	−	−	−	−	−	−	−	−	−	−	+
α-Methyl-d-mannoside	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	+
α-Methyl-d-glucoside	+	−	+	−	−	+	−	−	−	−	−	−	−	−	−	W
N-Acetyl glucosamine	+	−	+	+	+	−	+	+	+	−	−	+	−	−	−	+
Amygdalin	+	−	+	+	−	−	−	−	+	−	−	+	−	−	−	+
Arbutin	+	−	+	+	−	−	−	−	+	−	−	W	−	−	−	+
Esculin	+	−	+	+	+	−	−	W	+	−	−	W	−	−	−	+
Salicin	+	−	+	+	+	−	−	−	+	−	−	W	−	−	−	+
Cellobiose	+	−	+	+	−	−	+	+	+	−	−	+	−	−	−	+
Maltose	+	+	+	+	+	+	+	+	+	+	−	+	+	W	−	+
Lactose	−	−	+	+	−	+	W	+	−	−	−	+	−	−	−	+
Melibiose	+	+	−	+	−	+	−	−	−	+	−	−	+	+	−	+
Saccharose	+	+	+	+	+	+	−	−	W	+	−	+	+	+	+	+
Trehalose	+	−	+	+	−	−	−	−	+	−	−	+	−	−	−	+
Inulin	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
Melezitose	+	−	+	+	−	+	−	−	−	+	−	W	+	−	−	+
D-Raffinose	+	+	−	+	−	−	−	−	−	+	−	W	+	W	−	+
Starch	W	−	−	−	−	−	−	−	W	−	−	+	−	−	−	−

Table 2 (continued)

Item	FQ003	FQ002	FQ005	FQ011	FQ015	FQ027	FQ034	FQ072	FQ060	FQ066	FQ045	FQ103	FQ073	FQ084	FQ097	FQ076
Glycogen	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−
Xylitol	−	−	−	−	−	−	−	−	−	−	−	W	−	−	−	−
β-Gentiobiose	+	−	+	+	−	−	−	−	+	−	−	+	−	−	−	+
D-Turanose	W	−	+	+	−	+	−	−	−	−	−	−	−	−	−	+
D-Lyxose	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
D-Tagatose	W	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Fucose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
L-Fucose	W	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
D-Arabitol	−	−	−	W	−	−	−	−	−	+	−	−	−	−	−	−
L-Arabitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Gluconate	+	W	+	+	−	+	−	−	−	+	−	−	+	−	−	+
2-Keto-gluconate	−	−	−	−	−	W	−	−	−	W	−	−	−	−	−	−
5-Keto-gluconate	−	W	−	−	−	+	−	−	−	+	−	−	+	−	−	W

+, Positive; W, weakly positive; −, negative

FQ103 were unambiguously identified as *L. camelliae*, *L. manihotivorans*, *L. harbinensis*, *L. hilgardii*, *L. parafarraginis*, *L. rapi*, *L. buchneri*, *L. pontis*, *L. vaginalis*, *L. panis*, *L. fermentum*, and *L. amylovorus*, respectively (bootstrap between 92 and 100 %). FQ005 formed a very well-defined cluster with three type strains (*L. paracasei* subsp. *tolerans*, *L. paracasei* subsp. *paracasei*, and *L. casei*), and 94 % bootstrap values confirmed monophyly. Furthermore, this strain appeared to be more similar with *L. casei* and *L. paracasei* subsp. *paracasei* than *L. paracasei* subsp. *tolerans* based on the 16S rDNA sequence, and its 16S rDNA sequence showed a similarity of 99.59 % to *L. casei* and *L. paracasei* subsp. *paracasei*. Collins et al. (1989) reported that *L. casei* and *L. paracasei* subsp. *paracasei* could be classified by carbohydrate utilization. Comparison of the carbohydrate fermentation patterns revealed unambiguously that the pattern of strain FQ005 was an exact match to that of the *L. paracasei* subsp. *paracasei* type strain and different from that of the *L. casei* type strain. In fact, it appeared that although the *L. paracasei* subsp. *paracasei* type strain produced acid from ribose, α-methyl-d-glucoside, adonitol, dulcitol, inulin, and lactose, the *L. casei* type strain did not. On the other hand, unlike *L. paracasei* subsp. *paracasei*, *L. casei* was able to grow on media with glycerol as a carbon source (Table 2). FQ011 clustered with four type strains (*L. plantarum* subsp. *argentoratensis*, *L. paraplantarum*, *L. pentosus*, and *L. plantarum*), and 84 % bootstrap values confirmed monophyly. This strain also appeared to be more similar to *L. plantarum* and *L. pentosus* than to these other two type strains based on the 16S rDNA sequence, with its 16S rDNA sequence showing a similarity of 99.38 % to *L. plantarum* and *L. pentosus*. In our study, we referred to carbohydrate fermentation patterns that showed unambiguously that the pattern of strain FQ011 was an exact match to that of the *L. plantarum* type strain and different from that of the *L. pentosus* type strain. In fact, it appeared that although the *L. plantarum* type strain produced acid from melezitose, D-raffinose and α-methyl-d-mannoside, the *L. pentosus* type strain did not. On the other hand, unlike *L. plantarum*, *L. pentosus* could grow in media with glycerol or D-xylose as the sole carbon source (Table 2). FQ015 was distinctly clustered with *L. coryniformis* subsp. *torquens* and *L. coryniformis* with high bootstrap values of 100 %. However, based on its 16S rDNA sequence FQ015 was more similar to *L. coryniformis* subsp. *torquens* (99.52 %) than to *L. coryniformis* (98 %), so FQ015 was identified as *L. coryniformis* subsp. *torquens*. FQ072 appeared to be equally linked to both *L. helveticus* and *L. gallinarum* (bootstrap 97 %), but the carbohydrate fermentation pattern of strain FQ072 was unambiguously an exact match to that of the *L. helveticus* type strain and different from that of the *L. gallinarum* type strain.

To summarize, most of the isolated *Lactobacillus* strains were homofermentative species and some were heat- or cold-

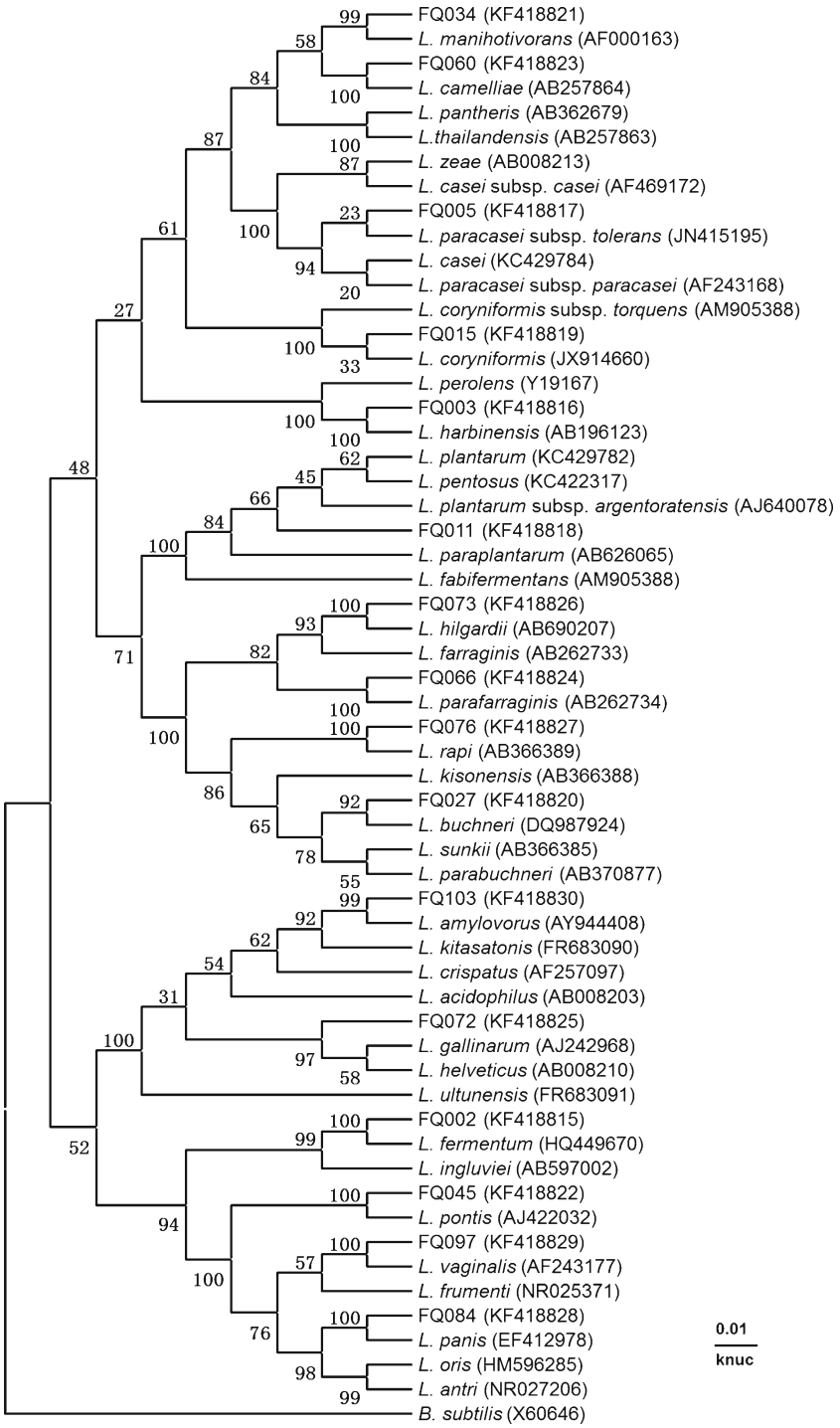

Fig. 1 Phylogenetic tree showing the relative positions of the representative isolates isolated from tomato pomace as inferred by the neighbor-joining tree based on our comparison of complete 16S rDNA sequences. Bootstrap values for 1,000 replicates are shown at the *nodes of the tree*. *Bacillus subtilis* is used as an outgroup. *Bar* indicates 1 % sequence divergence. *L. Lactobacillus*, *Knuc* nucleotide substitution rat

resistant. The heat- and cold-resistant strains could serve as good silage inoculants at extreme temperature conditions. In addition, this is the first report of the isolation of, *L. harbinensis*, *L. manihotivorans*, *L. helveticus*, *L. camelliae*, *L. pontis*, *L. amylovorus*, *L. hilgardii*, *L. panis*, *L. vaginalis*, and *L. rapi* from vegetable samples. A comprehensive analysis of the biochemical and physiological properties of both these extreme temperature-resistant isolates and the other isolates should hopefully provide a platform for the discovery and design of inoculants aimed at improving silage fermentation and quality under various prevailing conditions.

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