Lactobacillus shenzhenensis sp. nov., isolated from a fermented dairy beverage

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Two Lactobacillus strains, designated LY-73T and LY-30B, were isolated from a dairy beverage, sold in Shenzhen market, China. The two isolates were Gram-positive, non-spore-forming, non-motile, facultatively anaerobic rods that were heterofermentative and did not exhibit catalase activity. Sequencing of the 16S rRNA, pheS and rpoA genes revealed that the two isolates shared 99.5, 99.8 and 99.9 % sequence similarity, which indicates that they belong to the same species. Phylogenetic analysis demonstrated clustering of the two isolates with the genus Lactobacillus. Strain LY-73T showed highest 16S rRNA gene sequence similarities with Lactobacillus harbinensis KACC 12409T (97.73 %), Lactobacillus perolens DSM 12744T (96.96 %) and Lactobacillus selangorensis DSM 13344T (93.10 %). Comparative analyses of their rpoA and pheS gene sequences indicated that the novel strains were significantly different from other Lactobacillus species. Low DNA–DNA reassociation values (50.5 %) were obtained between strain LY-73T and its phylogenetically closest neighbours. The G+C contents of the DNA of the two novel isolates were 56.1 and 56.5 mol%. Straight-chain unsaturated fatty acids C18:1ω9c (78.85 and 74.29 %) were the dominant components, and the cell-wall peptidoglycan was of the L-Lys–D-Asp type. Based on phenotypic characteristics, and chemotaxonomic and genotypic data, the novel strains represent a novel species of the genus Lactobacillus, for which the name Lactobacillus shenzhenensis sp. nov. is proposed, with LY-73T (= CCTCC M 2011481T = KACC 16878T) as the type strain.

Lactic acid bacteria (LAB) are distributed in a variety of habitats rich in carbohydrate-containing substrates, including plants (Trias et al., 2008), naturally fermented products (Kacem et al., 2005), and the gastrointestinal tracts of humans and animals (Walter, 2008). Most LAB play an important role in food fermentation and preservation (Caplice & Fitzgerald, 1999; Bernardeau et al., 2008), and they are widely used for probiotics (Ziemer & Gibson, 1998; Fookes et al., 1999). The genus Lactobacillus, first proposed by Beijerinck (1901), consists of more than 170 recognized species (Euzéby, 2012), including eight species described in this journal recently (Dmitriy et al., 2012; Kaihei et al., 2012; Shiou-Huei et al., 2012; Yimin et al., 2012).

During the course of our study on beverage microbial diversity, a beverage was obtained from a company that specializes in fermenting and producing beverage in Shenzhen, China. The sample was spread on MRS (Difco, pH 6.2) plates and incubated under aerobic conditions at 37 °C for 3 days. Two rod-shaped bacterial strains, designated LY-73T and LY-30B, were isolated using the standard dilution plating technique on MRS plates at 37 °C (Chanal et al., 2006) and subjected to a polyphasic taxonomic study. The isolates were maintained in a glycerol suspension (20 %, v/v) at −80 °C and preserved by lyophilization at 4 °C.

Chromosomal DNA was extracted for phylogenetic analyses of strains LY-73T and LY-30B using the method described by Drancourt et al. (2000). The 16S rRNA gene was amplified...
using a pair of universal primers, 27f (5'-AGAGTTTGATCCTACGGGCTCA-3') and 1492r (5'-TACGGYTACCTGGTACGACTT-3') (Weisburg et al., 1991). The taxonomic position of strain LY-73T was also investigated by analysing the sequences of the phenylalanyl-tRNA synthase (pheS) and RNA polymerase (rpoA) genes using the primer pairs pheS-21-F (5' -CAYCCNGCHCGYGAYATGC-3') and pheS-22-R (5'-CCWARVCRAARGARCC-3'), and rpoA-21-F (5'-AGATGARTTTGAAAACC-3') and rpoA-23-R (5'-ACHGTRTTRATDCDGRCG-3'), respectively (Naser et al., 2005). The amplified 16S rRNA, pheS and rpoA genes were purified using a PCR Clean-Up System (Promega A9282- Wizard) and subsequently sequenced using a 3730 sequencer at BGI-Shenzhen. The sequence information was imported into the DNASTAR software program (DNASTAR) for assembly. The closest known relatives of the strains were determined using FastA and sequences of strains of closely related species were retrieved from the EzTaxon server, GenBank and DDBJ databases.

Approximately 1500 bp of the 16S rRNA gene sequences (approx. 800 bp for the rpoA gene and 450 bp for the pheS gene) (Fig. S1 available in IJSEM Online) of strains LY-73T and LY-30B, and of their related species, were analysed using the neighbour-joining method and the maximum-likelihood method implemented in MEGA 5 (Tamura et al., 2011) to construct phylogenetic trees (Fig. 1). The results showed that strain LY-73T is closely related to Lactobacillus

![Phylogenetic tree](image-url)
harbinensis KACC 12409T, Lactobacillus perolens DSM 12744T and Lactobacillus selangorensis DSM 13344T. 16S rRNA gene sequence similarities between strain LY-73T and L. harbinensis KACC 12409T, L. perolens DSM 12744T and L. selangorensis DSM 13344T were 97.73, 96.96 and 93.10 %, respectively. Although strains LY-73T and LY-30B were in the same branch, they formed a distinct branch within the genus Lactobacillus. The level of pheS gene sequence similarity between the novel strains and their closest neighbour was 84 %, whereas that for the rpoA gene was 93 %. On the basis of neighbour-joining analysis of the rpoA and pheS gene sequences (Fig. 2), the novel strains did not belong to any recognized species. Similar topologies were obtained by the minimum-evolution and maximum-parsimony methods (data not shown).

The strains grown on MRS agar at 37 °C for 3 days were used for physiological and biochemical tests. The Gram reaction was determined via a non-staining method using

![Phylogenetic trees based on pheS (a) and rpoA (b) gene sequences constructed using the neighbour-joining method showing the relationship between strains LY-73T and LY-30B and the type strains of closely related species. Bootstrap values (≥ 50 %) are indicated at branch points, Bar, 5 % nucleotide sequence divergence.](http://ijs.sgmjournals.org)
levels were performed between strain LY-73T and the type strain of the genus Lactobacillus. DNA was enzymically degraded, and the MRS broth culture incubated at 37 °C for 48 h in an anaerobic box (Bactron Anaerobic Chamber). Gas production from glucose was measured using a Durham tube in MRS broth, the presence of CO2 and ethanol indicating facultatively heterofermentative metabolism (Hammes & Vogel, 1995).

Catalase activity was determined using the method described by Cappuccino & Sherman (2002). Growth at different temperatures was evaluated in MRS broth after incubation at 4, 10, 15, 20, 30, 40, 45 and 50 °C for 7 days. The pH range for growth was assessed in MRS broth at pH 3.0–10.0 (at intervals of 0.5 pH units) for 7 days. Salt tolerance was determined in MRS broth containing 2.0–10.0 % (w/w) NaCl. API 50 CHL system (bioMérieux) tests were performed according to the manufacturer’s instructions. All physiological and biochemical tests in the study were performed under the same conditions.

Cells of strains LY-73T and LY-30B were Gram-positive, facultatively anaerobic, rod-shaped (0.6–0.8 x 3.5–4.0 μm), non-motile and catalase-negative. The temperature growth range was 10–45 °C, with optimum growth at 37 °C. The pH growth range was pH 4.0–9.0, with optimum growth at pH 6.2. The NaCl tolerance range was 0–6.0 % (w/w). The isolates were heterofermentative LAB and produced CO2 and ethanol from glucose. The phenotypic properties of strains LY-73T and LY-30B distinguished them from other members of the genus Lactobacillus. The main differences from closely related type strains of the genus are summarized in the species description and in Table 1.

DNA was extracted and purified from cells harvested from MRS broth culture incubated at 37 °C for 24 h using the traditional procedure (Saito & Miyura, 1963) to determine the G+C content and for DNA–DNA hybridization experiments. DNA was enzymically degraded, and the G+C content was measured by HPLC as described by Mesbah et al. (1989). Analyses of DNA–DNA hybridization levels were performed between strain LY-73T and the type strains of the most closely related species (L. harbinensis DSM 12409T and L. perolens DSM 12744T) according to the fluorometric microwell method (Ezaki et al., 1989; He et al., 2005).

The DNA G+C content of strains LY-73T and LY-30B was 56.1 and 56.5 mol%, respectively, values that are very high for members of the genus Lactobacillus (Kandler & Weiss, 1986; Liu & Dong, 2002). The DNA–DNA hybridization

### Table 1. Differential phenotypic features of strains LY-73T and LY-30B and the type strains of closely related species of the genus Lactobacillus

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<tr>
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<td>Salt tolerance</td>
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<tr>
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<tr>
<td>2-Ketogluconate</td>
<td>w</td>
<td>w</td>
<td>w</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>56.1</td>
<td>56.5</td>
<td>53*</td>
<td>51*</td>
<td>45–47*</td>
<td>50*</td>
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</table>

*DNA G+C content from Miyamoto et al. (2005), Back et al. (1999), Leisner et al. (2000) and Rogosa & Hansen (1971).
data showed that strain LY-73\textsuperscript{T} had a DNA–DNA relatedness value of more than 88.1 % with strain LY-30B, and of less than 50.5 % with \textit{L. harbinensis} KACC 12409\textsuperscript{T} and with \textit{L. perolens} DSM 12744\textsuperscript{T} (Table S1), which is well below the 70 % cut-off value for the delineation of genomic species (Stackebrandt & Goebel, 1994; Tindall \textit{et al.}, 2010).

Whole cells were hydrolysed with 4 M HCl at 100 °C for 15 h to analyse the peptidoglycan structure of strains LY-73\textsuperscript{T} and LY-30B. The hydrolysates were then subjected to TLC on cellulose plates using the solvent system of Rhuland \textit{et al.} (1955). Cell-wall composition analyses of strains LY-73\textsuperscript{T} and LY-30B showed lysine and aspartic acid as diagnostic amino acids, which indicates that the peptidoglycan structure is of the L-Lys–D-Asp type in the presence of Lys, Glu, Ala and Asp. Diaminopimelic acids were not detected in the peptidoglycans.

The fatty acid profiles of strains LY-73\textsuperscript{T} and LY-30B and of their related \textit{Lactobacillus} species were analysed under the same conditions. The strains were cultured at 37 °C for 2 days on MRS agar and fatty acid methyl esters were obtained from cells by saponification, methylation and extraction as described by Kämpfer & Kroppenstedt (1996) and were separated using GC (Agilent 6890N). The peaks were automatically integrated and the distribution of fatty acids was determined and analysed using the Sherlock Microbial Identification System (MIDI, TSBA6). The results were expressed as the percentage of the total fatty acids (Table 2).

Various phenotype characteristics of the novel strains distinguished them from \textit{L. harbinensis} KACC 12409\textsuperscript{T} (Miyamoto \textit{et al.}, 2005), \textit{L. perolens} DSM 12744\textsuperscript{T} (Back \textit{et al.}, 1999) and \textit{L. selangorensis} DSM 13344\textsuperscript{T} (Leisner \textit{et al.}, 2000; Haakensen \textit{et al.}, 2011), their phylogenetically most closely related species (Table 1). The strains isolated from beverage are considered to represent a novel species of the genus \textit{Lactobacillus} based on the obtained genotypic and phenotypic data, for which the name \textit{Lactobacillus shenzhenensis} sp. nov. is proposed.

\textbf{Description of \textit{Lactobacillus shenzhenensis} sp. nov.}

\textit{Lactobacillus shenzhenensis} (shen.zhen.en’sis. N.L. masc. adj. shenzhenensis of Shenzhen, referring to the city where the type strain was isolated).

Cells are rod-shaped, 3.5–4.0 μm long and 0.6–0.8 μm wide, Gram-positive, non-spore-forming and non-motile, occur singly or in pairs or chains, and are catalase-negative, facultatively anaerobic and heterofermentative. Two-day-old colonies on MRS agar are greyish white, opaque, smooth, circular and with entire edges (approx. 1.0–2.0 mm in diameter). In MRS broth, grows at 45 °C but not at 4 °C. Growth occurs in MRS broth containing 6 % (w/v) NaCl and at pH 4.0–9.0. Acid is produced from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-galactose, D-fructose, D-mannose, L-rihamnose, methyl \textit{x}-D-mannopyranoside, methyl D-glucopyranoside, \textit{N}-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, D-lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose, starch, gentiobiose, D-tagatose, gluconate and 2-ketogluconate, but not from D-xylose, L-xylose, D-adonitol, methyl \textit{β}-D-xlyopyranoside, D-glucose, L-sorbose, dulcitol, inositol, D-mannitol, D-sorbitol, glycollen, xylitol, turanose, D-lyxose, D-fucose, D-arabitol, L-arabitol or 5-ketogluconate. The cell-wall does not contain \textit{diamino}peptidoglycan structure is of the L-Lys–D-Asp type in the presence of Lys, Glu, Ala and Asp. Diaminopimelic acids were not detected in the peptidoglycans.

\begin{table}[h]
\centering
\caption{Cellular fatty acid composition of strains LY-73\textsuperscript{T} and LY-30B and the type strains of closely related species of the genus \textit{Lactobacillus}}
\begin{tabular}{lcccc}
\hline
Fatty acid & 1 & 2 & 3 & 4 & 5 \\
\hline
\textbf{Branched chain} & & & & & \\
iso-C\textsubscript{13:0} 3-OH & 1.81 & 0.62 & 0.66 & – & – \\
iso-C\textsubscript{14:0} & – & – & – & 1.21 & – \\
anteiso-C\textsubscript{15:0} & – & – & – & 26.98 & – \\
iso-C\textsubscript{15:0} & – & – & – & 26.79 & – \\
iso-C\textsubscript{16:0} & – & – & – & 3.71 & – \\
anteiso-C\textsubscript{17:0} & – & – & – & 8.41 & – \\
iso-C\textsubscript{17:0} & – & – & – & 10.48 & – \\
\hline
\textbf{Straight-chain saturated} & & & & & \\
C\textsubscript{14:0} & 2.49 & 5.10 & 6.40 & 1.88 & 9.63 \\
C\textsubscript{15:0} & 9.7 & 8.1 & 12.71 & 9.83 & 9.90 \\
C\textsubscript{16:0} & 7.19 & 2.58 & 4.07 & 2.71 & – \\
\hline
\textbf{Unsaturated} & & & & & \\
iso-C\textsubscript{15:1} F & – & – & 0.50 & 0.45 & – \\
C\textsubscript{18:1\(9\)} & 74.29 & 78.85 & 63.08 & 6.92 & 70.09 \\
\hline
\textbf{Summed features}\textsuperscript{*} & & & & & \\
1 & 0.69 & 0.34 & 0.26 & 0.35 & – \\
3 & – & 0.54 & 2.35 & – & 2.48 \\
8 & 3.26 & 2.87 & 9.77 & 0.42 & 3.67 \\
\hline
\end{tabular}
\textsuperscript{*}Summed feature 1: iso-C\textsubscript{15:1} H/C\textsubscript{13:0} 3-OH and C\textsubscript{13:0} 3-OH/iso-C\textsubscript{15:1} H; summed feature 3: C\textsubscript{16:1\(7\)}c/C\textsubscript{16:1\(6\)}c and C\textsubscript{16:1\(9\)}c/C\textsubscript{16:1\(9\)}c; summed feature 8: C\textsubscript{18:1\(7\)}c and C\textsubscript{18:1\(6\)}c.
\end{table}

The type strain is LY-73\textsuperscript{T} (=CCTCC M 2011481\textsuperscript{T}=KACC 16878\textsuperscript{T}), which was isolated from a fermented beverage obtained from Shenzhen, China, in 2011. LY-30B, isolated from a similar source, is a second strain of the species. The DNA G+C content of the type strain is 56.1 mol%.

\textbf{Acknowledgements}

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References


