Lactobacillus insicii sp. nov., isolated from fermented raw meat

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The analysis of the bacterial microbiota of retain samples of pork salami revealed an isolate (strain TMW 1.2011T) that could neither be assigned to typical genera of starter organisms nor to any other known meat-associated species. Cells were Gram-stain-positive, short, straight rods occurring singly, in pairs or short chains. Phylogenetic analysis of the 16S rRNA gene sequence and specific phenotypic characteristics showed that strain TMW 1.2011T belonged to the phylogenetic Lactobacillus alimentarius group, and the closest neighbours were Lactobacillus nodensis JCM 14932T (97.8 % 16S rRNA gene sequence similarity), Lactobacillus tucceti DSM 20183T (97.4 %), Lactobacillus ginsenosidimutans' EMML 3041 (97.3 %), Lactobacillus versmoldensis DSM 14857T (96.9 %) and Lactobacillus furfuricola JCM 18764T (97.2 %). Similarities using partial gene sequences of the alternative chronometers pheS, dnaK and rpoA also support these relationships. DNA–DNA relatedness between the novel isolate and L. nodensis JCM 14932T, L. versmoldensis DSM 14857T and L. tucceti DSM 20183T, L. furfuricola JCM 18764T and 'L. ginsenosidimutans' EMML 3041 were below 70 % and the DNA G + C content was 36.3 mol%. The cell-wall peptidoglycan type is L-Lys-Gly-D-Asp. Based on phylogenetic, chemotaxonomic and physiological evidence, strain TMW 1.2011T represents a novel species of the genus Lactobacillus, for which the name Lactobacillus insicii sp. nov. is proposed. The type strain is TMW 1.2011T (=CECT 8802T=DSM 29801T).

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Abbreviation: LAB, lactic acid bacteria.

The GenBank/EMBL/DDBJ accession numbers for the partial 16S rRNA gene, dnaK, pheS and rpoA sequences of strain TMW 1.2011T are KP677494, KP677490, KP677493 and KP677495, respectively; those for the dnaK, partial pheS and rpoA sequences of ‘Lactobacillus ginsenosidimutans’ JCM 16719 are KP677485, KP677488 and KP677486, respectively. The GenBank/EMBL/DDBJ accession numbers for the dnaK sequence of Lactobacillus furfuricola JCM 18764T, Lactobacillus versmoldensis DSM 14857T, Lactobacillus tucceti DSM 20183T, Lactobacillus nodensis JCM 14932T and Lactobacillus furfuricola DSM 20184T are KP677487, KP677488, KP677489, KP677491 and KP677492, respectively.

Two supplementary figures are available with the online Supplementary Material.

Fermented meat products are produced in a wide variety and are consumed globally (Aymerich et al., 2003). Regardless of differences in regional recipes and technologies, the microbiota of these products mainly consists of lactic acid bacteria (LAB) and coagulase-negative cocci (Rantsiou & Cocolin, 2006). Only a few highly specialized species of the genera Lactobacillus and Pediococcus are the dominating groups of LAB that are favoured by the substrate, anaerobic environment and the presence of NaCl, nitrate and nitrite. Lactobacillus sakei, Lactobacillus curvatus, Lactobacillus plantarum, Pediococcus pentosaceus and Pediococcus acidilactici are the species that have been most frequently found (Aquilanti et al., 2007; Leroy et al., 2006; Cocolin et al., 2011). These species are considered as technologically important during the fermentation period and during ripening of sausages as they reduce the pH by formation of organic acids (lactic acid and acetic acid) (Hammes et al., 1990; Hammes & Knauf, 1994). Additionally, they are jointly responsible for flavour formation by their proteolytic activity on meat proteins (Sanz et al., 1999; Pereira et al., 2001; Fadda et al., 2010) and production of aromatic substances (Urso et al., 2006; Demeyer et al., 1986; Bacus, 1986). Therefore, various strains of these species are successfully used as commercial starter cultures.

Aside from these normally dominating species, additional lactobacilli occur sporadically in low, or even in high, cell...
numbers. They have been isolated as members of the autochthonous microbiota regardless of whether the fermented meat products were spontaneously acidified or inoculated with a starter. In 1970, Reuter described *Lactobacillus farcininis* and *Lactobacillus alimentarius* isolated from meat and meat products (Reuter, 1970). Also from the same study stems strain *Lactobacillus* sp. CECT 5920 (= DSM 20183), originally isolated as strain R19c and later described, and subsequently validly published, as *Lactobacillus tucetti* (Chenoll et al., 2006). Closely related to *L. tucetti* is *Lactobacillus versmoldensis* that occurs sporadically in high numbers dominating the LAB populations of fermented raw meat products (Kröckel et al., 2003).

Although, LAB are usually present in high hygienic quality raw meat at low numbers (10^2–10^3 c.f.u. g⁻¹), this indicates that there exists an autochthonous lactobacilli microbiota in the raw material composed of a couple of species that successfully compete in raw fermented sausages and grow to high numbers under certain meat fermentation and storage conditions.

During routine analyses of retain samples of an industrial-type pure pork salami (grain size 2–4 mm, pH 4.9 ± 0.1 at the time of analysis), an unusually diverse microbiota was suggested by the colony morphologies on Rogosa agar. Colonies showing different morphologies were selected, cultured on MRS agar according to De Man et al. (1960) at 30 °C and maintained as glycerol stocks at −80 °C. Most isolates were identified by partial 16S rRNA gene sequencing (100 % similarity to the type strain sequences) as *L. plantarum*, *Lactobacillus furfuricola*, *Lactobacillus brevis* and *Lactobacillus senmazukei* (data not shown). The 16S rRNA gene sequence of one isolate (strain TMW 1.2011T) present in cell numbers of 10⁶ c.f.u. g⁻¹, indicates that there exists an autochthonous lactobacilli microbiota in the raw material composed of a couple of species that successfully compete in raw fermented sausages and grow to high numbers under certain meat fermentation and storage conditions.

Chromosomal DNA from bacterial strains was prepared using the E.N.Z.A. bacterial DNA kit from Omega-Biotek (VWR) according to the manufacturer’s instructions. The DNA was used as a template for amplification of the 16S rRNA gene of strain TMW1.2011T, and parts of the genes encoding the phenylalanyl-tRNA synthase alpha subunit (*pheS*), the RNA polymerase alpha subunit (*rpoA*) and the molecular chaperone DnaK. The complete 16S rRNA gene sequence (1565 nt) was amplified with primers *616V* (5’-AGAGTTTGATCTTGGCTCAG-3’) and *630R* (5’-CAKAAAGGATGATCC-3’), and primer sets *pheS-21-F* (5’-CCWARVCRAAGCAAARCC-3’) and *rpoA-21-F* (5’-ATGATGAR1TTTGA1AAAACC-3’)/*rpoA-23-R* (5’-ACH-GTRTRWATDCDCGCRG-3’) (Naser et al., 2005, 2007).

As a third phylogenetic chronometer providing more phylogenetic resolution than the 16S rRNA gene, we included *dnaK* sequence analysis. Partial sequences were obtained from PCR products generated with the previously published primers *dnaK500F3* (5’-CGGTCTTTRCTRATCR-TCRAA-3’) and *dnaK1710R5* (5’-GAAAYAAGTGG-HGAAGT-3’) (Huang et al., 2010; Huang & Lee, 2011). PCR products were purified using the QIAquick PCR purification kit (Qiagen) and sequenced by the chain-termination method using the ABI Prism Dye Terminator Cycle Sequencing method. Approximately 1565 nt of the 16S rRNA gene, 430 nt of the *pheS*, 800 nt of the *rpoA* and 1130 nt of the *dnaK* sequences were determined.

A 16S rRNA gene phylogenetic tree-based similarity matrix was constructed by the neighbour-joining method (Saitou & Nei, 1987) using the Bioinformatics software package (Applied Maths). Unknown bases were discarded for the analyses. Bootstraping analysis was undertaken to test the statistical reliability of the topology of the tree using 1000 bootstrap resamplings of the data (Fig. 1).

The 16S gene sequence tree showed that strain TMW 1.2011T belonged to the phylogenetic *L. alimentarius* group, and the closest neighbours were *L. nodensis* JCM 14932T (97.8 % 16S rRNA gene sequence similarity), *L. tucetti* DSM 20183T (97.4 %), ‘*L. ginsenosidimutans*’ EMML 3041 (97.3 %), *L. versmoldensis* DSM 14857T (96.9 %) and *L. furfuricola* JCM 18764T (97.2 %). As a 16S rRNA gene sequence similarity lower than 98.7 % has been approved as sufficient evidence that organisms belong to different species (Stackebrandt & Ebers, 2006), we supposed that strain TMW 1.2011T was a novel species. Additional neighbour-joining trees reconstructed with partial sequences of *pheS*, *rpoA* and *dnaK* genes (Fig. 2) also supported the relationships as obtained with the 16S rRNA gene sequences.

Phylogenetic trees based on 16S rRNA gene sequences were also reconstructed using maximum-likelihood analysis and the maximum-parsimony method (Figs S1 and S2, available in the online Supplementary Material). Whereas the branching position of strain TMW 1.2011T is stable with the maximum-parsimony algorithm, the topology with the maximum-likelihood method is slightly different for the 16S rRNA gene. However, the independent phylogenetic position of strain TMW 1.2011T remains.

The DNA G+C content of strain TMW 1.2011T was determined by HPLC analyses by the Leibniz Institut Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany) following the protocol previously described by Tamaoka & Komagata (1984). The G+C value (mol%) was determined according to Mesbah et al. (1989); the measurements were performed with six replications. The DNA G+C content of strain TMW 1.2011T was 36.3 ± 0.06 mol%.
According to Mattarelli et al. (2014), for species delineation within the genus Lactobacillus, DNA–DNA hybridization might be needed if the 16S rRNA gene sequence similarity to the closest known species is higher than 97 %. DNA–DNA relatedness values were determined by using chromosomal DNA of strain TMW 1.2011T and its closest 16S rRNA gene sequence neighbours. Cells were disrupted by using a Constant Systems TS 0.75 kW device (IUL Instruments). DNA in the crude lysate was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). Renaturation kinetics were performed by the DSMZ after the protocol of De Ley et al. (1970) with modifications described by Huss et al. (1983) using a Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multichannel changer and a temperature controller with in situ temperature probe (Varian). All experiments were performed in duplicate.

Genomic DNA–DNA relatedness values of strain TMW 1.2011T to the strains of L. nodensis, L. versmoldensis and L. tucceti and L. furfuricola and 'L. ginsenosidimutans' were 68.8 %, 55.0 %, 42.6 %, 24.1 % and 15.5 %, respectively. These are below the 70 % boundary value normally recommended for allocating isolates to the same species (Rossello-Mora & Amann, 2001; Stackebrandt & Goebel, 1994; Wayne et al., 1987).

The cell-wall peptidoglycan structure of strain TMW 1.2011T was determined as described by Schumann (2011). The cross-linkage type is L-Lys–Gly–D-Asp (A11.42) according to the nomenclature described by Schumann (2011).

Cell morphology, absence of spores and Gram staining of 1–2-day-old cultures of strain TMW 1.2011T grown in MRS broth at 30 °C were examined by phase-contrast and normal light microscopy, respectively, at ×1200 magnification.

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**Fig. 1.** 16S rRNA gene phylogenetic tree demonstrating the relationship of Lactobacillus insicii sp. nov. to the closest related lactobacilli. The tree was reconstructed using the neighbour-joining method. Bootstrap values >50 % are shown on each node and were calculated from 1000 replications. Lactococcus lactis ATCC 19435T was used as an outgroup. Bar, 1 % sequence divergence.
Fig. 2. Phylogenetic trees based on rpoA (a), pheS (b) and dnaK (c) partial sequences demonstrating the relationship of *Lactobacillus insicii* sp. nov. to the closest related lactobacilli. The trees were reconstructed using the neighbour-joining method. Bootstrap values >50 % are shown on each node and were calculated from 1000 replications. *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081T was used as an outgroup. Bars, 10 % sequence divergence.
magnification. Cells were Gram-stain-positive, short, straight rods occurring singly, in pairs or short chains. Beige colonies of 1–2 mm appeared on MRS plates within 48 h. Colonies were circular, convex and smooth-edged. By growing the strain on MRS agar, no motility was detected.

Growth in Durham tubes using MRS broth with 20 g l⁻¹ glucose showed no gas production. Gluconate was not fermented. Growth in MRS broth occurred at 8–8°C (within 10 days) and 45°C (within 24 h), but not at 4°C; optimal growth was at 30°C. Salt tolerance examined in MRS broth containing 0–20 % (w/v) NaCl revealed good growth up to 10 %, weak growth at 11 % and no growth at 12 % NaCl. Growth occurred between pH 3.9 and pH 8.0. Catalase activity was tested by the addition of 5 % (v/v) H₂O₂ to cells. The lack of bubble formation indicated that strain TMW 1.2011¹ was catalase-negative. Arginine and aesculin were hydrolysed.

The enantiomers of the lactic acid produced were determined enzymically (R-Biopharm). D- and L-lactic acid were produced in a ratio of 10 : 90.

Carbohydrate fermentation of additional sugars was determined using API 50 CHL strips (bioMérieux) in duplicate as recommended by the manufacturer. Strain TMW 1.2011¹ produced acid from D-glucose, D-fructose, D-mannose, maltose, turanose and N-acetylglucosamine.

Enzyme activity patterns of the novel strain and all reference strains mentioned in Table 1 were determined using API ZYM test strips (bioMérieux). All strains including the novel strain were positive for β-glucosidase, and negative for esterase (C₄), lipase (C₁₄), cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase.

Characteristics that differentiate strain TMW 1.2011¹ from its closest relatives are summarized in Table 1. Strain TMW

<table>
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<td>Hydrolysis of aesculin</td>
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<td>Enzyme activity:</td>
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<td>β-Galactosidase</td>
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<td>+</td>
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<tr>
<td>DNA G+C content (mol%)*</td>
<td>36.3</td>
<td>40.6</td>
<td>ND</td>
<td>40.5</td>
<td>40–40.8</td>
<td>38.3</td>
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</table>

*Data from: Irishawa et al. (2014); Chenoll et al. (2006); Kröckel et al. (2003); Kashiwagi et al. (2009); Jung et al. (2013).
1.2011T differs from its closest phylogenetic neighbours by the ability to grow at 45 °C and the production of acid from turanose. Moreover, the novel strain possesses the lowest DNA G+C content (36.3 mol%). The peptidoglycan type (L-Lys-Gly-D-Asp) is, with the exception of L. tuceti, unique within the L. alimentarius/L. farciminis subgroup.

Based on phylogenetic, chemotaxonomic and biochemical considerations, strain TMW 1.2011T represents a novel species of the genus Lactobacillus for which the name Lactobacillus insicii sp. nov. is proposed.

Description of Lactobacillus insicii sp. nov.

Lactobacillus insicii (in.s‘i.c.i.i. L. gen. neut. n. insicii from minced meat or sausage).

Cells are rod-shaped, 3–6 × 1 μm in size, Gram-stain-positive, non-spore-forming and non-motile. Grows aerobically and anaerobically on RÖGOSA and MRS agar, with better growth under anaerobic conditions. Grows better in MRS broth than on MRS agar. Colonies on MRS agar after incubation for 3 days at 30 °C are small (up to 1 mm in diameter), circular, convex with entire edges, beige and catalase-negative. Grows in MRS broth at 8–45 °C, with optimum growth at 30 °C. Both D- and L-lactic acid are produced (ratio D : L of 10 : 90). Cells are catalase-negative and homofermentative. Gas is not produced from glucose or from gluconate. Arginine is hydrolysed, but ascorbin gives only a weak reaction. Acid is produced from N-acetylglucosamine, D-glucose, D-fructose, maltose, turanose and D-mannose. Acid is weakly produced from D-ribose, arbutin, D-arabitol, salicin and 5-ketogluconate. Acid is not produced from glycerol, erythritol, D-arabino-bose, L-arabinose, D-xyllose, L-xyllose, D-xylo-nybose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, amygdalin, cellobiose, D-lactose, melibiose, sucrose, trehalose, inulin, D-melezitose, D-rhamnose, starch, glycyrrhizin, xylitol, gentiobiose, D-lyxose, D-tagatose, D-fucose, L-fucose, L-arabitol, gluconate or 2-ketogluconate. The peptidoglycan type is L-Lys-Gly-D-Asp.

The type strain is TMW 1.2011T (=CECT 8802T=DSM 29801T), isolated from fermented sausage. The DNA G+C content of the type strain is 36.3 mol%.

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References


