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Larkinella bovis sp. nov., isolated from fermented bovine products, and emended descriptions of the genus *Larkinella* and of *Larkinella insperata* Vancanneyt *et al.* 2006

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A novel bacterial strain, designated M2T2B15^T, was isolated from fermented bovine products and was characterized by using a polyphasic approach. Colonies were reddish pink and circular with entire margins. Cells were strictly aerobic, Gram-reaction-negative, oxidase- and catalase-positive rods that lacked flagella and were motile by gliding. Flexirubin-type pigments were absent. 16S rRNA gene sequence analysis indicated that strain M2T2B15^T was related most closely to *Larkinella insperata* LMG 22510^T (94.4 % similarity) but shared <87 % similarity with other members of the phylum *Bacteroidetes*. The major cellular fatty acids were $C_{16:1}\omega5c$, iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. The polar lipids were phosphatidylethanolamine, phosphatidylserine, two unidentified aminophospholipids and two unidentified polar lipids. Menaquinone 7 (MK-7) was the major respiratory quinone. The G+C content of the DNA of strain M2T2B15^T was 52 mol%. The phenotypic, genotypic and phylogenetic data presented clearly indicate that strain M2T2B15^T represents a novel species of the genus *Larkinella*, for which the name *Larkinella bovis* sp. nov. is proposed. The type strain is M2T2B15^T (=KACC 14040^T =NBRC 106324^T). Emended descriptions of the genus *Larkinella* and of *Larkinella insperata* Vancanneyt *et al.* 2006 are also proposed.

Panchakavya is a natural fertilizer prepared by organic farmers in Tamil Nadu, India, and is used widely for various agricultural and horticultural crops. Panchakavya is a combination of five products [dung, urine, milk, yogurt and ghee (clarified butter)] obtained from cows. Application of Panchakavya has significantly increased the yield of maize, sunflower and greengram and has increased crop resistance to pests and diseases (Belina *et al.*, 2005; Somasundaram *et al.*, 2007). To the best of our knowledge, no previous studies have addressed the microbial diversity in Panchakavya. Accordingly, in the present study, we report the taxonomic characterization of strain M2T2B15^T isolated from modified Panchakavya. The results provide evidence that strain M2T2B15^T represents a novel species of the genus *Larkinella*. On the basis of the new chemotaxonomic data obtained from this study, emended descriptions of the genus *Larkinella* and of *Larkinella insperata* are also proposed.

The genus *Larkinella* was first proposed to accommodate a bacterial strain isolated from water of a steam generator in a pharmaceutical company, with *Larkinella insperata* as the type species (Vancanneyt *et al.*, 2006). According to Ludwig *et al.* (2008), the genus *Larkinella* is placed in the family *Cytophagaceae*, phylum *Bacteroidetes*.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain M2T2B15^T is GQ246692.

A supplementary figure is available with the online version of this paper.

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Modified Panchakavya was prepared by using the following ingredients: cow dung (1.5 g l^{-1}), cow urine (4.5 ml l^{-1}), cow's milk (3.0 ml l^{-1}), yogurt (3.0 ml l^{-1}), molasses (4.5 ml l^{-1}) and potato (3.0 g l^{-1}). The mixture was incubated on a rotary shaker (120 r.p.m.) for 15 days at 30 °C. The cultivable bacteria associated with modified Panchakavya were isolated by using serial dilution plating on trypticase soy agar (TSA), Luria–Bertani agar and R2A agar (all from Difco). Plates were incubated for 4 days at 30 °C. A total of 37 bacterial strains were isolated (data not shown), of which strain M2T2B15^T appeared on R2A agar and could be distinguished from other bacteria based on its reddish pink colony colour.

Genomic DNA was isolated according to the method described by Ausubel *et al.* (1987), except that the lysates were extracted twice with chloroform to remove residual phenol. The 16S rRNA gene was amplified by using the universal primers fD1 and rP2 (Weisburg *et al.*, 1991), and was sequenced as described by Kwon *et al.* (2003). The alignment of 16S rRNA gene sequences was performed with the CLUSTAL W program (Thompson *et al.*, 1994) following identification of phylogenetic neighbours via the EzTaxon server (http://www.eztaxon.org/; Chun *et al.*, 2007). Phylogenetic trees were reconstructed with the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Fitch, 1971) and maximum-likelihood (Felsenstein, 1981) methods by using the program MEGA3 (Kumar *et al.*, 2004), with bootstrap values based on 1000 replications (Felsenstein, 1985). An almost-complete 16S rRNA gene sequence of strain M2T2B15^T was obtained (1429 bp). Preliminary comparisons with 16S rRNA gene sequences deposited in the GenBank database indicated that strain M2T2B15^T belongs to the family *Cytophagaceae*, phylum *Bacteroidetes*. 16S rRNA gene sequence analysis revealed that strain M2T2B15^T was related most closely to *L. insperata* LMG 22510^T (94.4 % similarity) and exhibited less than 87 % sequence similarity to other members of the phylum *Bacteroidetes*. The overall topologies of the phylogenetic trees were similar irrespective of the phylogenetic methods used (data not shown). Strain M2T2B15^T formed a separate clade from *L. insperata* LMG 22510^T, with 100 % bootstrap support (Fig. 1).

Basic phenotypic tests were examined simultaneously for strain M2T2B15^T and *L. insperata* LMG 22510^T (=KACC 11764^T) by using the same culture conditions. Cell morphology was examined by using phase-contrast (AXIO; Zeiss) and transmission electron (LEO model 912AB) microscopy with cells grown for 3 days on R2A agar at 30 °C. For transmission electron microscopy, cells were negatively stained with 0.5 % (w/v) uranyl acetate. Gram staining, catalase and oxidase activities, and hydrolysis of CM-cellulose, casein, chitin from crab shells, hypoxanthine, pectin, starch, tyrosine, Tween 20 and xanthine were assessed with the methods described by Smibert & Krieg (1994). Degradation of DNA was investigated on DNase test agar (Difco) supplemented with 0.01 % toluidine blue (Merck). The presence of flexirubin-type pigments and gliding motility were assessed by using the methods recommended by Bernardet *et al.* (2002). The pH range for growth was determined in R2A broth at 30 °C. The pH of the medium was adjusted with citrate phosphate buffer or Tris/HCl buffer (Breznak & Costilow, 1994) to pH 4.0–10.0 at increments of 1.0 pH unit. Growth at 5, 10, 15, 20, 30, 35, 40 and 45 °C was measured in R2A broth. Salt tolerance was tested in R2A broth supplemented with 0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 % (w/v) NaCl after 7 days of incubation at 30 °C. Anaerobic growth on R2A agar was assessed at 30 °C for 10 days by using a BBL anaerobic jar (Becton Dickinson). The strain was also characterized by using API 20 NE, API ID 32 GN and API ZYM test strips (bioMérieux), according to the manufacturer's recommendations. Susceptibility to the antibiotics rifampicin, ampicillin, oleandomycin, chloramphenicol, tetracycline, novobiocin, streptomycin, spectinomycin, kanamycin, trimethoprim, hygromycin, polymyxin B and nystatin (each at 50 μg ml^{-1}) was tested on R2A agar plates. Three-day-old colonies of strain M2T2B15^T grown on R2A agar were reddish pink and circular with entire margins. Cells were strictly aerobic, Gram-reaction-negative, oxidase- and catalase-positive rods that lacked flagella but were motile by gliding. Strain M2T2B15^T could be differentiated from *L. insperata* LMG 22510^T on the basis of colony colour, cell shape, hydrolysis of CM-cellulose and tyrosine, and assimilation of D-glucose, L-arabinose, D-mannitol, *N*-acetylglucosamine, sucrose and salicin. Other differentiating characteristics between strain M2T2B15^T and *L. insperata* LMG 22510^T are listed in Table 1.

Cellular fatty acid methyl esters were prepared from cells of strain M2T2B15^T and *L. insperata* LMG 22510^T grown on R2A agar for 3 days at 30 °C, and were analysed by GC according to the instructions of the Microbial Identification System (MIDI). The isoprenoid quinones of strain M2T2B15^T were analysed by HPLC as described by Groth *et al.* (1996). The polar lipid profiles of strain

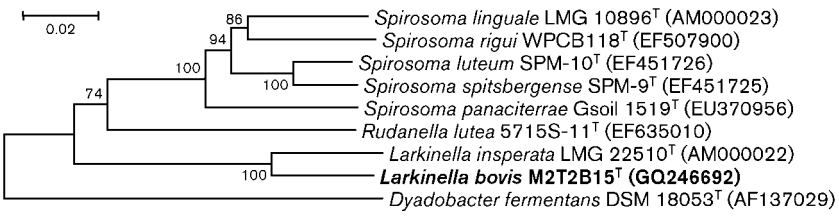

Fig. 1. Neighbour-joining tree based on almost-complete 16S rRNA gene sequences showing the phylogenetic position of strain M2T2B15^T. Numbers at nodes indicate percentages of 1000 bootstrap resamplings; only values >70 % are given. Bar, 0.02 substitutions per nucleotide position.

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Table 1. Differential characteristics between strain M2T2B15^T and *Larkinella insperata* LMG 22510^T

Strains: 1, M2T2B15^T; 2, *L. insperata* LMG 22510^T. The two strains hydrolyse aesculin, starch and Tween 20 but not casein, chitin, DNA, hypoxanthine, pectin, gelatin or xanthine. They are negative for the Gram reaction, nitrate reduction, indole production and glucose fermentation. The two strains assimilate maltose, glycogen and melibiose, but not D-mannose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, phenylacetic acid, L-rhamnose, D-ribose, inositol, itaconic acid, suberic acid, sodium malonate, sodium acetate, lactic acid, L-alanine, potassium 5-ketogluconate, 3-hydroxybenzoic acid, L-serine, L-fucose, D-sorbitol, propionic acid, valeric acid, L-histidine, potassium 2-ketogluconate, 3-hydroxybutyric acid, 4-hydroxybenzoic acid or L-proline. They are positive for gliding motility, and catalase, oxidase, β-galactosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase activities. They are negative for esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, α-fucosidase, arginine dihydrolase and urease activities. The two strains are susceptible to rifampicin, ampicillin and novobiocin, but resistant to streptomycin, spectinomycin, kanamycin, trimethoprim, hygromycin and nystatin. All the data were obtained from this study. +, Positive; −, negative; W, weakly positive; S, sensitive; R, resistant.

Characteristic	1	2
Colony colour on R2A agar	Reddish pink	Pale pink
Cell morphology on R2A agar, cultured for 3 days at 30 °C	Rods	Ring-like and horseshoe
Temperature range for growth (°C)	10–35	10–40
NaCl range for growth (%)	0–1	0–3
Hydrolysis of:		
CM-cellulose	+	−
Tyrosine	+	−
Assimilation of:		
D-Glucose	+	−
L-Arabinose	+	−
D-Mannitol	+	−
N-Acetylglucosamine	+	−
Sucrose	+	−
Salicin	+	−
Enzyme activity (API ZYM)		
Alkaline phosphatase	+	−
Esterase (C4)	−	+
Trypsin	W	−
Acid phosphatase	W	−
α-Galactosidase	−	W
Response to antibiotics (50 μg ml^{-1})		
Oleandomycin	S	R
Chloramphenicol	S	R
Tetracycline	S	R
Polymyxin B	R	S

M2T2B15^T and *L. insperata* LMG 22510^T were determined according to the method of Minnikin *et al.* (1984). The DNA G+C content of strain M2T2B15^T was determined by HPLC of deoxyribonucleosides as described by Mesbah *et al.* (1989), by using a reversed-phase column (Supelcosil LC-18-S; Supelco). The fatty acid profiles of strain M2T2B15^T and *L. insperata* LMG 22510^T were almost identical. However, strain M2T2B15^T could be differentiated from *L. insperata* LMG 22510^T based on lower amounts of iso-$C_{15:0}$ (23.9 %) and iso-$C_{17:0}$ 3-OH (9.9 %), a higher amount of $C_{16:1}\omega5c$ (45.5 %) and the absence of summed feature 4 (Table 2). The major respiratory quinone of strain M2T2B15^T was menaquinone 7 (MK-7), in agreement with data for *L. insperata* LMG 22510^T (Vancanneyt *et al.*, 2006). Strain M2T2B15^T and *L. insperata* LMG 22510^T displayed similar polar lipid profiles. Their major polar lipids were phosphatidylethanolamine, phosphatidylserine, two unidentified aminophospholipids and two unidentified polar lipids (see Supplementary Fig. S1 in IJSEM Online). The DNA G+C content of strain M2T2B15^T was 52 mol%, a value close to that reported for *L. insperata* LMG 22510^T (53 mol%; Vancanneyt *et al.*, 2006). On the basis of the phenotypic and phylogenetic data presented, we propose that strain M2T2B15^T represents a novel species of the genus *Larkinella*, for which the name *Larkinella bovis* sp. nov. is proposed. Also, emended descriptions of the genus *Larkinella* and of *Larkinella insperata* Vancanneyt *et al.* 2006 are provided.

Table 2. Cellular fatty acid compositions (%) of strain M2T2B15^T and *Larkinella insperata* LMG 22510^T

Strains: 1, M2T2B15^T; 2, *L. insperata* LMG 22510^T. Fatty acids amounting to <1 % of the total fatty acids in both strains are not shown. tr, Trace (<1 %); −, not detected. Prior to fatty acid extraction the two strains were cultivated on R2A agar medium for 3 days at 30 °C.

Fatty acid	1	2
iso-$C_{15:0}$	23.9	32.2
iso-$C_{15:0}$ 3-OH	1.2	1.2
anteiso-$C_{15:0}$	2.5	1.6
$C_{16:0}$	3.2	4.1
$C_{16:0}$ 3-OH	1.6	tr
$C_{16:1}\omega5c$	45.5	29.6
iso-$C_{17:0}$	1.6	3.3
iso-$C_{17:0}$ 3-OH	9.9	15.3
Summed feature 3*	5.1	2.3
Summed feature 4*	−	1.7
Unknown ECL 13.565†	4.6	5.6
Unknown ECL 16.852†	1.0	1.8

*Summed features are groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 comprised $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH. Summed feature 4 comprised iso-$C_{17:1}$ and/or anteiso-$C_{17:1}$ B.

†Unknown fatty acids are designated by their equivalent chain-length (ECL).

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Emended description of the genus *Larkinella* Vancanneyt *et al.* 2006

The description remains as given by Vancanneyt *et al.* (2006) with the following additions. The predominant polar lipids are phosphatidylethanolamine, phosphatidylserine, two unidentified aminophospholipids and two unidentified polar lipids. The DNA G+C content is 52–53 mol%. The genus is a member of the family *Cytophagaceae*, phylum *Bacteroidetes*.

Emended description of *Larkinella insperata* Vancanneyt *et al.* 2006

The description remains as given by Vancanneyt *et al.* (2006) with the following additions or modifications. Growth occurs on TSA, R2A agar and nutrient agar but not on MacConkey agar. Hydrolyses aesculin and starch, but not hypoxanthine, pectin, gelatin or xanthine. Negative for glucose fermentation. Assimilates maltose, glycogen and melibiose but not D-mannose, potassium gluconate, capric acid, adipic acid, malic acid, phenylacetic acid, L-rhamnose, D-ribose, inositol, itaconic acid, suberic acid, sodium malonate, sodium acetate, lactic acid, L-alanine, potassium 5-ketogluconate, 3-hydroxybenzoic acid, L-serine, L-fucose, propionic acid, valeric acid, L-histidine, potassium 2-ketogluconate, 3-hydroxybutyric acid, 4-hydroxybenzoic acid, L-proline, D-glucose, L-arabinose, D-mannitol, *N*-acetylglucosamine, sucrose or salicin. Positive for esterase (C4), leucine arylamidase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase activities. Negative for alkaline phosphatase, esterase lipase (C8), trypsin, acid phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, α-fucosidase, arginine dihydrolase, α-galactosidase, β-galactosidase and urease activities. Susceptible to rifampicin, ampicillin, polymyxin B and novobiocin, but resistant to streptomycin, oleandomycin, chloramphenicol, tetracycline, spectinomycin, kanamycin, trimethoprim, hygromycin and nystatin.

Description of *Larkinella bovis* sp. nov.

Larkinella bovis (bo′vis. L. gen. n. *bovis* of a cow, of bovine).

Cells are strictly aerobic, Gram-reaction-negative, oxidase- and catalase-positive rods (1.2–3.2 μm long and 0.9–1.2 μm wide), that lack flagella and are motile by gliding. Colonies on R2A agar are reddish pink, and convex with entire margins. Flexirubin-type pigments are not produced. Optimum growth occurs at 30 °C and pH 7.0. The temperature, pH and NaCl concentration ranges for growth are 10–35 °C, pH 6.0–7.0 and 0–1 %. Growth occurs on TSA, R2A agar and nutrient agar but not on MacConkey agar. Hydrolyses CM-cellulose, tyrosine, aesculin, starch and Tween 20, but not casein, chitin, DNA, hypoxanthine, pectin, gelatin or xanthine. Negative for nitrate reduction, indole production and glucose fermentation. Assimilates D-glucose, L-arabinose, D-mannitol, *N*-acetylglucosamine, sucrose, salicin, maltose, glycogen and melibiose, but not D-mannose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, phenylacetic acid, L-rhamnose, D-ribose, inositol, itaconic acid, suberic acid, sodium malonate, sodium acetate, lactic acid, L-alanine, potassium 5-ketogluconate, 3-hydroxybenzoic acid, L-serine, L-fucose, D-sorbitol, propionic acid, valeric acid, L-histidine, potassium 2-ketogluconate, 3-hydroxybutyric acid, 4-hydroxybenzoic acid or L-proline. Positive for alkaline phosphatase, β-galactosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and *N*-acetyl-β-glucosaminidase activities. Weakly positive for trypsin and acid phosphatase. Negative for esterase (C4), esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, α-mannosidase, α-fucosidase, arginine dihydrolase and urease activities. Susceptible to rifampicin, ampicillin, oleandomycin, chloramphenicol, tetracycline and novobiocin, but resistant to streptomycin, spectinomycin, kanamycin, trimethoprim, hygromycin, polymyxin B and nystatin. The major cellular fatty acids are $C_{16:1}\omega5c$, iso-$C_{15:0}$ and iso-$C_{17:0}$ 3-OH. The detailed fatty acid composition of the type strain is given in Table 2. The DNA G+C content of the type strain is 52 mol%.

The type strain, M2T2B15^T (=KACC 14040^T =NBRC 106324^T), was isolated from fermented bovine products, Suwon, Republic of Korea.

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