

Listeria fleischmannii sp. nov., isolated from cheeseDavid Bertsch,¹ Jörg Rau,² Marcel R. Eugster,³ Martina C. Haug,¹
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A study was performed on three isolates (LU2006-1^T, LU2006-2 and LU2006-3), which were sampled independently from cheese in western Switzerland in 2006, as well as a fourth isolate (A11-3426), which was detected in 2011, using a polyphasic approach. The isolates could all be assigned to the genus *Listeria* but not to any known species. Phenotypic and chemotaxonomic data were compatible with the genus *Listeria* and phylogenetic analysis based on 16S rRNA gene sequences confirmed that the closest relationships were with members of this genus. However, DNA–DNA hybridization demonstrated that the isolates did not belong to any currently described species. Cell-wall-binding domains of *Listeria monocytogenes* bacteriophage endolysins were able to attach to the isolates, confirming their tight relatedness to the genus *Listeria*. Although PCR targeting the central portion of the flagellin gene *flaA* was positive, motility was not observed. The four isolates could not be discriminated by Fourier transform infrared spectroscopy or pulsed-field gel electrophoresis. This suggests that they represent a single species, which seems to be adapted to the environment in a cheese-ripening cellar as it was re-isolated from the same type of Swiss cheese after more than 5 years. Conjugation experiments demonstrated that the isolates harbour a transferable resistance to clindamycin. The isolates did not exhibit haemolysis or show any indication of human pathogenicity or virulence. The four isolates are affiliated with the genus *Listeria* but can be differentiated from all described members of the genus *Listeria* and therefore they merit being classified as representatives of a novel species, for which we propose the name *Listeria fleischmannii* sp. nov.; the type strain is LU2006-1^T (=DSM 24998^T =LMG 26584^T).

The aim of this study was the phenotypic, chemotaxonomic and genotypic description, as well as the phylogenetic characterization, of four isolates from cheese belonging to a hitherto-undefined species of the genus *Listeria*. The absence of virulence factors and human pathogenic properties and, especially, the discrimination from *Listeria monocytogenes* are of general interest for food control measures and consumer protection.

Strains LU2006-1^T, LU2006-2 and LU2006-3 were sampled individually in 2006 during screening of cheeses and ripening cellars for the genus *Listeria* in Switzerland. Strain

A11-3426 was detected in September 2011 on the same type of Swiss hard cheese sold in Germany. The strains and PCR primers used in this study are listed in Tables S1 and S2, respectively (available in IJSEM Online). PCR analysis showed that the isolates could be assigned to the genus *Listeria* but not to any species with a validly published name, including *Listeria marthii* (Graves *et al.*, 2010) and *Listeria rocourtiae* (Leclercq *et al.*, 2010), indicating that they represent a novel species.

The isolates showed good growth both in liquid broth and on agar of brain heart infusion (BHI; Biolife Italiana) and tryptone soya broth (TSB; Oxoid) at 22–42 °C (optimum 30–37 °C). No growth was observed at 47 °C and only very weak growth was seen at 4 °C after 2 weeks. Colonies were green-blue without haloes on ALOA agar (Biolife Italiana) and chromogenic Brilliance *Listeria* agar, grey-green with black haloes on PALCAM agar, brown to black on Oxford agar (all from Oxoid) and white with yellow haloes on RAPID[®] *L.mono* agar (Bio-Rad). With this appearance on

Abbreviations: CBD, Cell-wall-binding domain; GFP, green fluorescent protein.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LU2006-1^T, LU2006-2 and LU2006-3 are JN093101–JN093103, respectively.

Four supplementary figures and three supplementary tables are available with the online version of this paper.

the selective agars, the isolates could clearly be discriminated from the human pathogen *L. monocytogenes*. On BHI and TSB agar, colonies appeared bluish grey by normal illumination and had a bluish appearance when applying the Henry oblique transmitted-light viewing technique (Lachica, 1990). Haemolysis was not visible on Columbia sheep blood agar (Oxoid) or after performing a CAMP test (Christie *et al.*, 1944). The isolates were Gram-positive (Gram-colour set; Merck) and displayed catalase activity (Haas *et al.*, 1991).

API *Listeria* (incubated at 37 °C for 24 h; Bille *et al.*, 1992) and API 50 CH (incubated at 37 °C for 48 h without

mineral oil cover) with API 50 CHL medium (all from bioMérieux) were applied for identification of the isolates and to study carbohydrate metabolism. Members of the genus *Listeria* and *Brochothrix thermosphacta* DSM 20171^T (incubated at 30 °C) were used as reference strains; Table 1). Test kits (Fluka) were used to determine sodium hippurate hydrolysis, nitrate reduction and hydrogen sulfide production, and umbrella-like growth was tested both in semi-solid BHI medium and on sulfide indole motility agar (Becton Dickinson) at 22, 30 and 37 °C (Table 1). As motility was not observed for either the isolates or *L. rocourtiae* DSM 22097^T under our test conditions, the presence of flagella was investigated by

Table 1. Characteristics differentiating *Listeria fleischmannii* sp. nov. from other members of the genus *Listeria* and *Brochothrix thermosphacta*

Strains: 1, *Listeria fleischmannii* sp. nov. LU2006-1^T; 2, *L. monocytogenes* DSM 20600^T; 3, *L. grayi* DSM 20601^T; 4, '*L. grayi* subsp. *murrayi*' DSM 20596; 5, *L. innocua* DSM 20649^T; 6, *L. ivanovii* subsp. *ivanovii* DSM 20750^T; 7, *L. ivanovii* subsp. *londoniensis* DSM 12491^T; 8, *L. seeligeri* DSM 20751^T; 9, *L. welshimeri* DSM 20650^T; 10, *L. marthii* DSM 23813^T; 11, *L. rocourtiae* DSM 22097^T; 12, *B. thermosphacta* DSM 20171^T. All data were taken from this study. All strains are positive for acid production from aesculin ferric citrate, amygdalin, arbutin, D-arabitol, cellobiose, D-fructose*, D-glucose*, maltose*, D-mannose, trehalose*, gentiobiose, methyl α -D-glucopyranoside, N-acetylglucosamine and salicin. All strains are negative for acid production from D-adonitol*, D-arabinose, D-fucose, raffinose, D-sorbitol, dulcitol*, erythritol, glycogen, inulin, L-arabinose*, L-arabitol, L-fucose, L-xylose, methyl β -D-xylopyranoside, potassium 2-ketogluconate, potassium 5-ketogluconate and potassium gluconate. Differing fermentation patterns in other publications may be due to different incubation times and temperatures compared to the conditions applied in this study. Of the taxa tested, only *Listeria fleischmannii* sp. nov. is positive for acid production from both D-xylose and D-mannitol. +, Positive; (+), weakly positive; –, negative.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12
Acid production from:												
Starch	–	–	+	+	+	–	–	–	–	–	–	–
D-Galactose*	–	–	+	+	–	–	–	–	–	–	–	–
Lactose*	+	+	+	+	+	–	+	+	+	+	+	–
D-Lyxose	–	–	+	+	–	–	–	–	–	–	–	–
D-Mannitol*	+	–	+	+	–	–	–	–	–	–	–	+
Melezitose*	+	+	+	–	+	–	+	+	+	–	–	–
Melibiose*	–	–	–	–	–	–	–	–	–	–	+	–
D-Ribose	+	–	+	+	–	+	–	–	–	–	+	+
D-Tagatose	–	–	–	–	+	–	–	–	+	–	–	+
Turanose	+	–	–	–	+	–	–	–	–	–	–	–
D-Xylose*	+	–	–	–	–	+	+	+	+	–	+	–
Glucose 1-phosphate	–	+	–	+	–	–	+	–	–	–	–	–
Glycerol	–	+	+	+	+	–	–	–	–	–	–	+
Inositol	–	–	–	–	–	–	–	–	–	–	–	+
L-Rhamnose*	+	+	–	+	+	–	–	–	–	–	+	–
L-Sorbose	+	–	–	+	–	–	–	–	–	–	–	–
Methyl α -D-glucopyranoside	+	+	+	+	+	–	–	+	+	+	–	–
Sucrose	(+)	–	–	–	–	–	–	–	–	–	–	+
Xylitol	+	+	+	+	+	+	+	+	+	+	–	–
Arylamidase (DIM test)	–	–	+	+	+	+	+	+	+	–	–	–
α -Mannosidase	–	+	+	+	+	–	–	–	+	+	–	–
Hydrolysis of hippurate	+	+	–	(+)	+	+	+	(+)	(+)	+	+	–
Nitrate reduction	+	–	–	+	–	–	–	–	–	–	+	–
H ₂ S production	(+)	–	–	–	–	–	–	–	–	–†	–	–
Motility (at 22 and 30 °C)	–	+	+	+	+	+	+	+	+	+	–	+

*Acid production confirmed with the carbohydrate fermentation test using rhamnose broth (Fluka).

†H₂S production was reported for *L. marthii* by Graves *et al.* (2010).

immunostaining of the flagellar H antigen with *Listeria* antisera H-AB (Denka Seiken) and visualization by fluorescence microscopy (data not shown). Furthermore, the isolates were analysed by transmission electron microscopy (JEM-1011; JEOL) after growth on sheep blood agar for 24 h at both 17 and 30 °C (Fig. S1). Both tests revealed the absence of flagella; however, from the novel isolates the central portion (approx. 450 bp) of *flaA*, encoding the *Listeria* flagellin protein, could be amplified and was shown to be identical to the corresponding sequence of *L. monocytogenes* DSM 20600^T. Non-motile members of the genus *Listeria* have been described previously (Farber *et al.*, 1991; Leifson & Palen, 1955).

Phylogenetic analysis was performed with the isolates and members of the genus *Listeria* based on PCR-amplified partial sequences of the 16S rRNA gene (*rrs*) (1305 bp), the housekeeping genes *gap* (glyceraldehyde-3-phosphate dehydrogenase) and *ldh* (lactate dehydrogenase) and the 23S rRNA gene. Sequences were aligned using CLUSTAL X (Thompson *et al.*, 1997) and edited manually to remove terminal regions that were not represented by all members (Nicholas *et al.*, 1997). Jukes–Cantor evolutionary distances were calculated (Jukes & Cantor, 1969) and neighbour-joining (Saitou & Nei, 1987) phylogenetic trees were reconstructed with MEGA4 (Tamura *et al.*, 2007). Stability of the groupings was estimated by bootstrap analysis (1000 replications) using the same programs. All

major branching nodes were confirmed by maximum parsimony (data not shown). Analysis of the *rrs* sequences showed that the four isolates were highly related to each other (100 % 16S rRNA gene sequence similarity) and formed a clade supported by a high bootstrap value (100 %). The isolates were phylogenetically related to the genus *Listeria*, even though they were well separated from all members with validly published names and they represented a distinct line of descent at the base of the *Listeria* cluster (Fig. 1). However, 16S rRNA gene sequence similarities between members of the same genus can be much lower than 97 % (Tindall *et al.*, 2010). In this case, the 16S rRNA gene sequence similarities ranged from 91.7 to 95.0 %, the highest similarity being with *L. grayi* CECT 931^T. There is no precise correlation between 16S rRNA gene sequence divergence and species delineation, but it is generally recognized that divergence values of ≥ 3 % are significant (Stackebrandt & Goebel, 1994). The tree topology and sequence divergence values (>5 %) showed that the isolates warrant classification in a novel species. Analysis of concatenated sequences of *gap* (719 bp) and *ldh* (483 bp), as well as of the 23S rRNA gene, displayed a topology similar to the *rrs* sequences, with minor perturbations in the branching order (data not shown).

Cell-wall-binding domains (CBDs) of *L. monocytogenes* bacteriophage endolysins were applied to detect *Listeria*

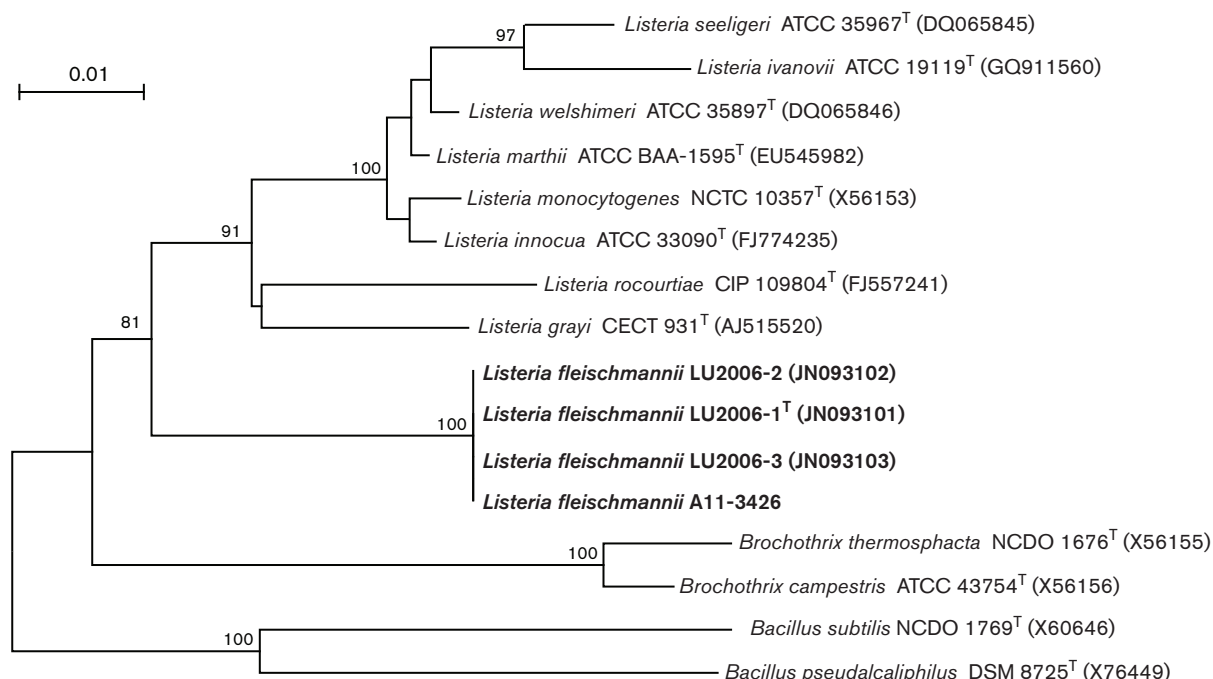


Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strains LU2006-1^T, LU2006-2, LU2006-3 and A11-3426 and other members of the genus *Listeria*. 16S rRNA gene sequence similarities between all four *L. fleischmannii* isolates were 100%. Bootstrap values (>80 %) based on 1000 replications are shown at branch nodes. Members of the genera *Bacillus* and *Brochothrix* were used as outgroups. Bar, 0.01 substitutions per site.

cell-wall carbohydrates. Binding patterns were determined by direct visual comparison to control strains with known binding intensities using fluorescence microscopy (TCS SPE; Leica). Production of His-tagged CBDs fused to green fluorescent protein (GFP) was carried out as published earlier (Eugster *et al.*, 2011). All seven tested GFP-CBD constructs were able to decorate the LU2006 isolates (Table S3), confirming their tight relatedness to the genus *Listeria*, as CBDs generally feature a high degree of specificity and only single CBDs sporadically attach to members of other genera such as *Bacillus* and *Staphylococcus* (Schmelcher *et al.*, 2010). The binding pattern of the LU2006 isolates has not been detected before and allows discrimination from all other members of the genus *Listeria*, including the recently characterized species *L. marthii* and *L. rocourtiae* (M. R. Eugster, unpublished data).

Fatty acid analysis of strain LU2006-1^T was carried out by the DSMZ (Braunschweig, Germany), using the Sherlock Microbial Identification System (MIDI) after growth for 24 h at 37 °C on TSB agar supplemented with sheep blood. Major peaks for anteiso-C_{15:0} (40.31 %) and anteiso-C_{17:0} (47.15 %) were detected, which are characteristic for the genus *Listeria* when grown at 37 °C (McLauchlin & Rees, 2009). Furthermore, iso-C_{15:0} (1.82 %), iso-C_{16:0} (2.69 %), C_{16:0} (4.35 %), iso-C_{17:0} (1.3 %) and C_{18:1ω9c} (1.03 %) were identified. iso-C_{14:0}, C_{14:0}, C_{15:0} and C_{18:0} were present in trace amounts (<1 %). This profile is in agreement with *L. marthii* (Graves *et al.*, 2010) and other members of the genus *Listeria* (Püttmann *et al.*, 1993). Freeze-drying, extraction and analysis of respiratory quinones and polar lipids by TLC (DSMZ and Dr Brian Tindall) showed that only MK-7 was present, which is the major quinone in the genus *Listeria* (McLauchlin & Rees, 2009). The polar lipids diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine, which are phospholipids typical of the genus *Listeria* (McLauchlin & Rees, 2009; Crandall & Montville, 1998), as well as unknown lipids, could be extracted (Fig. S2). In summary, all chemotaxonomic data from this study are consistent for the genus *Listeria*. The G + C content of the genomic DNA of strain LU2006-1^T, determined by HPLC at the DSMZ, was 39.0 mol%. This is within the range for the genus *Listeria* (36.0–42.5 mol%), but not the genus *Brochothrix* (34.6–38.0 mol%; McLauchlin & Rees, 2009). DNA–DNA relatedness was determined in duplicate by hybridization at 66 °C (DSMZ) (De Ley *et al.*, 1970; Huss *et al.*, 1983); DNA–DNA relatedness values between strain LU2006-1^T and *L. monocytogenes* DSM 20600^T, *L. grayi* DSM 20601^T and '*L. grayi* subsp. *murrayi*' DSM 20596 were 34.2 ± 1.3, 29.2 ± 2.5 and 39.0 ± 0.9 %, respectively. Considering the recommendation of a threshold value of 70 % DNA–DNA relatedness for the definition of bacterial species (Wayne *et al.*, 1987), strain LU2006-1^T did not belong to any of the tested species.

Fourier transform IR spectroscopy was applied as described previously (Wortberg *et al.*, 2012) to create phenotypic fingerprints of the four isolates after growth for 24 h at

30 °C, which were compared with spectra of members of the genus *Listeria* in a reference database at the CVUAS. Cluster analysis (Rau *et al.*, 2009) revealed that the IR spectra of the isolates were not distinguishable (indicating their congruence in biochemical composition), but they could be separated from all other members of the genus *Listeria* (Fig. S3). Furthermore, genotypic fingerprinting of the isolates by pulsed-field gel electrophoresis after cleaving with the restriction endonucleases *AscI* and *Apal* according to the protocol of Morot-Bizot *et al.* (2003) also demonstrated their identity at the genetic level (Fig. S4).

Assessing the pathogenic potential and virulence of a novel species is of utmost importance. By application of previously published PCR protocols, it was shown that *inlA*, encoding the *L. monocytogenes* cell-surface protein internalin A which is necessary to invade host cells that express E-cadherin (Lecuit *et al.*, 1997), and all virulence genes located on the *Listeria* pathogenicity island 1, namely *actA*, *plcA*, *plcB*, *prfA*, *hly* and *mpl*, were not present in the isolates. Absence of the six virulence genes was confirmed by sequencing the corresponding gene cluster as described before (Schmid *et al.*, 2005), using a primer-walking strategy. Alignment using Finch TV (Geospiza) and BioEdit (Hall, 1999) produced a sequence of about 10 kb (accession number JN118555). The prediction tool FGENESB (Softberry) indicated the presence of eight open reading frames between *prs* and *ldh*, all showing the highest similarity (>50 %) with protein sequences from members of the genus *Listeria* (Fig. 2). ORFB (function unknown) seems to be present in *prs*–*ldh* intergenic regions of all *Listeria* species (Cai & Wiedmann, 2001) and five successive open reading frames encode a putative phosphotransferase system.

Caco-2 invasion by the isolates was tested in duplicate from three successive passages as previously described (Gaillard *et al.*, 1987). Confluent monolayers of Caco-2 cells (DSM ACC 169) were infected with 0.5 ml inoculum (approx. 4 × 10⁷ c.f.u. ml⁻¹) for 60 min at 37 °C. Afterwards, extracellular bacteria were killed by a treatment with 150 µg gentamicin ml⁻¹ for 45 min, Caco-2 cells were dissociated with trypsin/EDTA (0.05 %/53 mM), lysed with 0.1 % Triton X-100 and appropriate dilutions were spread onto BHI agar. All LU2006 isolates as well as *L. innocua* DSM 20649^T showed very low invasiveness (<0.001 %) compared with *L. monocytogenes* 10403S (1.36 ± 0.2 %), suggesting that the novel organism could not enter human epithelial cells. Altogether, there is no indication for human pathogenicity or virulence.

Antibiotic resistance phenotypes of the four isolates were determined three times by broth microdilution susceptibility testing based on the guidelines M45–A2 for *L. monocytogenes* (Jorgensen *et al.*, 2010). After incubation at 37 °C for 48 h, the MICs (µg ml⁻¹) showed that the isolates were sensitive or intermediately sensitive to amoxicillin (0.25–0.50), ampicillin (0.25–0.50), chloramphenicol (4–8), ciprofloxacin (1–2), erythromycin (0.5–1.0), gentamicin (0.5–1.0), kanamycin (1–4), norfloxacin (4–8), ofloxacin

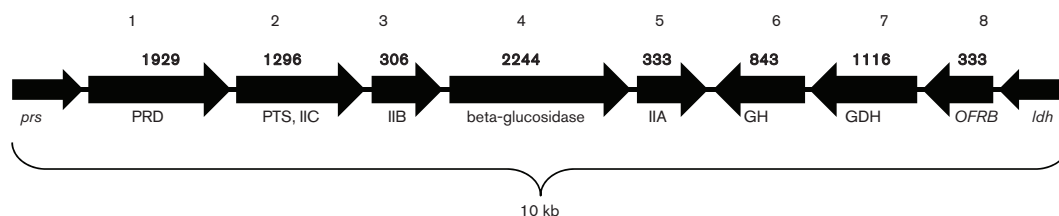


Fig. 2. Schematic genetic organization of the *prs*–*ldh* intergenic region of strain LU2006-1^T showing open reading frames 1–8 and their lengths (bp, above) and putative domains (below). GDH, Putative glutathione dehydrogenase; GH, putative S-formyl-glutathione hydrolase; IIA–IIC, putative PTC components; PRD, putative PTS regulation domain; PTS, putative phosphotransferase system.

(2), penicillin (0.25), rifampicin (0.25), streptomycin (8–16), tetracycline (1–2), trimethoprim (0.063–0.125) and vancomycin (0.5–1.0), but were resistant to nalidixic acid (>256), which is typical for the genus *Listeria*, and additionally to clindamycin (32). To understand the resistance mechanism for clindamycin, the efflux pump inhibitor reserpine (Alfa Aesar) was dissolved in DMSO and added to the setup for microdilution testing at a final concentration of 10 µg ml^{−1} as described before (Montagnani *et al.*, 2007). As the MIC was not influenced, an efflux mechanism is unlikely. Genotypic antibiotic resistance profiling was performed by hybridization of biotin-labelled DNA on a microarray targeting antibiotic resistance genes of Gram-positive bacteria (Perreten *et al.*, 2005). Except for the controls, no hybridization was detected which indicates a clindamycin-resistance mechanism unknown in Gram-positive bacteria. The resistance could be transferred by a filter-mating technique (Haug *et al.*, 2010) to *L. monocytogenes* 10403S and *Enterococcus faecalis* JH2-2 (Jacob & Hobbs, 1974). Spontaneous mutation could be excluded, as both recipients did not grow on agar for selection of transconjugants in control experiments. The observation that no plasmid could be isolated using either the protocol of Anderson & McKay (1983) or the Plasmid Maxi kit (Qiagen), adapted for Gram-positive bacteria, suggests that the resistance determinant is encoded on a transferable transposon which remains to be identified.

The isolates represent a deeply branching lineage and a somewhat-loose phylogenetic association with the genus *Listeria*, which might open a debate about creating a novel genus. However, phenotypic and chemotaxonomic data, which are of prior importance (Kämpfer, 2012), were consistent with members of this genus. In addition to a unique 16S rRNA gene sequence, the isolates could be differentiated from other members of this genus by characteristics shown in Table 1, including the ability to ferment both D-mannitol and D-xylose. Additionally, the isolates could be clearly distinguished from the closely related genus *Brochothrix* by a variety of characteristics, among others by G+C content and conditions for growth.

Based on phenotypic and phylogenetic evidence that demonstrate the isolates' separateness from all described

Listeria species, we consider that they merit classification in a novel species within the genus *Listeria*, for which the name *Listeria fleischmannii* sp. nov. is proposed.

Description of *Listeria fleischmannii* sp. nov.

Listeria fleischmannii (fleisch.man'ni.i. N.L. gen. masc. n. *fleischmannii* of Fleischmann, named after the Agricultural Scientist Professor Wilhelm Fleischmann, who performed important general and bacterial research on milk and dairy products and is considered the founder of dairy science and technology).

Cells are regular short rods, occurring singly or in short chains (0.4–0.6 µm in diameter and 0.7–1.2 µm in length) with a Gram-positive cell wall. Aerobic and facultatively anaerobic. After incubation on BHI or TSB agar at 37 °C for 24 h, colonies are 0.4–1.0 mm in diameter, translucent, circular and low convex with entire margins and smooth surfaces. Colonies appear bluish grey by normal illumination and have a bluish appearance when applying the Henry oblique transmitted-light viewing technique. Non-motile but harbours the central portion of the flagellin gene *flaA*. Positive for nitrate reduction, hydrolysis of hippurate and aesculin and production of hydrogen sulfide. Non-haemolytic, not invasive into Caco-2 cells and does not harbour virulence genes on the *Listeria* pathogenicity island 1. Resistant to clindamycin and nalidixic acid. Other characteristics are indicated in Table 1. Can be differentiated from all other members of the genus *Listeria* based on its unique ability to ferment both D-mannitol and D-xylose.

The type strain is LU2006-1^T (=DSM 24998^T =LMG 26584^T), isolated from a hard cheese in western Switzerland in 2006. The DNA G+C content of the type strain is 39.0 mol% (HPLC).

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Wangen im Allgäu, Germany) for providing isolate A11-3426. Furthermore, we thank Professor Martin J. Loessner for providing CBDs and for helpful discussions, Esther Marty for assistance during pulsed-field gel electrophoresis analysis, Dr Mélanie Gagnon and Alexandra Dostal for help during the tissue culture invasion assay, and Dr Markus Schuppler and Carmen Saez for help during phenotypic characterization (all from the Institute of Food, Nutrition and Health at the ETH Zurich). We also thank Claudia Geiger for taking TEM pictures, Mandy Hailer for phenotypic characterization and Fourier transform infrared spectroscopy (both from the CVUAS) and Professor Vincent Perreten (Institute of Veterinary Bacteriology at the University of Bern) for helping with genotypic antibiotic resistance profiling. This project ('BactFlow') was funded by the Competence Center Environment and Sustainability (CCES) directed by ETH Zurich.

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