IJSEM paper no. IJS/2012/044123: second revision

Lactobacillus porcinae sp. nov. isolated from traditional Vietnamese nem chua

Doan Thi Lam Nguyen[1,2], Margo Cnockaert[1], Koenraad Van Hoorde[1,3], Evie De Brandt[1], Isabel Snauwaert[1], Cindy Snauwaert[4], Luc De Vuyst[5], Binh Thanh Le[6], and Peter Vandamme[1]

[1]Laboratory of Microbiology, and [4]BCCM/LMG Bacteria Collection, Department of Biochemistry and Microbiology, Gent University, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium.

[2] Department of Biochemistry and Food Biotechnology, Faculty of Food Science and Technology, Hanoi University of Agriculture, Trauquy - Gialam, Hanoi, Vietnam.

[3]Faculty of Applied Bioscience Engineering, University College Gent, Schoonmeersstraat 52, 9000 Gent, Belgium

[5]Research Group of Industrial Microbiology and Food Biotechnology (IMDO), Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Pleinlaan 2, B-1050 Brussels, Belgium

[6] Institute of Biotechnology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Hanoi, Vietnam

Key words: *Lactobacillus porcinae* sp. nov., nem chua, Vietnam, taxonomy, identification

Running title: *Lactobacillus porcinae* sp. nov.

Subject category: New Taxa **Subsection:** Gram-positive bacteria

Corresponding author: Peter Vandamme, Laboratorium voor Microbiologie, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium, Tel: +32.9.2645114, Fax: +32.9.2645092, email: Peter.Vandamme@ugent.be

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene and *pheS* gene sequences of LMG 26767^T are HE616585 and HE616586, respectively. The accession number for *pheS* gene sequences of *Lactobacillus manihotivorans* LMG 18011, *Lactobacillus camelliae* LMG 24277^T, and *Lactobacillus thailandensis* LMG 24278^T are HE798569, HE798567, HE798568, respectively.

A species diversity study of lactic acid bacteria occurring in traditional Vietnamese nem chua yielded an isolate, LMG 26767^T, that could not be assigned to a validly named species. The isolate was initially investigated by 16S rRNA gene sequence analysis, which revealed that it belonged to the genus *Lactobacillus*, with *Lactobacillus manihotivorans* and *Lactobacillus camelliae* as the closest relatives (98.9% and 96.9% gene sequence similarity towards the type strains, respectively). Comparative $(GTG)_5$-PCR genomic fingerprinting confirmed the unique taxonomic status of the novel strain. DNA-DNA hybridization experiments, DNA G+C content determination, sequence analysis of the phenylalanyl-tRNA synthase (*pheS*) gene, and physiological and biochemical characterization demonstrated that strain LMG 26767^T (= CCUG 62266^T) represents a novel species, for which the name *Lactobacillus porcinae* sp. nov. is proposed. Biochemically, *Lb. porcinae* can be distinguished from *Lb. manihotivorans* and *Lb. camelliae* by its carbohydrate fermentation profile, absence of growth at 45°C, and production of D- and L- lactate as end products of glucose metabolism.

Meat fermentation is an ancient process originally used to extend the shelf life of perishable raw meat. During fermentation complex biochemical and physical reactions take place that result in a significant change of the initial characteristics of the raw materials. Moreover, production of aromatic substances during fermentation defines the sensorial characteristics of the final products which are significantly different from the ones of the raw materials used (Rantsiou & Cocolin, 2008). In Vietnam, fermentation is the main process for preserving meat. Nem chua is a traditional fermented food product that consists of lean ground pork mixed with spices and boiled pig skin cut into thin strings. The meat paste is shaped into cubes which are partly wrapped in a leaf 'Oi' of the plant *Psidium guajava* for decoration and flavour. The cubes are wrapped in banana leaves to provide the anaerobic environment for the fermentation process and to inhibit entry of potentially pathogenic micro-organisms. The fermentation takes place without addition of a starter culture or prior cooking or heating, and proceeds for 2 to 4 days at ambient temperature. Nem chua has a shelf life of five days when preserved at room temperature. However, the shelf life can be prolonged up to one month at refrigerator temperatures (Nguyen *et al.*, 2011).

Despite a growing knowledge on the lactic acid bacteria (LAB) ecology of many fermented foods, little is known on the native LAB communities associated with the production of nem chua. Nguyen *et al.* (submitted) recently studied the nem chua LAB communities in various households in Hanoi and Thanhhoa, two cities in Northern Vietnam, through culture-dependent and culture-independent approaches (Nguyen *et al.*, submitted). They reported a dominance of lactobacilli, with *Lactobacillus plantarum*, *Lactobacillus farciminis*, and *Lactobacillus pentosus* as predominant species. The study also yielded a large variety of other LAB, including some less common species, such as *Pediococcus stilesii*, and an isolate, LMG 26767^{T}, that could not be assigned to a validly named species. In the present study, the taxonomic position of this isolate is determined.

Strain LMG 26767^{T} was isolated from Thanhhoa nem chua in Northern Vietnam from a sample collected after approximately 48 h of fermentation at ambient temperature (about 32 $^{\circ}$C); it was assigned the research collection number R-42633 originally. About 25 gram of nem chua (pH 4.4; 42x10^{8} CFU LAB per gram nem chua) was homogenized in 225 ml maximum recovery diluent (MRD) (Oxoid,

Erembodegem-Aalst, Belgium) by using a Stomacher Lab Blender 80 (Seward Medical, London, UK), after which 10-fold serial dilutions were prepared in MRD. MRS agar (Oxoid) was used for the isolation of LMG 26767^{T}. MRS plates were incubated for 48 h at 28 °C in aerobic conditions. The isolate was checked for bacteriological purity by successive plating on MRS agar and stored in Microbank^{TM} vials (Pro-Lab Diagnostics, Richmond Hill, ON, Canada) at -80 ^{o}C until further analysis. Gram stain reaction, cell morphology, catalase activity, and all further experiments were performed by using cultures grown for 48 h in MRS medium at 28 ^{o}C under aerobic conditions.

The phylogenetic position of strain LMG 26767^{T} was first determined by analysis of its 16S rRNA gene sequence analysis. Genomic DNA was obtained using the phenol-chloroform extraction method described by Gevers and co-workers (Gevers *et al.*, 2001). Quality and purity of the obtained DNA were checked by spectrophotometric measurements at 234, 260, and 280 nm (SpectraMax Plus384, Molecular Devices, California, USA), and visually by electrophoresis of 5 µL DNA mixed with 2 µL loading dye (4 g sucrose and 2.5 mg bromophenol blue dissolved in 6 mL TE buffer) on a 1% w/v agarose (Result LE General Purpose Agarose, BIOzym group, Landgraaf, Holland) gel, run for 30 min at 100 V in 1 L TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0), flanked by a molecular mass marker (SmartLadder, Eurogentec, Seraing, Belgium). 16S rRNA gene amplification, purification and sequencing were performed as described by De Bruyne *et al.* (De Bruyne *et al.*, 2008), using the following modifications. Sequencing reactions were purified using a BigDye® XTerminator^{TM} Purification Kit according to the protocol of the supplier (Applied Biosystems). The ARB software package (Ludwig *et al.*, 2004) and the corresponding SILVA SSURef 102 database (Pruesse *et al.*, 2007) were used to align the obtained 16S rRNA gene sequence and these of the type strains of all established *Lactobacillus* species, its nearest phylogenetic neighbours (see below). These aligned sequences were imported into the software package MEGA (Molecular Evolutionary Genetics Analysis) version 5.0 (Tamura *et al.*, 2011) and analyzed using the neighbour-joining, maximum-likelihood, and maximum-parsimony methods. The statistical reliability of tree topologies was evaluated by bootstrapping analysis based on 1000 tree replicates. The maximum-parsimony tree and the neighbour-joining tree revealed topologies similar to those obtained in the phylogenetic tree constructed

using the maximum-likelihood approach (Fig.1). Sequence similarity calculations performed using the ARB software package indicated that the closest relatives of strain LMG 26767^T were *Lactobacillus manihotivorans* OND 32^T (98.9%) and *Lactobacillus camelliae* MCH3-1^T (96.9%). Lower sequence similarities (< 96.0%) were found towards other validly named species of the genus *Lactobacillus*.

The taxonomic position of strain LMG 26767^T was further studied using phenylalanyl-tRNA synthase (*pheS*) gene sequencing analysis (Naser *et al.*, 2005a). Analysis of *pheS* gene sequences proved to be an excellent tool for the identification of *Lactobacillus* isolates and the delineation of novel taxa (Naser *et al.*, 2007; Naser *et al.*, 2005b; Švec *et al.*, 2005b; Vancanneyt *et al.*, 2006). The primer combinations pheS-21-F (5'-CAYCCNGCHCGYGAYATGC-3') and pheS-22-R (5`-CCWARVCCRAARGCAAARCC- 3`) are used for *pheS* gene amplification for most strains; if they fail to generate an amplicon, the alternative primer set pheS-21-F and pheS-23-R (5`-GGRTGRACCATVCCNGCHCC-3`) can be used. The primers used for amplification of *pheS* of strain LMG 26767^T and *Lb. camelliae* LMG 24277 ^{T} were pheS-21-F and pheS-23-R, whereas primers used for amplification of *pheS* of strains *Lb. manihotivorans* LMG 18011 and *Lb. thailandensis* LMG 24278^T were pheS-21-F and pheS-22-R. The primers used for sequencing of *pheS* were pheS-21-F and pheS-23-R. Amplification and sequencing were performed as described by Naser and co-workers (Naser *et al.*, 2005a). *PheS* gene sequences of other *Lactobacillus* reference strains were available from previous studies (Naser *et al.*, 2007) Sequences were subsequently imported into the BioNumerics version 5.1 software package. The software package MEGA version 5.0 (Tamura *et al.*, 2011) was used to align the translated gene sequences and to analyze the nucleotide sequences using the neighbour-joining, maximum-likelihood, and maximum-parsimony methods. The statistical reliability of tree topologies was evaluated by bootstrapping analysis based on 1000 tree replicates. The maximum-parsimony tree and the neighbour-joining tree revealed topologies similar to those obtained in the phylogenetic tree constructed using the maximum-likelihood approach for both analyses (Fig. 2). Strain LMG 26767^T again clustered most closely with *Lb. manihotivorans* (88.0% sequence similarity towards both strains LMG 18010^T and LMG 18011) and *Lb. camelliae* (LMG 24277^T at a very low value: 80.0% sequence similarity) (Fig. 2). This low *pheS* gene sequence similarity value strongly suggests that strain LMG 26767^T represents a

distinct *Lactobacillus* species. To confirm this, DNA-DNA hybridizations were performed between strain LMG 26767^T and *Lb. manihotivorans* LMG 18010^T, the type strain of its nearest phylogenetic neighbour. DNA was extracted from 0.75-1.25 g cell mass using the protocol described by Gevers et al. (Gevers *et al.*, 2001), with the following modifications. Volumes were increased tenfold for large-scale application. After the addition of 20% SDS and glass beads, cells were mixed for 30 s. Subsequently, 16.5 ml TE buffer (10 mM Tris/HCl, 100 mM EDTA, pH 8.0) and 5 ml 5 M NaCl were added, followed by 10 min of incubation at 65 ^oC. Chloroform/isoamyl alcohol extraction and ethanol and RNase treatment were performed as described by Marmur (Marmur, 1961). DNA-DNA hybridizations were performed with biotin-labeled probes in microplate wells (Ezaki *et al.*, 1989), by using an HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 44 ^oC in the presence of 50% formamide. The average level of DNA-DNA relatedness between strain LMG 26767^T and *Lb. manihotivorans* LMG 18010^T was 39% (the reciprocal values were 38 and 40%). This value is well below the threshold of 70% for species delineation, confirming that strain LMG 26767^T represents a novel species in the genus *Lactobacillus*.

$(GTG)_5$–PCR fingerprinting was used as a genotypic tool to distinguish strain LMG 26767^T from its closest relatives, *Lb. manihotivorans* and *Lb. camelliae* as described previously (Gevers *et al.*, 2001; Švec *et al.*, 2005a). In short, PCR amplification was performed as described by Versalovic and co-workers (Versalovic, 1994) in a DNA thermal cycler (Perkin Elmer 9600, Massachusetts, USA). Subsequently, PCR amplicons were separated on a 1.5% w/v agarose gel (20 cm x 15 cm) in 1 x TAE buffer at 55 V for 16 h. After staining with ethidium bromide, $(GTG)_5$-PCR profiles were visualized under ultraviolet light and digitally captured using a CCD camera. The resulting fingerprints were analyzed using the BioNumerics software (Applied Maths). Fig. 3 illustrates that the $(GTG)_5$-PCR profiles of strain LMG 26767^T, and the *Lb. manihotivorans* and *Lb. camelliae* reference strains are clearly different.

The mol percentage G + C content of the DNA of strain LMG 26767^T was determined. DNA was enzymically degraded into nucleosides as described by Mesbah and co-workers (Mesbah *et al.*, 1989). The DNA nucleotide mixture was

chromatographically analyzed using a Waters Breeze HPLC system. A thermostable XBridge Shield RP18 column was used at 37 ^{o}C. The solvent was 0.02 M $(NH_4)H_2PO_4$ (pH = 4.0) with 1.5% (v/v) acetonitrile. Non-methylated λ phage DNA (Sigma) was used as the calibration reference. The DNA G + C content of strain LMG 26767^{T} was 47.6 mol%, which is similar to the value determined for *Lb. manihotivorans* (48.4 mol%) (Morlon-Guyot *et al.*, 1998) and somewhat lower than that of *Lb. camelliae* (51.9 mol%) (Tanasupawat *et al.*, 2007).

The preparation of the peptidoglycan and the analysis of the peptidoglycan structure were performed according to Schumann {Schumann, 2011 #3153}. The hydrolysate (6N HCl, 120°C, 15h) contained the peptide L-Lys – D-Asp and the amino acids alanine, glutamic acid, lysine and aspartic acid in the approximate molar ration: 1.7 Ala : 0.9 Asp : 1.0 Glu : 0.9 Lys. Milder hydrolysis (4N HCl, 100°C, 0.75h) yielded additionally the peptides L-Ala – D-Glu, L-Lys – D-Ala and D-Ala – L-Lys – D-Asp. From these data it was concluded that strain LMG 26767^{T} shows the peptidoglycan type A4α L-Lys – D-Asp.

Finally, the cellular morphology and growth characteristics of strain LMG 26767^{T} were determined in MRS broth (pH = 6.2; Oxoid CM 0359). Growth was tested at 15, 20, 37, 45 and 52 ^{0}C, in the presence of 5, 6, 7, and 8% NaCl, and at pH 3.9 and 5.4 (at 28 ^{0}C) (the pH of nem chua samples normally ranges from 4.3 to 5.0). The production of gas from 2% glucose and 2% gluconate in MRS broth (pH 6.2, without the addition of triammonium citrate) was also determined, along with production of D- and L-lactate from glucose, as determined enzymically by using the R-Biopharm assay. The carbohydrate fermentation pattern of the strains was determined using the API 50 CHL system (bioMérieux) following the manufacturer`s instructions (strains were cultivated at 28 ^{o}C). A detailed phenotypic description is given below. Characteristics that differentiate the novel species represented by strain LMG 26767^{T} from *Lb. manihotivorans* include absence of growth at 45°C, acid production from arbutin, no acid production from L-rhamnose, methyl-α-D-glucopyranoside, D-lactose, D-melibiose, and D-raffinose, and production of D- and L- lactate from glucose. Similarly, biochemical differences between the novel species represented by strain LMG 26767^{T} and *Lb. camelliae* include acid production from D-saccharose and D-trehalose, but not from L-arabinose, D-mannitol, or D-xylose and production of D- and L- lactate from glucose. In conclusion, data from the present

study demonstrate that strain LMG 26767^T represents a novel *Lactobacillus* species which can be distinguished from its nearest neighbours, *Lb. manihotivorans* and *Lb. camelliae*, by genotypic and phenotypic criteria. We therefore propose to formally classify this strain as *Lactobacillus porcinae* sp. nov., with LMG 26767^T (= CCUG 62266^T) as the type strain.

Description of *Lactobacillus porcinae* sp. nov.

Lactobacillus porcinae (por'ci.nae L. n. *porcina*, pork; *porcinae*, of pork).

Cells are small coccobacilli, 1.5 – 2.0 µm in length and about 1.0 µm wide, and occur singly or in pairs. They are Gram-positive and catalase-negative, do not form spores, and are non motile. After 48 h of incubation on MRS agar at 28 ^oC, colonies are greyish white, opaque, smooth and circular with a convex elevation, entire margin, and approximately 0.5 – 0.7 mm in diameter. Cells grow well in liquid or on solidified MRS under aerobic and anaerobic conditions and grow at 15, 20 and 37 ^oC, but not at 45 or 52 ^oC. The maximum NaCl concentration for growth is 6% (w/v). Able to grow at pH values of 3.9 and 5.4. Strain LMG 26767^T produces D and L isomers of lactic acid in a ratio of 1:9 from glucose. Gas is not produced from glucose or gluconate. Acid is produced from D-galactose, D-glucose, D-fructose, D-mannose, N-acetylglucosamine, amygdalin, arbutin, esculin, ferric citrate, salicin, D-cellobiose, D-maltose, D-saccharose, D-trehalose, amidon, and gentiobiose, but not from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, D-lactose, D-melibiose, inulin, D-melezitose, D-raffinose, glycogen, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate, or potassium 5-ketogluconate.

The type strain, LMG 26767^T (=CCUG 62266^T), was isolated from nem chua in Thanhhoa city, Vietnam in 2009. Strain LMG 26767^T was the only *Lb. porcinae* isolate out of a total of 273 LAB that were picked from 10 nem chua samples from Northern Vietnam (Nguyen et al. submitted). The DNA G + C content of the type strain is 47.6 mol% and its peptidoglycan type is A4α L-Lys – D-Asp.

Acknowledgments

This work was performed in a doctoral framework programme funded by the Belgian Technical Cooperation (BTC).

References

De Bruyne, K., Franz, C. M. A. P., Vancanneyt, M., Schillinger, U., Mozzi, F., de Valdez, G. F., De Vuyst, L. & Vandamme, P. (2008). *Pediococcus argentinicus* sp. nov. from Argentinean fermented wheat flour and identification of *Pediococcus* species by *pheS*, *rpoA* and *atpA* sequence analysis. *Int J Syst Evol Micr* **58**, 2909-2916.

Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224-229.

Gevers, D., Huys, G. & Swings, J. (2001). Applicability of rep-PCR fingerprinting for identification of *Lactobacillus* species. *FEMS Microbiol Lett* **205**, 31-36.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363-1371.

Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *J Mol Biol* **3**, 208-IN201.

Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G+C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159-167.

Morlon-Guyot, J., Guyot, J. P., Pot, B., de Haut, I. J. & Raimbault, M. (1998). *Lactobacillus manihotivorans* sp. nov., a new starch-hydrolysing lactic acid bacterium isolated during cassava sour starch fermentation. *Int J Syst Bacteriol* **48**, 1101-1109.

Naser, S. M., Thompson, F. L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M. & Swings, J. (2005a). Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. *Microbiology* **151**, 2141-2150.

Naser, S. M., Dawyndt, P., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Vancanneyt, M. & Swings, J. (2007). Identification of lactobacilli by *pheS* and *rpoA* gene sequence analyses. *Int J Syst Evol Micr* **57**, 2777-2789.

Naser, S. M., Vancanneyt, M., De Graef, E., Devriese, L. A., Snauwaert, C., Lefebvre, K., Hoste, B., Švec, P., Decostere, A. & other authors (2005b). *Enterococcus canintestini* sp. nov., from faecal samples of healthy dogs. *Int J Syst Evol Micr* **55**, 2177-2182.

Nguyen, H., Elegado, F., Librojo-Basilio, N., Mabesa, R. & Dizon, E. (2011). Isolation and characterisation of selected lactic acid bacteria for improved processing of nem chua, a traditional fermented meat from Vietnam. *Beneficial Microbes* **1**, 67-74.

Nguyen, T. L. D., Van Hoorde, K., Cnockaert, M., De Brandt, E., De Bruyne, K., Le, T. B. & Vandamme, P. (submitted). A culture-dependent and -independent approach for the identification of lactic acid bacteria associated with the production of nem chua, a Vietnamese fermented meat product. *Food Res Int*.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B. M., Ludwig, W., Peplies, J. & Glöckner, F. O. (2007). SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Res* **35**, 7188-7196.

Rantsiou, K. & Cocolin, L. (2008). Fermented meat products. In *Molecular Techniques in the Microbial Ecology of Fermented Foods*, pp. 91-118. Edited by L. Cocolin & D. Ercolini. New York: Springer

Schumann, P. (2011) Peptidoglycan Structure. *Methods in Microbiology, Vol 38: Taxonomy of Prokaryotes (ed. Rainey, FOA)* 101-129.

Švec, P., Vancanneyt, M., Seman, M., Snauwaert, C., Lefebvre, K., Sedlacek, I. & Swings, J. (2005a). Evaluation of $(GTG)_5$-PCR for identification of *Enterococcus* spp. *FEMS Microbiol Lett* **247**, 59-63.

Švec, P., Vancanneyt, M., Devriese, L. A., Naser, S. M., Snauwaert, C., Lefebvre, K., Hoste, B. & Swings, J. (2005b). *Enterococcus aquimarinus* sp. nov., isolated from sea water. *Int J Syst Evol Micr* **55**, 2183-2187.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731-2739.

Tanasupawat, S., Pakdeeto, A., Thawai, C., Yukphan, P. & Okada, S. (2007). Identification of lactic acid bacteria from fermented tea leaves (miang) in

Thailand and proposals of *Lactobacillus thailandensis* sp. nov., *Lactobacillus camelliae* sp. nov., and *Pediococcus siamensis* sp. nov. *J Gen Appl Microbiol* **53**, 7-15.

Vancanneyt, M., Naser, S. M., Engelbeen, K., De Wachter, M., Van der Meulen, R., Cleenwerck, I., Hoste, B., De Vuyst, L. & Swings, J. (2006). Reclassification of *Lactobacillus brevis* strains LMG 11494 and LMG 11984 as *Lactobacillus parabrevis* sp. nov. *Int J Syst Evol Micr* **56**, 1553-1557.

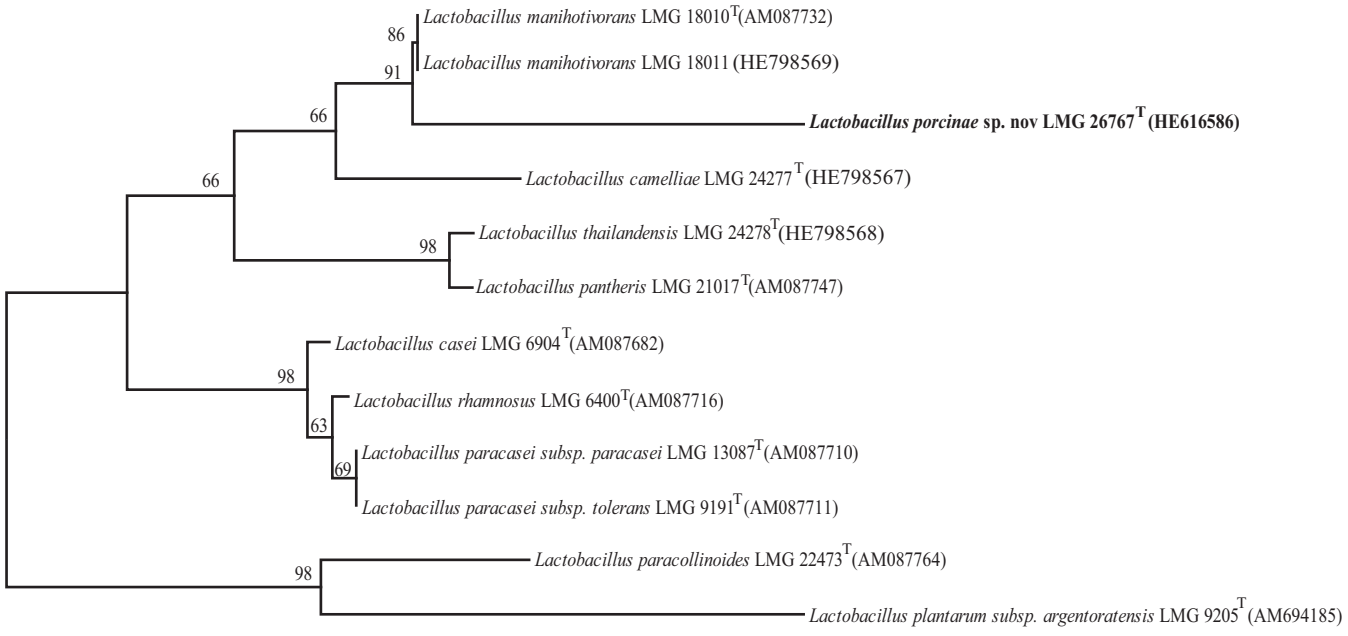
Versalovic, J., Schneider, M., de Bruijn, F. J and Lupski, J. R. (1994). Genomic fingerprinting of bacteria using repetitive sequence based PCR (rep-PCR). *Methods Mol Cell Biol* **5**, 25-40.

Figures:

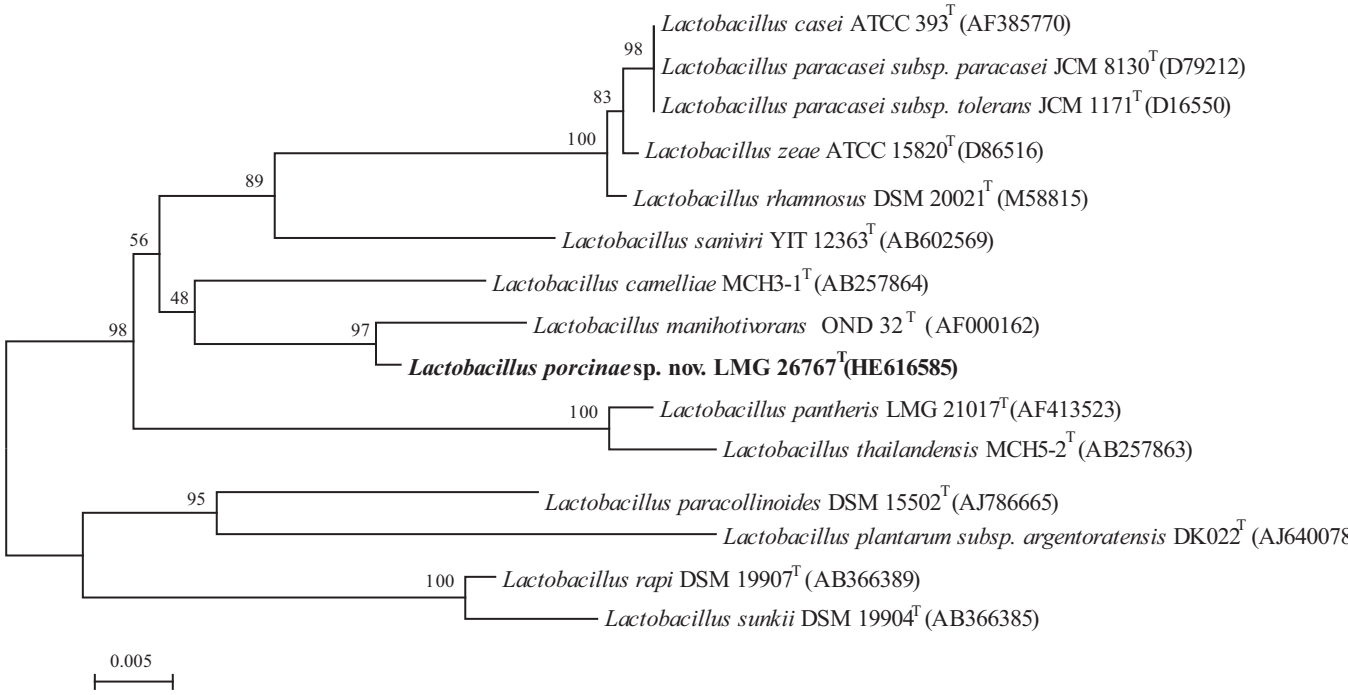
Fig. 1. Maximum-likelihood tree based on 16S rRNA gene sequences showing the phylogenetic relationships of strain LMG 26767^T among the type strains of its nearest neighbours. Bootstrap values (%) based on 1000 replications are shown at the branch points. The substitution model used is the general time reversible model and the aligned sequence has a length of 1426 bp. The bar indicates 0.005 % sequence divergence.

Fig. 2. Maximum-likelihood tree based on *pheS* gene sequences showing the phylogenetic relationships of strain LMG 26767^T among the type strains of its nearest neighbours. Bootstrap values (%) based on 1000 replications are shown at the branch points. The substitution model used is the general time reversible model and the aligned sequence has a length of 390 bp. The bar indicates 0.05 % sequence divergence.

Fig. 3. $(GTG)_5$-PCR pattern and dendrogram based on the UPGMA linkage of Dice coefficients showing the phylogenetic relationships of strain LMG 26767^T among the type strains of its nearest neighbours.


Lactobacillus manihotivorans LMG 18010T(AM087732)
Lactobacillus manihotivorans LMG 18011 (HE798569)
Lactobacillus porcinae sp. nov LMG 26767T (HE616586)
Lactobacillus camelliae LMG 24277T (HE798567)
Lactobacillus thailandensis LMG 24278T(HE798568)
Lactobacillus pantheris LMG 21017T(AM087747)
Lactobacillus casei LMG 6904T(AM087682)
Lactobacillus rhamnosus LMG 6400T(AM087716)
Lactobacillus paracasei subsp. paracasei LMG 13087T(AM087710)
Lactobacillus paracasei subsp. tolerans LMG 9191T(AM087711)
Lactobacillus paracollinoides LMG 22473T(AM087764)
Lactobacillus plantarum subsp. argentoratensis LMG 9205T (AM694185)
86
91
66
66
98
98
63
69
98
0.05

1


Lactobacillus casei ATCC 393T (AF385770)
Lactobacillus paracasei subsp. paracasei JCM 8130T(D79212)
Lactobacillus paracasei subsp. tolerans JCM 1171T (D16550)
Lactobacillus zeae ATCC 15820T (D86516)
Lactobacillus rhamnosus DSM 20021T (M58815)
Lactobacillus saniviri YIT 12363T (AB602569)
Lactobacillus camelliae MCH3-1T (AB257864)
Lactobacillus manihotivorans OND 32T (AF000162)
Lactobacillus porcinae sp. nov. LMG 26767T(HE616585)
Lactobacillus pantheris LMG 21017T(AF413523)
Lactobacillus thailandensis MCH5-2T (AB257863)
Lactobacillus paracollinoides DSM 15502T(AJ786665)
Lactobacillus plantarum subsp. argentoratensis DK022T (AJ640078
Lactobacillus rapi DSM 19907T (AB366389)
Lactobacillus sunkii DSM 19904T (AB366385)
98
83
100
89
56
48
98
97
100
95
100
0.005

2

1


20
40
60
80
100
Lactobacillus manihotivorans LMG 18011
Lactobacillus manihotivorans LMG 18010T
Lactobacillus porcinae sp. nov. LMG 26767T
Lactobacillus camelliae LMG 24277 T