Pediococcus argentinicus sp. nov. from Argentinean fermented wheat flour and identification of *Pediococcus* species by *pheS*, *rpoA* and *atpA* sequence analysis

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A Gram-positive, small coccus-shaped lactic acid bacterium, strain LMG 23999^T, was isolated from Argentinean wheat flour. 16S rRNA gene sequence analysis revealed that the phylogenetic position of the novel strain was within the genus *Pediococcus*, with *Pediococcus stilesii, Pediococcus pentosaceus* and *Pediococcus acidilactici* as its closest relatives (97.7, 97.3 and 96.9 % gene sequence similarity, respectively). Fluorescent amplified fragment length polymorphism fingerprinting of whole genomes and whole-cell protein electrophoresis confirmed the unique taxonomic status of the novel strain. DNA–DNA hybridizations, DNA G+C content determination, comparative sequence analysis of the *pheS*, *rpoA* and *atpA* genes and physiological and biochemical characterization demonstrated that strain LMG 23999^T (=CCUG 54535^T=CRL 776^T) represents a novel species for which the name *Pediococcus argentinicus* sp. nov. is proposed. Multi-locus sequence analysis based on *pheS*, *rpoA* and *atpA* genes was found to be a suitable method for the identification of species of the genus *Pediococcus*.

Pediococci are homolactic acid-fermentative, non-motile, catalase-negative, facultative anaerobes of the family *Lactobacillaceae*. They inhabit fermentable-sugar-rich niches such as plant materials and fermented foods. Strains of the genus *Pediococcus* are used in the food industry as starter cultures for fermented meat or fish products and dairy products (Leroy & De Vuyst, 2004; Simpson & Taguchi, 1995). However, quality problems in beer and wine due to pediococci have also been reported (Renouf *et al.*, 2007; Sakamoto & Konings, 2003). In the present study, we describe a novel species of the genus *Pediococcus* isolated from Argentinean fermented wheat flour. Despite their importance for the food industry, the correct identification of *Pediococcus* strains is complicated by their ambiguous response in traditional physiological tests and methods. Nowadays, speciation is based on habitat, DNA–DNA hybridizations and tolerance of temperature, pH and NaCl (Dobson *et al.*, 2002; Garvie, 1974, 1986). In this study, sequence analysis of the genes encoding the alpha subunits of phenylalanyl-tRNA synthase (*pheS*), RNA polymerase (*rpoA*) and ATP synthase

Abbreviations: FAFLP, fluorescent amplified fragment length polymorphism; LAB, lactic acid bacteria; T_{OR}, temperature of optimal renaturation.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of LMG 23999^T is AM709786. The accession numbers for the *pheS*, *rpoA* and *atpA* gene sequences reported in this paper are AM899805–AM899946, as indicated in Supplementary Fig. S2a–c.

Supplementary figures showing a cluster analysis of whole cell protein profiles with a UPGMA dendrogram and additional neighbour-joining phylogenetic trees based on *pheS*, *rpoA* and *atpA* gene sequences are available with the online version of this paper. A supplementary table giving details of the genomes used for the design of primers rpo-21-F* and rpoA-23-R* is also provided.

(*atpA*) was developed as an alternative approach for the identification of species of the genus *Pediococcus*. Special attention was give to *Pediococcus dextrinicus* as it represents a distantly related species. Konstantinidis *et al.* (2006) considered that three was the minimum number of genes needed for multilocus, sequence-based differentiation between species. This number of genes is required in order to exclude possible horizontal gene transfer or recombination events. The three genes used in this study have already given satisfactory results with several genera of lactic acid bacteria (De Bruyne *et al.*, 2007; Naser *et al.*, 2005a, b, 2007).

Strain LMG 23999^T (=CRL 776^T) was isolated in 1990 in the Centro de Referencia para Lactobacilos (CERELA, CONICET), San Miguel de Tucumán, Argentina. Wheat flour was mixed with warm sterilized tap water and after a fermentation of 4–6 days at 30 °C, samples of the dough were taken. Samples were diluted to 10^{-6} in peptone water (0.1 % w/v) and plated onto LAPTg agar using the pour plate technique. Plates were incubated aerobically at 37 °C for 48 h. The strain was subcultured onto MRS agar at 37 °C, unless indicated otherwise.

The phylogenetic position of strain LMG 23999^T was first determined by analysis of its 16S rRNA gene sequence, as described by Vancanneyt *et al.* (2004) using the following modifications. The PCR-amplified 16S rRNA gene was purified by using a NucleoFast 96 PCR Clean-up kit (Macherey-Nagel). Sequencing reactions were purified using a Montage SEQ96 Sequencing Reaction Clean-up kit (Millipore). Electrophoresis of sequence reaction products was performed by using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). FASTA analysis of the 16S rRNA gene sequence of strain LMG 23999^T (a continuous stretch of 1492 bp) revealed that *Pediococcus stilesii*, *Pediococcus pentosaceus* and *Pediococcus acidilactici* were the closest relatives (with 97.7, 97.3 and 96.9 % sequence similarity, respectively). The 16S rRNA gene sequence of strain LMG 23999^T and sequences of reference strains (retrieved from EMBL) were aligned using CLUSTAL_X. A neighbour-joining phylogenetic tree was constructed using the BioNumerics software package, version 4.61 (Applied Maths). The statistical reliability of the tree topology was evaluated by bootstrapping analysis (Fig. 1). The phylogenetic tree of the genus *Pediococcus* consisted of two separate clades. The clade containing strain LMG 23999^T also included *Pediococcus claussenii*, *P. stilesii*, *P. acidilactici* and *P. pentosaceus*. The second clade comprised *Pediococcus ethanolidurans*, *Pediococcus siamensis*, *Pediococcus cellicola*, *Pediococcus damnosus*, *Pediococcus inopinatus* and *Pediococcus parvulus*. The species *P. dextrinicus* represented a divergent line which was not surprising as this species is atypical of the genus *Pediococcus* and it has been suggested that *P. dextrinicus* may represent a novel genus (Holzapfel *et al.*, 2005).

SDS-PAGE of whole-cell proteins and fluorescent amplified fragment length polymorphism (FAFLP) analysis were used to compare strain LMG 23999^T with other strains of the genus *Pediococcus*, especially strains belonging to its nearest phylogenetic neighbours *P. stilesii*, *P. pentosaceus* and *P. acidilactici*. The protein profiles of all reference strains used were from a previous study (Franz *et al.*, 2006). SDS-PAGE of cellular proteins from strain LMG 23999^T was performed as described by Pot *et al.* (1994). Densitometric analysis, normalization and interpolation of protein profiles and numerical analysis were performed by the use of BioNumerics software package, version 4.61 (Applied Maths). When compared with those of the *Pediococcus* reference strains, the whole-cell protein profile of strain LMG 23999^T was well separated from *P. stilesii*, *P. pentosaceus* and *P. acidilactici*, its nearest phylogenetic

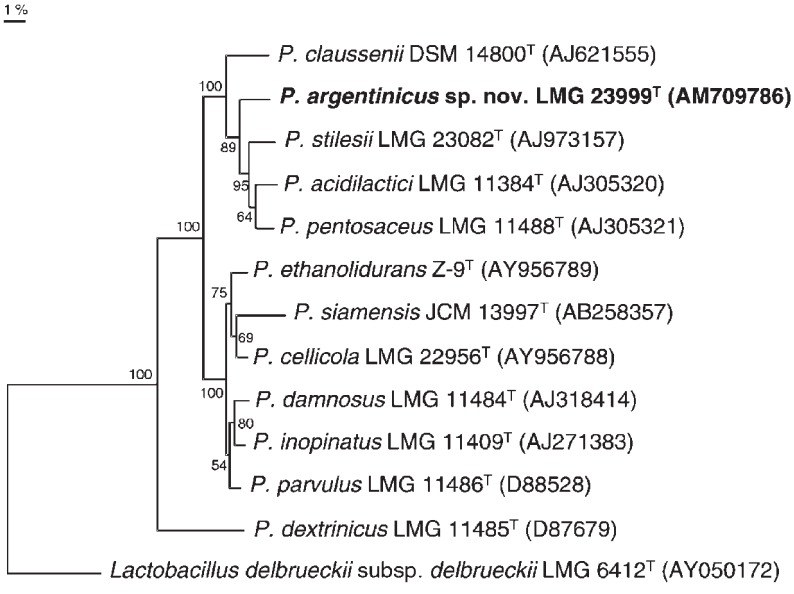

Fig. 1. Phylogenetic neighbour-joining tree based on 16S rRNA gene sequence analysis showing the phylogenetic relationships of *Pediococcus argentinicus* sp. nov. LMG 23999^T within the genus *Pediococcus*. *Lactobacillus delbrueckii* subsp. *delbrueckii* LMG 6412^T was used as the outgroup organism. Bootstrap percentage values (>50) based on 500 tree replications are indicated at the branching points. Bar, 1 % sequence divergence.

neighbours (see Supplementary Fig. S1, available in IJSEM Online). The unique position of strain LMG 23999^T was confirmed by FAFLP analysis. Already available FAFLP fingerprint patterns of *Pediococcus* reference strains were used (Franz *et al.*, 2006) and additional patterns for *P. ethanolidurans*, *P. siamensis* and *P. argentinicus* were generated as described by Franz *et al.* (2006). The resulting electrophoretic patterns were tracked and normalized using the GENESCAN 3.1 software package (Applera). Normalized tables of peaks were transferred into the BioNumerics software package, version 4.61 (Applied Maths). The FAFLP fingerprint of strain LMG 23999^T was compared with reference profiles of its closest phylogenetic neighbours and with those of the type strains of all recognized species within the genus *Pediococcus* (Fig. 2). The results confirmed that strain LMG 23999^T was very different from its nearest neighbours and from other recognized species of the genus *Pediococcus*.

Genomic DNA of strain LMG 23999^T and of *P. stilesii* LMG 23082^T, *P. pentosaceus* LMG 11488^T and *P. acidilactici* LMG 11384^T was isolated according to Marmur (1961), as modified by Stackebrandt & Kandler (1979). DNA–DNA hybridizations were performed using the spectrophotometric method as described by De Ley *et al.* (1970). Hybridization values of strain LMG 23999^T towards strains LMG 23082^T [T_{OR} (temperature of optimal renaturation): 68.4 °C], LMG 11488^T (T_{OR}: 67.8 °C) and LMG 11384^T (T_{OR}: 69.7 °C) were 24, 25 and 21 %, respectively, confirming that strain LMG 23999^T represents a novel species (Wayne *et al.*, 1987). The DNA G+C content of strain LMG 23999^T was determined as described by Mesbah *et al.* (1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column. The solvent used was 0.02 M $NH_4H_2PO_4$ (pH 4.0), 1.5 % (v/v) acetonitrile. Non-methylated lambda phage (Sigma) and *Escherichia coli* LMG 2093 DNA were used as the calibration reference and control, respectively. The DNA G+C content of strain LMG 23999^T was 40.8 mol%. This value was consistent with the DNA G+C contents observed in the genus *Pediococcus*, which are 37–42 mol% (Dobson *et al.*, 2002; Liu *et al.*, 2006; Sneath *et al.*, 1986; Tanasupawat *et al.*, 2007; Zhang *et al.*, 2005).

As already investigated for the genera *Lactobacillus* (Naser *et al.*, 2007), *Enterococcus* (Naser *et al.*, 2005a, b) and *Leuconostoc* (De Bruyne *et al.*, 2007), we evaluated whether *pheS*, *rpoA* and *atpA* gene sequence analysis could be used to differentiate species of the genus *Pediococcus*. In order to assess inter- and intra-species variability among the loci, a total of 44 *Pediococcus* strains was examined: 18 reference strains and 26 field isolates which were additionally identified through AFLP and SDS-PAGE of whole-cell proteins. Several representative strains per species were included when possible. Bacterial strains, depositors and sources are listed in Table 1. The design of the primers, amplification conditions and sequencing reactions were as described by Naser *et al.* (2005a, b). The primer combinations pheS-21-F/pheS-23-R, rpoA-21-F/rpoA-23-R and atpA-20-F/atpA-26-R amplified the target genes of most strains. When no amplification product for the *atpA* gene was obtained, an alternative primer set atpA-22-F/atpA-26-R was used. For the amplification of the *rpoA* genes of *P. pentosaceus* strains LMG 13561, LMG 13562,

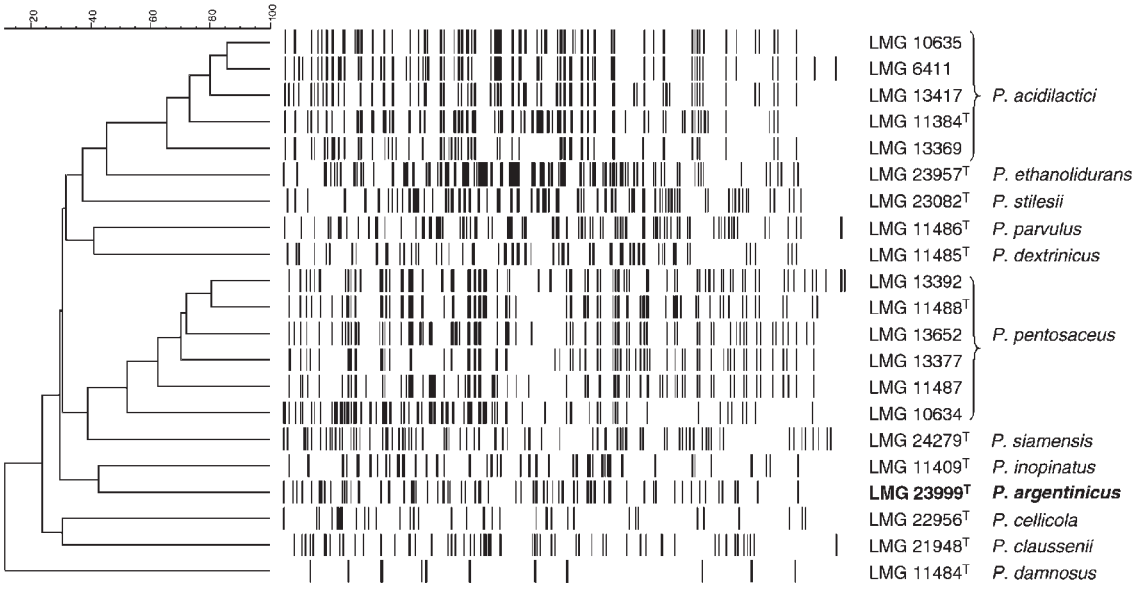

Fig. 2. FAFLP patterns and dendrogram based on the UPGMA linkage of Dice coefficients (expressed as percentage values for convenience) of *Pediococcus argentinicus* sp. nov. LMG 23999^T and of reference strains of all recognized species of the genus *Pediococcus*.

Table 1. List of *Pediococcus* strains used in this study

Strains	Depositor*	Source
P. argentinicus		
LMG 23999^T	Own isolate	Commercial wheat flour (1990, Argentina)
P. acidilactici		
LMG 6411	DSMZ	Not known
LMG 10635	A. Ledeboer	Plant
LMG 11384^T	Rijkszuivelstation, Melle	Barley
LMG 13362	NV Radar, Deinze	Grass sample (Belgium)
LMG 13369	NV Radar, Deinze	Grass sample (Belgium)
LMG 17680	G. Rusul	Chili bo (Malaysia)
P. cellicola		
LMG 22956^T	CGMCC	Distilled-spirit-fermenting cellar wall (2004, China)
P. claussenii		
LMG 21948^T	DSMZ	Spoiled beer (Canada)
LAB 990	Heineken	Beer (1994, The Netherlands)
LAB 994	Heineken	Beer (1994, The Netherlands)
LAB 1004	Heineken	Beer (1994, The Netherlands)
LAB 1014	Heineken	Beer (1994, The Netherlands)
LAB 1231	Heineken	Beer (1994, The Netherlands)
LAB 1232	Heineken	Beer (1994, The Netherlands)
LAB 1329	Heineken	Beer (1994, The Netherlands)
P. damnosus		
LMG 11484^T	NCFB	Lager beer yeast
LAB 1330	Heineken	Beer (1994, Belgium)
LAB 1362	W. Simpson	Not known
LAB 1449	I. Bohack	Not known
P. dextrinicus		
LMG 11485^T	NCFB	Silage
P. ethanolidurans		
LMG 23957^T	CGMCC	Distilled-spirit-fermenting cellar wall (2004, China)
LMG 13386	NV Radar, Deinze	Grass sample (Belgium)
LMG 13387	NV Radar, Deinze	Grass sample (Belgium)
P. inopinatus		
LMG 11409^T	DSMZ	Brewery yeast
LMG 11410	DSMZ	Beer
LMG 22104	J. Leisner	Beer (Czech Republic)
LMG 22105	J. Leisner	Beer (Czech Republic)
LAB 1451	I. Bohack	Not known
LAB 1454	I. Bohack	Not known
P. parvulus		
LMG 11486^T	NCFB	Silage
LMG 16740	ATCC	Wine (Australia)
LAB 194	R. Vogel	Wine
P. pentosaceus		
LMG 10634	Unilever, Vlaardingen	Fermented milk
LMG 11488^T	NCFB	Dried American beer yeast (1931)
LMG 13392	NV Radar, Deinze	Grass sample (Belgium)
LMG 13561	TNO-Voeding, Zeist	Not known
LMG 13562	TNO-Voeding, Zeist	Not known
LMG 13652	L. A. Devriese	Cat (Belgium)
LMG 13377	NV Radar, Deinze	Grass sample (Belgium)
LMG 17236	L. A. Devriese	Sow (Belgium)
LMG 17237	L. A. Devriese	Sow (Belgium)
P. siamensis		
LMG 24279^T	JCM	Fermented tea leaves (2007, Thailand)
P. stilesii		
LMG 23082^T	W. Holzapfel	White maize grains (1997, Nigeria)

Table 1. cont.

*ATCC, American Type Culture Collection; I. Bohack, Lehrstuhl für Technologie der Brauerei I, Technische Universität München, Germany; CGMCC, China General Microbiological Culture Collection Centre; L. A. Devriese, Ghent University, Ghent, Belgium; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; W. Holzapfel, Institute of Hygiene and Toxicology, Karlsruhe, Germany; JCM, Japan Collection of Microorganisms; A. Ledeboer, Unilever, Vlaardingen, The Netherlands; J. Leisner, Royal Veterinary and Agricultural University, Frederiksberg Copenhagen, Denmark; LMG, Belgian Co-ordinated Collections/Laboratory of Microbiology Ghent, Ghent, Belgium; NCFB, National Collection of Food Bacteria (now NCIMB); NCIMB, National Collections of Industrial, Food and Marine Bacteria, Aberdeen, UK; G. Rusul, University Putra, Malaysia; W. Simpson, BRF International, Surrey, UK; R. Vogel, Universität Hohenheim, Stuttgart, Germany.

LMG 11488^T, LMG 13377 and LMG 17237 and *P. stilesii* LMG 23082^T, no amplicon was obtained despite using different sets of primers and amplification conditions as described by Naser *et al.* (2005b). The objective of identifying a maximum number of lactic acid bacteria (LAB) strains using the same amplification protocol (Naser *et al.*, 2005a, b) has led to the use of a standard protocol, which is probably not the most optimal protocol for some members of the genus *Pediococcus*. Failure due to DNA sequence variability in these genes, which prevented annealing of the PCR primers to their target sequences, was solved by designing new primers. The primers rpoA-21-F and rpoA-23-R were evaluated against 53 LAB genomes (see Supplementary Table S1 in IJSEM Online) containing 32 *Streptococcus* strains, 12 *Lactobacillus* strains, three *Lactococcus* strains, two *Enterococcus* strains, two *Oenococcus* strains and one strain of *Leuconostoc mesenteroides* subsp. *mesenteroides* and, most important for the present study, *P. pentosaceus* ATCC 25745 (GenBank accession no. CP000422). The evaluation was performed using the genomic BLAST tool of NCBI (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). Starting from the *rpoA* amino acid sequence of *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893^T translated from the gene sequence GenBank accession no. AM711294, a BLASTP sequence similarity search was performed against protein databases of genomes of all the members of the order *Lactobacillales* present in the database at time of writing (strain information and accession numbers are listed in Supplementary Table S1). The *rpoA* gene identities obtained were used to retrieve the *rpoA* nucleotide sequences from the whole genomes. Together with the primer sequences, all *rpoA* gene sequences were used to perform sequence alignments using CLUSTAL_X. The position of primers within the alignment was verified and substitutions were checked manually. An A/G substitution specific for the *Pediococcus* strains could be found in primer sequence rpoA-21-F and a T/A and a C/G substitution were found in primer sequence rpoA-23-R. Taking into account these substitutions and the information from the other 52 LAB genomes, primers rpoA-21-F and rpoA-23-R were modified to rpoA-21-F* (5′-ATGATYGARTTTGARAARCC-3′) and rpoA-23-R* (5′-ACHSTRTTRATACCDGCNCG-3′). Using these modified primers, *rpoA* products for all the remaining strains were obtained.

The phylogenetic trees constructed for the *pheS*, *rpoA* and *atpA* gene sequences are based on the neighbour-joining method and were obtained by importing the external sequence alignments from CLUSTAL_X into the BioNumerics software package. For each of the genes, the intra-species diversity was smaller than the inter-species diversity and, therefore, numerical analysis yielded species-specific clusters (see Supplementary Figs S2a–c in IJSEM Online). The topologies of the neighbour-joining phylogenetic trees roughly resembled those of the 16S rRNA gene-based phylogeny. To resolve the ambiguous position of *P. dextrinicus*, for which suggestions for it to be reclassified next to the *Lactobacillus casei* group have already been made (Collins *et al.*, 1990, 1991; Stiles & Holzapfel, 1997), sequence data of the type strains of all species of the *Lactobacillus casei* group were included in the sequence analyses (Fig. 3, Supplementary Fig. S2a–c). *P. dextrinicus* represented the most divergent lineage in each of the analyses. This observation strongly supported the two options already suggested in the literature: to transfer *P. dextrinicus* to a new genus (Holzapfel *et al.*, 2005) or to reclassify this species as a novel species of the genus *Lactobacillus*, close to the *Lactobacillus casei* group (Collins *et al.*, 1990, 1991; Stiles & Holzapfel, 1997). To date, no official proposal has been made for this change (Dobson *et al.*, 2002). For the remaining pediococci species, both *rpoA* and *atpA* gene analysis yielded the same division into two clades, corresponding to the 16S rRNA gene sequence phylogeny. Analysis of the *pheS* gene showed a slightly different topology; it was however the most discriminatory gene for the identification of species of the genus *Pediococcus*. By integrating information from different molecular markers, the simultaneous use of several housekeeping genes offers a higher reliability for bacterial identification (Fig. 3) (Konstantinidis *et al.*, 2006). Using the concatenated gene sequences, all *Pediococcus* isolates could be classified into species-specific clusters. In addition, two previously unidentified *Pediococcus* isolates from grass silage in Belgium were identified as *P. ethanolidurans* in this study (Fig. 3), an observation that was confirmed by AFLP analysis and SDS-PAGE of whole-cell proteins.

Morphological, physiological and biochemical tests were performed according to Schillinger & Lücke (1987). Lactate enantiomer production was determined using an enzymic test kit (Roche Diagnostics). After 48 h growth, culture

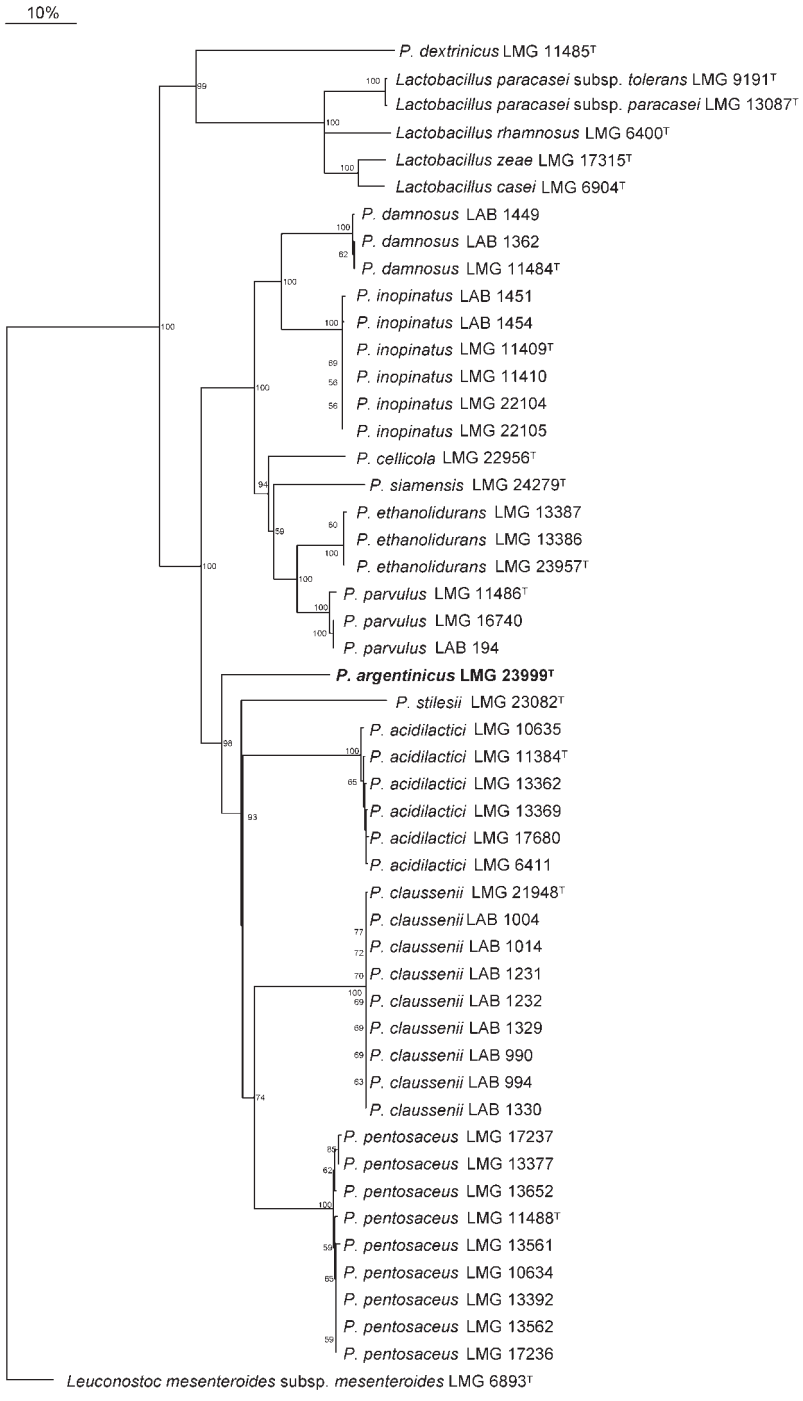

Fig. 3. Concatenated tree based on *pheS* (see Supplementary Fig. S2a), *rpoA* (see Supplementary Fig. S2b) and *atpA* (see Supplementary Fig. S2c) gene sequences of *Pediococcus* strains and the type strains of all species belonging to the *Lactobacillus casei* group. Bootstrap percentages (>50) after 500 simulations are shown. *Leuconostoc mesenteroides* subsp. *mesenteroides* LMG 6893^T was included as the outgroup organism. Bar, 10 % sequence divergence.

supernatants comprised more than 90 % L-lactate. After a prolonged incubation of 4 days, a DL-lactate mixture was obtained (87.6 % L-lactate, 12.4 % D-lactate). The API 50 CHL *Lactobacillus* identification system (bioMérieux) was used to determine the carbohydrate fermentation profile. Morphological and physiological characteristics are given in the species description. An overview of the physiological differences between the novel species and the most closely related species is presented in Table 2. It is evident from these summarized physiological data that strain LMG 23999^T can be distinguished from related species of the genus *Pediococcus* by a combination of acid production tests from sugars (arabinose, galactose, lactose, maltose, mannitol, methyl α-D-mannopyranoside and xylose), NaCl tolerance tests and by the lactic acid configuration.

The results of the polyphasic analysis demonstrate that strain LMG 23999^T represents a novel species within the genus *Pediococcus*, for which we propose the name *Pediococcus argentinicus* sp. nov. Sequence analysis of the

Table 2. Phenotypic characteristics that differentiate *Pediococcus argentinicus* sp. nov. from the most closely related species of the genus *Pediococcus*

Taxa: 1, *P. argentinicus* sp. nov. LMG 23999^T; 2, *P. acidilactici*; 3, *P. pentosaceus*; 4, *P. claussenii*; 5, *P. stilesii* LMG 23082^T. +, 90 % or more strains positive; −, 90 % or more strains negative; d, 11–89 % of strains positive; ND, not determined. Some data were taken from Simpson & Taguchi (1995), Holzapfel *et al.* (2005) and Franz *et al.* (2006).

Characteristic	1	2	3	4	5
Growth at					
pH 9.0	−	−	−	−	+
40 °C	+	+	d	+	+
45 °C	−	+	d	−	+
48 °C	−	+	−	−	−
Lactic acid configuration	L(+)	DL	DL	L(+)	DL
Acid from:					
Arabinose	−	d	+	−	−
Galactose	+	+	+	−	+
Lactose	−	d	+	−	−
Maltose	+	−	+	d	+
Mannitol	+	−	−	d	−
Methyl α-D-mannopyranoside	+	ND	ND	−	−
Xylose	−	+	d	−	−
Max. NaCl concentration for growth (% w/v)	6	10	10	5	8
DNA G+C content (mol%)	40.8	42.0	38.0	40.5	38.0

pheS, *rpoA* and *atpA* genes proved to be a valuable technique for the differentiation of recognized species of the genus *Pediococcus*.

Description of *Pediococcus argentinicus* sp. nov.

Pediococcus argentinicus (ar.gen′ti.ni.cus. N.L. masc. adj. *argentinicus* pertaining to Argentina).

After 24 h growth, cells are small cocci (0.7–1.0 μm) and occur singly or in pairs. They are Gram-positive, do not form spores and no gliding motility is observed. Colonies are greyish white, opaque, smooth and circular with a convex elevation and an entire margin. Both D- and L-lactate are produced as end products of glucose metabolism. Able to grow at pH values of 4–8 and at temperatures up to 40 °C. The maximum NaCl concentration for growth is 6 % (w/v). Acid is produced from glucose, ribose, galactose, fructose, mannose, mannitol, methyl α-D-mannopyranoside, *N*-acetylglucosamine, amygdalin, arbutin, aesculin, salicin, cellobiose, maltose, sucrose, gentiobiose and tagatose. Acid is not produced from glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl *β*-D-xylopyranoside, sorbose, rhamnose, dulcitol, inositol, sorbitol, methyl α-D-glucopyranoside, lactose, melibiose, trehalose, inulin, melezitose, raffinose, glycogen, xylitol, turanose, lyxose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate.

The type strain, LMG 23999^T (=CCUG 54535^T=CRL 776^T), was isolated from Argentinean fermented wheat flour. The DNA G+C content of the type strain is 40.8 mol%.

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