Brief Report

Reclassification of probiotic *Lactobacillus acidophilus* NCIMB 30184 as *Lactobacillus helveticus* and *Lactobacillus casei* NCIMB 30185 as *Lacticaseibacillus paracasei*

Rodenes, A[1]., Casinos, B.[1], Alvarez, B.[1], Martinez-Blanch, J.F.[1], Vijayakumar, V.[2*], Superti, G.[3], Day, R.[2], Chenoll, E[1*].

[1] ADM-BIOPOLIS. ADM Health & Wellness, 46980 Paterna, Valencia, Spain

[2]ADM-PROTEXIN, ADM Health & Wellness, Lopen Head TA13 5JH, UK

[3]ADM do Brasil ADM Health & Wellness, Sãu Paulo, SP, Brazil

*Corresponding authors:

maria.chenoll@adm.com. ADM-BIOPOLIS, 46980 Paterna, Valencia, Spain

vineetha.vijayakumar@adm.com ADM Protexin, Lopen Head TA13 5JH, UK

ABSTRACT

The strains NCIMB 30184 and NCIMB 30185, originally designated as *Lactobacillus acidophilus and Lactobacillus casei* respectively, have been studied in published pre-clinical and clinical studies. Their nucleotide sequences of the 16S rRNA were compared with public databases and were preliminarily found to have the highest similarity with *Lactobacillus helveticus* and *Lacticaseibacillus paracasei* species respectively. Subsequently, total 16S and 23S rRNA genes were compared with their most similar taxa, and the preliminary identification as *Lactobacillus helveticus* and *Lacticaseibacillus paracasei* were confirmed.

INTRODUCTION

Initially proposed by Beijerinck in 1901 as Gram-positive, homofermentative, thermophilic and non-spore- forming rods, genus *Lactobacillus* has experienced multiple taxonomical reassignments. Initially based on phenotypic traits [1], its taxonomic assignment criteria has been modified throughout the last decades mostly based on molecular approaches. Sequencing of 16S rRNA genes has been established in bacterial taxonomy as a marker gene in phylogenetic studies for classification and nomenclature purposes [2]. However, *Lactobacillus* is a very complex genus, in which the initial phenotypic differentiations are highly variable within clades [3,4] with an ever increasing number of new species and re-designations [5,6]. This heterogeneity was finally studied as a whole by Zheng and coworkers [7], who divided the genus in 23 different genera. All of these reassignments and changes have made new taxonomical evaluation of the

41 strains necessary and have reinforced the taxonomical complexity of this genus in its
42 identification.

43 Strain NCIMB 30184, originally designated as lactic acid bacteria *Lactobacillus*
44 *acidophilus*, has been studied in published pre-clinical and clinical studies [8,9]. An isolate
45 from human origin, probiotic strain PXN35 was deposited at National Collection of
46 Industrial, Food and Marine Bacteria (NCIMB, Aberdeen, United Kingdom) on the 7th of
47 April 2006 as *L. acidophilus*.

48 Strain NCIMB 30185 was identified initially as *Lactobacillus casei* and its functionality
49 was informed in [8,9]. The probiotic strain PXN37 was deposited at NCIMB collection on
50 the 7th of April 2006 as *L. casei*.

51

52 In both NCIMB 30184 and NCIMB 30185 strains, *in vitro* microbiological and molecular
53 evaluation performed showed that the genetic characteristics presented by the isolates
54 differed from their original designation. In order to determine the correct identification,
55 the strains were revived from the original master cell banks and 16s rRNA and 23s rRNA
56 gene sequencing analysis were performed to allow comparison with closely related
57 species and determine the correct classification of these probiotic strains.

58 **MATERIALS AND METHODS**

59 **Culture and growth conditions** The long-term storage stock of the strains from NCIMB
60 (vial dated 22nd October 2009 for PXN 35 and 04th November 2002 for PXN 37)) were
61 revived by culturing in MRS (Oxoid) at 30 °C under microaerophilic conditions. Working

62 cell banks were conserved in 20 % (v/v) glycerol at -80 °C. Microbiological evaluation
63 was applied by standard methodology.

64 **Nucleotide sequence analysis.** DNA from pure culture was extracted following the
65 guanidium thiocyanate method isolation [10], spectrophotometrically quantified
66 (Nanodrop) and adjusted to the final concentration with ultra-pure water (Sigma-Aldrich,
67 Madrid, Spain). 16s rRNA gene sequencing was run following Chenoll et al [11]. Almost
68 full-length 16S rRNA gene fragment was amplified from genomic DNA by using the
69 following primers: 616 Valt (5'-AGA GTT TGA TYMTGG CTC AG-3') and 630R (5'-CAK AAA
70 GGA GGT GATCC-3'). PCR amplifications were conducted in a solution containing 1× PCR
71 buffer, 100 µmol/l of each dNTP, 1 µmol/l of each primer, 1.5 mmol/l $MgCl_2$, 0.5 U of
72 thermoestable Biotaq™ DNA polymerase (BioLine, Randolph, MA) and 5 µl of DNA
73 template, in a final volume of 50 µl. Amplification conditions were: 5 min at 94 °C, 35
74 cycles of 30 sec at 94 °C, 60 sec at 56 °C and 90 sec at 72 °C, and a final extension of 10
75 min at 72 °C. Almost full-length 23S rRNA gene was in vitro amplified using three
76 universal primer sets following Elizaquivel and coworkers [12]: 1027V/504R, and Ibrahim
77 et al. 2001 [13]: 1104F/1930R and 1522F/130R (all amplification primers are listed in
78 Table 1). PCR amplifications were carried as described above. Amplification conditions
79 were 5 min at 94 °C, 35 cycles of 30s at 94°C, 45 s at 52 °C and 45 s (with a 5 s increment
80 in each cycle) at 72 °C for elongation, and a final extension of 10 min at 72 °C. All
81 reactions were carried out in Mastercycler nexus 2x system thermal cycler (Eppendorf).
82 Controls devoid of DNA were simultaneously included in the amplification process. The
83 integrity of the PCR products was checked by single bands detection following
84 electrophoresis for 1 h at 100 V in 1.4% (wt/vol) agarose gels in Tris-borate-EDTA buffer.

85 Table 1. Primers sequences to amplify 16S and 23S rRNA.

ID primer	rRNA	Sequence (5'-3')
616 Valt	16S rRNA	5'-AGAGTTTGATYMTGGCTCAG-3'
630R	16S rRNA	5'-CAKAAAGGAGGTGATCC-3'
1522F	23S rRNA	5'-TGCGGTGGATCACCTCCTT-3'
130R	23S rRNA	5'-CCTTGCCCCATTCGG-3'
1104F	23S rRNA	5'-TGCGTAATAGCTCAC-3'
1930R	23S rRNA	5'-CGACAAGGAATTTCGCTAC-3'
1027V	23S rRNA	5'-AAACCGACACAGGTRG-3'
504R	23S rRNA	5'-SWGTTCGRVAWGGGA-3'

86

87 **Sequencing of the 16S and 23S rRNA genes and sequence data analysis**. Almost full
88 sequences of the 16S and 23S rRNA genes were amplified separately as indicated above.
89 Purification of amplification products was applied by QIAquick PCR purification kit
90 (QIAGEN) and subsequent sequencing reactions were performed using the Big Dye
91 Terminator (version 3.1) cycle sequencing kit (Applied Biosystems) in a premixed format.
92 The 16S rRNA and 23S rRNA sequences determined in this study have been deposited in
93 the GenBank data base (PXN35 accession reference ON495975 and ON495977
94 respectively, and PXN37 accession reference OP623495 and OP623508 respectively).

95 The 16S and 23S rRNA sequences were aligned separately and initially inspected by eye
96 and compared by the use of the online tool BLAST
97 (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The strains were tentatively identified on the
98 basis of highest scores. For each strain, two phylogenetic trees were constructed based
99 on 16S and 23 rRNA genes with sequences from type strains (Tables 2-5). Sequences
100 were downloaded from NCBI database and *Bifidobacterium longum* ATCC 15707 was
101 used as outgroup. T-Coffee multiple sequence alignment software [14] was used to align
102 the selected sequences. The jModelTest algorithm [15,16] was used to statistically select
103 the best nucleotide substitution model. In this case, the selected model was TPM2uf+G.

104 PhyML software [17] was then used to check and build the 16S rRNA phylogenetic tree.
105 Finally, the iTOL v5 interactive platform [18] was used for its visualization. Percentage of
106 identity among sequences was obtained by Clustal Omega v2.1 package with default
107 parameters [19].

108 Table 2. PXN35 16s rRNA gene sequences used for the study.

Strain	Sequence ID
L. iners DSM 13335^T	NR036982
L. hominis DSM 23910^T	NR125548
L. gasseri ATCC 33323^T	NR075051
L. delbrueckii subsp *bulgaricus* ATCC 11842^T	NR075019
L. kefiranofaciens subsp *kefiranofaciens* DSM 5016	AM113781
L. acidophilus TMPC 32111	OM281001
L. acidophilus 10058	OK447646
L. acidophilus JCM 1132^T	HM162411
L. acidophilus ATCC 4356^T	AB008203
L. crispatus ATCC 33820^T	NR041800
L. amylovorus DSM 20531^T	AY944408
L. kitasatonis JCM 1039^T	AB107638
L. ultunensis DSM 10647^T	AY253660
L. helveticus IDCC 3801	GU121903
PXN35	ON495975
L. helveticus D5401	EU183494
L. gallinarum ATCC 33199^T	NR042111
L. helveticus DSM 20075^T	NR042439
L. helveticus JCM 1120^T	LC062899
L. helveticus C09	EU377824
L. pasteurii DSM 23907^T	NR117058
L. amylolyticus DSM 11664^T	NR029352

109

110 Table 3. PXN37 16s rRNA gene sequences used for the study.

Strain	Sequence ID
PXN37	PXN37
Lacticaseibacillus absianus YH-lac23	MT009028.2
Lacticaseibacillus chiayiensis BCRC81062	MF446960.1
Lacticaseibacillus chiayiensis NCYUAS	NZ_MSSM01000072.1
Lacticaseibacillus brantae DSM23927^T	NZ_AYZQ01000010.1
Lacticaseibacillus casei ATCC393^T	AF469172.1
Lacticaseibacillus casei BIO5773	NZ_WBOC01000018.1
Lactobacillus crispatus ATCC33820^T	NZ_AZCW01000112.1
Lacticaseibacillus paracasei LOCK919	NC_021721.1
Lacticaseibacillus paracasei subsp *paracasei* JCM8130^T	D79212.1

Lacticaseibacillus rhamnosus NCTC13764^T	NZ_LR134331.1
Lacticaseibacillus rhamnosus NRRL B-442	NZ_PKQF01000030.1
Lacticaseibacillus saniviri CJNU3004	NZ_JAIZVU010000062.1
Lacticaseibacillus saniviri DSM24301^T	AB602569.1

111

112 Table 4. PXN35 23S rRNA gene sequences used for the study.

Strain	Sequence ID
L. iners DSM 13335^T	NZ_GG700803.1
L. gasseri 63 AM	NR_076437.1
L. hominis DSM 23910	NZ_AYZP01000029.1
L. delbrueckii subsp *bulgaricus* ATCC 11842^T	NR_077006.1
L. amylolyticus DSM 11664^T	Y17360.1
L. pasteurii DSM 23907^T	NZ_PUFQ01000021.1
L. kefiranofaciens subsp *kefiranofaciens* JCM 6985	NZ_BAMG01000080.1
L. ultunensis Kx293C1^T	NZ_CP059829.1
L. acidophilus NCFM	NR_076355.1
L. amylovorus GRL 1112	NR_076802.1
L. kitasatonis JCM1039^T	NZ_BALU01000025.1
L. crispatus ST1	NR_077026.1
L. gallinarum DSM 10532^T	NZ_AZEL01000029.1
L. helveticus DPC 4571	NR_076471.1
PXN35	PXN35

113

114 Table 5. PXN37 23S rRNA gene sequences used for the study.

Strain	Sequence ID
PXN37	PXN37
Lacticaseibacillus absianus YH-lac23	NZ_JABEKX010000006.1
Lacticaseibacillus chiayiensis BCRC81062	NZ_NOXN01000109.1
Lacticaseibacillus chiayiensis NCYUAS	NZ_MSSM01000072.1
Lacticaseibacillus brantae DSM23927^T	NZ_AYZQ01000009.1
Lacticaseibacillus casei ATCC393^T	NZ_AP012544.1
Lacticaseibacillus casei BIO5773	NZ_WBOC01000014.1
Lactobacillus crispatus ATCC33820^T	NZ_AZCW01000100.1
Lacticaseibacillus paracasei LOCK919	NC_021721.1
Lacticaseibacillus paracasei subsp *paracasei* JCM8130^T	NZ_AP012541.1
Lacticaseibacillus rhamnosus NCTC13764^T	NZ_LR134331.1
Lacticaseibacillus rhamnosus NRRL B-442	NZ_PKQF01000030.1
Lacticaseibacillus saniviri CJNU3004	NZ_JAIZVU010000042.1
Lacticaseibacillus saniviri DSM24301^T	NZ_JQCE01000031.1

115

116 **RESULTS AND DISCUSSION**

117 **Strain PXN35**

118 Strain PXN35 was originally designated as *L. acidophilus* and deposited at National
119 Collection of Industrial, Food and Marine Bacteria (NCIMB, Aberdeen, United Kingdom)
120 as NCIMB 30184.

121 Due to *in vitro* microbiological and molecular evaluation discrepancies with *L.*
122 *acidophilus* designation, almost full 16s rRNA gene sequencing was applied with a
123 sample from the revived original master cell bank. Initially, *on line* tool BLAST grouped
124 the strain with *Lactobacillus helveticus* sequences (data not shown), as a preliminary
125 tentative identification. In order to confirm BLAST identification, a phylogenetic tree was
126 constructed based on 16S rRNA with its closest phylogenetic neighbors. For the analysis,
127 we selected the closest members of *Lactobacillus* phylogenetic group, using the most
128 recent reclassification available and scientifically accepted [7]. The analysis grouped
129 PXN35 sequence with all the *L. helveticus* sequences studied, including the species type
130 strain sequence [7]. *Lactobacillus acidophilus* grouped separately. PXN35 and *L.*
131 *helveticus* shared 99.43% 16s rRNA gene on average, whereas strain PXN35 only showed
132 97.83% homology with *L. acidophilus* representatives (Figure 1, Table 6).

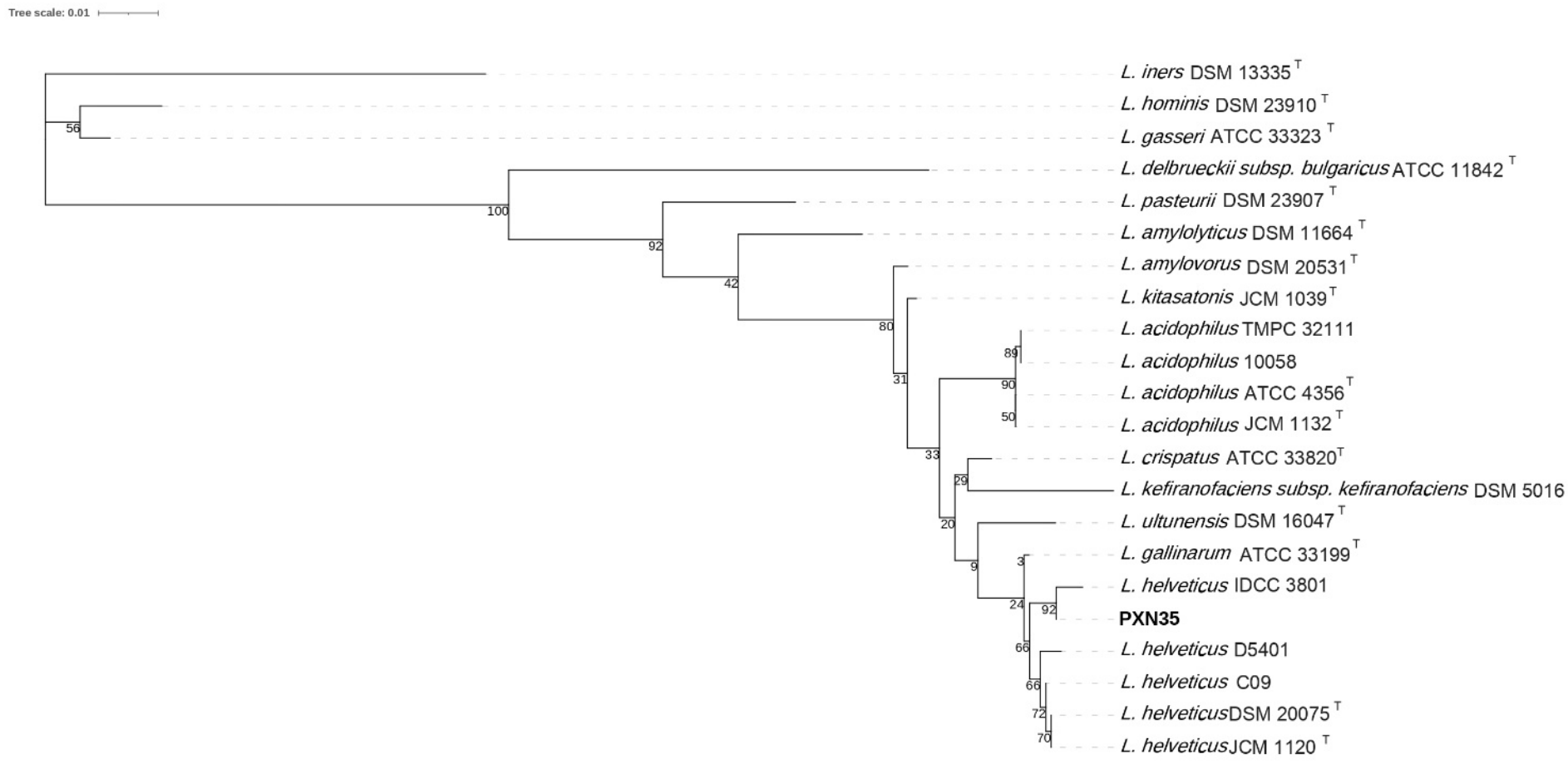


Figure 1. Phylogenetic tree constructed using the neighbour-joining method based on 16S rRNA gene sequences. Strain PXN35 sequence is close to *L. helveticus*. Bar, 10% nucleotide substitution.

136 Table 6. PXN35 16s rRNA gene % homology.

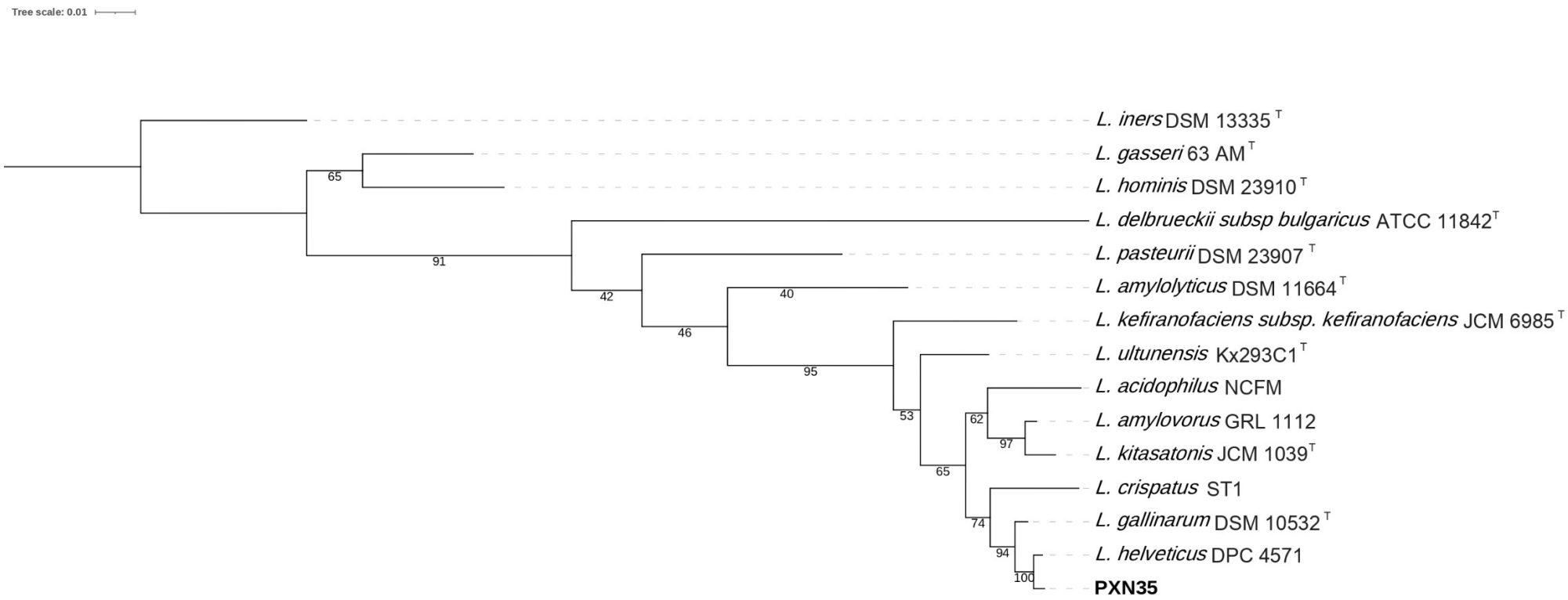
	L. iners strain DSM 13335	*L. hominis* DSM 23910	*L. gasseri* ATCC 33323	L. delbrueckii subsp bulgaricus ATCC 11842	*L. kefiranofaciens* subsp *kefiranofaciens* DSM 5016	*L. acidophilus* strain TMPC 32111	*L. acidophilus* strain 10058	*L. acidophilus* ATCC 4356	*L. crispatus* strain ATCC 33820	*L. amylovorus* strain DSM 20531	*L. kitasatonis*	*L. ultunensis*	*L. gallinarum* strain ATCC 33199	*L. helveticus* DSM 20075	*L. helveticus* strain C09	*L. pasteurii* DSM 23907	*L. amylolyticus* DSM 11664	PXN35
L. iners strain DSM 13335	100.00																	
L. hominis DSM 23910	94.50	100.00																
L. gasseri ATCC 33323	95.00	98.38	100.00															
L. delbrueckii subsp *bulgaricus* ATCC 11842	89.29	90.77	90.85	100.00														
L. kefiranofaciens subsp *kefiranofaciens* DSM 5016	89.52	91.00	90.93	92.06	100.00													
L. acidophilus strain TMPC 32111	89.57	91.27	91.13	92.33	96.60	100.00												
L. acidophilus strain 10058	89.87	91.70	91.57	93.05	97.24	99.29	100.00											
L. acidophilus ATCC 4356	89.94	91.78	91.64	93.13	97.31	99.22	99.93	100.00										
L. crispatus strain ATCC 33820	90.16	91.92	91.78	92.98	97.88	97.66	98.30	98.37	100.00									
L. amylovorus strain DSM 20531	89.66	91.71	91.57	92.70	97.45	97.52	98.16	98.23	98.73	100.00								
L. kitasatonis	89.52	91.71	91.57	92.77	97.66	97.87	98.51	98.58	98.80	99.50	100.00							
L. ultunensis	90.30	91.99	91.86	92.63	97.31	97.09	97.73	97.81	98.30	98.23	98.23	100.00						
L. helveticus strain IDCC 3801	89.57	91.27	91.21	91.70	95.96	97.30	97.02	96.95	97.09	96.88	96.88	96.95						
L. gallinarum strain ATCC 33199	90.30	92.20	92.07	93.13	97.31	97.73	98.37	98.44	98.58	98.23	98.37	98.44	100.00					
L. helveticus DSM 20075	90.16	92.06	91.93	92.84	97.03	97.59	98.23	98.30	98.30	97.95	98.09	98.16	99.58	100.00				

L. helveticus strain C09	90.23	92.13	92.00	92.77	97.10	97.66	98.30	98.37	98.37	98.02	98.16	98.23	99.65	99.93	100.00			
L. pasteurii DSM 23907	91.80	93.07	93.30	93.91	96.09	96.17	96.17	96.17	95.86	96.32	96.24	96.17	96.09	95.79	95.86	100.00		
L. amylolyticus DSM 11664	90.15	91.91	91.85	92.48	95.54	95.25	95.89	95.96	96.03	96.60	96.60	95.54	95.82	95.54	95.61	96.84	100.00	
PXN35	90.16	92.06	91.93	92.70	97.10	97.38	98.02	98.09	98.23	98.02	98.02	98.09	99.50	99.36	99.43	95.79	95.68	100.00

137

138 After that, we ran a further phylogenetic analysis with the same group of strains by
139 comparing the almost full-length large ribosomal subunit 23S. Both 16S and 23S
140 phylogenies are described to be in good agreement and provide an advantage over short
141 ones like 5S, as local non-random rearrangements do not disturb the overall result [20].
142 The 23S phylogenetic tree structure closely resembled the 16S, with PXN35 grouped
143 together with *L. helveticus* representatives and separated from *L. acidophilus*. When
144 percentage homologies were studied, the analysis showed the highest nucleotide
145 sequence homology between PXN35 and *L. helveticus* 23S rRNA gene (99.61%), whereas
146 strain PXN35 showed 97.26% homology with *L. acidophilus* representatives (Figure 2,
147 Table 7).

148



149

150 Figure 2. Phylogenetic tree constructed using the neighbour-joining method based on 23S rRNA gene sequences. Strain PXN35 sequence is close

151 to *L. helveticus*. Bar, 10% nucleotide substitution.

152 Table 7. 23s rRNA gene % homology (only reference sequence is considered).

	L. iners DSM 13335	*L. gasseri* 63 AM	*L. hominis* DSM 23910	*L. delbrueckii* subsp *bulgaricus* strain ATCC 11842	*L. amylolyticus* DSM 11664	*L. pasteurii* DSM 23907	*L. kefiranofaciens* subsp *Kefiranofaciens* JCM 6985	*L. ultunensis* Kx293C1	*L. acidophilus* strain NCFM	*L. amylovorus* GRL 1112	*L. kitasatonis* JCM1039	*L. crispatus* ST1	*L. gallinarum* DSM 10532	*L. helveticus* DPC 4571	PXN35
L. iners DSM 13335	100.00														
L. gasseri 63 AM	93.05	100.00													
L. hominis DSM 23910	92.27	95.65	100.00												
L. delbrueckii subsp *bulgaricus* ATCC 11842	89.47	88.97	89.24	100.00											
L. amylolyticus DSM 11664	89.24	90.15	90.07	89.49	100.00										
L. pasteurii DSM 23907	89.89	90.45	91.02	90.75	92.62	100.00									
L. kefiranofaciens subsp *kefiranofaciens* JCM 6985	89.46	90.33	90.47	89.24	92.98	92.12	100.00								
L. ultunensis Kx293C1	89.81	91.32	90.54	88.84	93.01	92.15	96.52	100.00							
L. acidophilus strain NCFM	89.63	90.89	90.37	88.40	92.18	91.71	95.52	97.26	100.00						
L. amylovorus GRL 1112	89.81	91.29	90.47	89.15	92.84	92.64	96.00	96.87	97.43	100.00					
L. kitasatonis JCM1039	89.72	91.33	90.77	88.93	92.84	92.72	95.87	96.69	97.35	99.17	100.00				
L. crispatus ST1	89.72	90.76	90.51	89.15	92.89	92.29	95.78	96.30	96.69	97.00	97.13	100.00			
L. gallinarum DSM 10532	90.07	91.10	90.32	89.31	92.66	92.19	95.99	97.17	97.21	97.82	97.47	97.74	100.00		
L. helveticus DPC 4571	90.07	91.27	90.58	89.44	92.66	92.23	95.86	96.95	97.26	97.65	97.26	97.43	99.22	100.00	
PXN35	90.11	91.22	90.58	89.52	92.48	92.14	95.95	96.91	97.26	97.69	97.26	97.43	99.17	99.61	100.00

153

154 Isolated from a human individual and deposited in NCIMB collection as NCIMB 30184,
155 strain PXN35 is a gram-positive, catalase negative strain. Cells are nonmotile and non-
156 spore-forming, rod-shaped, isolated or tend to form pairs, with 1.0 µm x 5-15 µm. Strain
157 is obligately homofermentative and grows well in commercially available media for lactic
158 acid bacteria (MRS, Mann Rogosa and Sharpe) at 37°C. Colonies of strain PXN35 grown
159 on MRS agar plates are grey, smooth, slightly domed, and round. The strain PXN35 has
160 been shown to inhibit growth of *Salmonella Typhimurium*, *Clostridium difficile*,
161 *Staphylococcus aureus*, *Escherichia coli* and *Enterococcus faecalis*. Strain PXN35
162 produces organic acids and significantly reduces pH [21, 22] . Originally designated as *L.*
163 *acidophilus*, the present study demonstrates its unequivocal identification as *L.*
164 *helveticus*.

165

166 **Strain PXN37**

167 Originally identified as *L. casei,* strain PXN37 was deposited at NCIMB collection as
168 NCIMB 30185. Further molecular evaluation with almost full 16s rRNA gene sequencing
169 with the revived original master cell bank showed some divergences with *L. casei* species
170 and BLAST tool tentatively grouped the strain with *Lacticaseibacillus paracasei*
171 sequences (data not shown). The initial BLAST identification was checked by a
172 phylogenetic tree based on 16S rRNA with is closest phylogenetic neighbors as Zheng
173 and coworkers established [7]. Strain PXN37 was clearly grouped with all the *L. paracasei*
174 sequences including the species type strain sequence [7] sharing 100% 16s rRNA gene

175 on average with *L. paracasei* whereas *L. casei* representatives grouped separately
176 (Figure 3, Table 6).

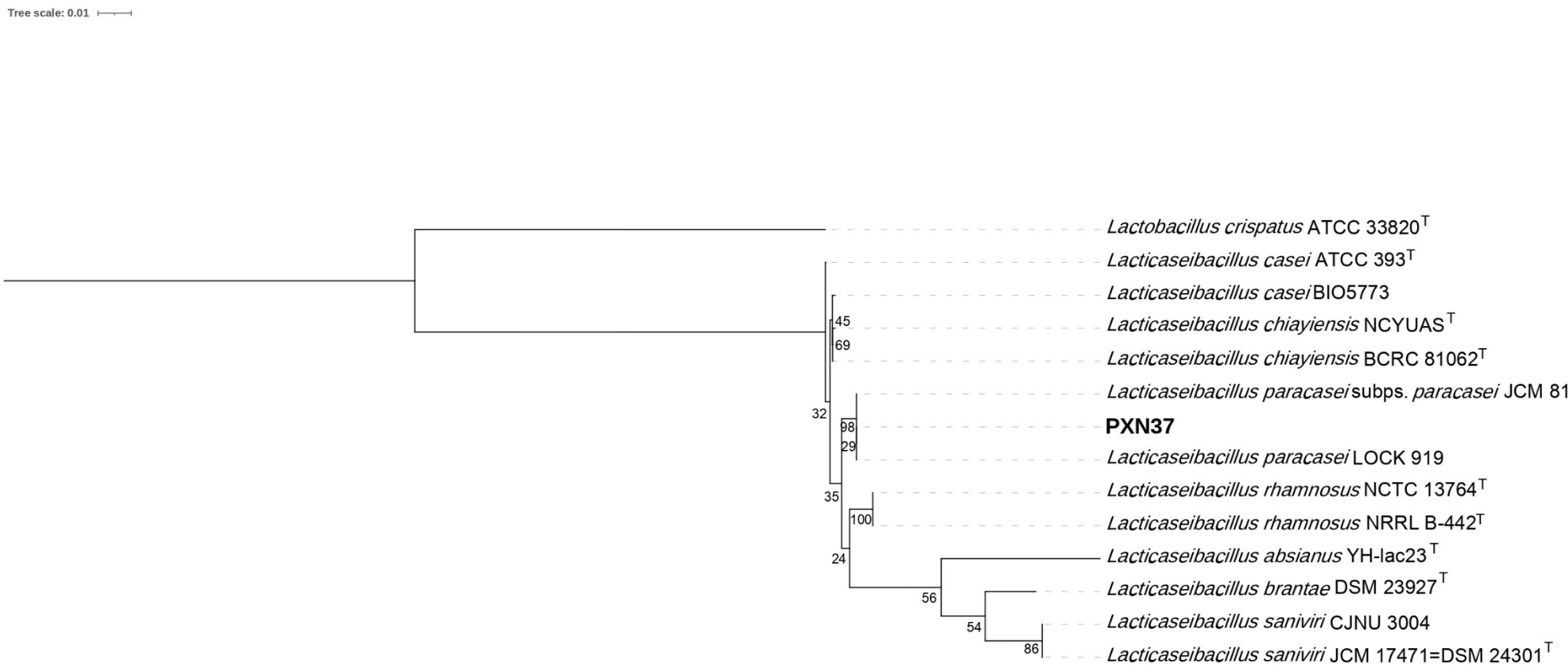


Figure 3. Phylogenetic tree constructed using the neighbour-joining method based on 16S rRNA gene sequences. Strain PXN37 sequence is close to *L. paracasei*. Bar, 10% nucleotide substitution.

180 Table 8. PXN37 16s rRNA gene % homology.

	L. crispatus ATCC33820^T	*L. absianus* YH-lac23	*L. rhamnosus* NCTC13764^T	*L. rhamnosus* NRRL B-442	*L. paracasei* subsp. *paracasei* JCM8130T	*L. paracasei* LOCK919	*L. casei* ATCC393T	*L. chiayiensis* NCYUAS	*L. chiayiensis* BCRC81062	*L casei* BIO5773	*L. saniviri DSM* 24301^T	*L. saniviri* CJNU3004	*L. brantae* DSM23927T	PXN37
Lactobacillus crispatus ATCC33820^T	100.00													
Lacticaseibacillus absianus YH-lac23	86.38	100.00												
Lacticaseibacillus rhamnosus NCTC13764^T	86.37	94.03	100.00											
Lacticaseibacillus rhamnosus NRRL B-442	86.37	94.03	100.00	100.00										
Lacticaseibacillus paracasei subsp. *paracasei* JCM8130^T	86.37	93.83	98.85	98.85	100.00									
Lacticaseibacillus paracasei LOCK919	86.37	93.83	98.85	98.85	100.00	100.00								
Lacticaseibacillus casei ATCC393^T	86.51	93.83	98.78	98.78	99.19	99.19	100.00							
Lacticaseibacillus chiayiensis NCYUAS	86.43	93.96	98.92	98.92	99.19	99.19	99.73	100.00						
Lacticaseibacillus chiayiensis BCRC81062	86.43	93.89	98.92	98.92	99.19	99.19	99.73	99.93	100.00					
Lacticaseibacillus casei BIO5773	86.5	93.96	98.92	98.92	99.19	99.19	99.8	99.86	99.86	100.00				
Lacticaseibacillus saniviri DSM 24301^T	87.4	93.96	94.65	94.65	94.51	94.51	94.44	94.58	94.51	94.51	100.00			
Lacticaseibacillus saniviri CJNU3004	87.4	93.96	94.65	94.65	94.51	94.51	94.44	94.58	94.51	94.51	100.00	100.00		
Lacticaseibacillus brantae DSM23927^T	86.99	94.09	94.65	94.65	94.65	94.65	94.38	94.58	94.51	94.44	97.43	97.43	100.00	
PXN37	86.37	93.83	98.85	98.85	100.00	100.00	99.19	99.19	99.19	99.19	94.51	94.51	94.65	100.00

181

182 Further phylogenetic analysis with full-length large ribosomal subunit 23S was run as
183 previously and as in the case of 16s rRNA analysis, PXN37 grouped together with *L.*
184 *paracasei* representatives and separated from *L. casei*. When percentage homologies
185 were studied, the analysis showed the highest nucleotide sequence homology between
186 PXN37 and *L. paracasei* 23S rRNA gene (100%), and lower (98.59%) with *L. casei*
187 representatives (Figure 4, Table 8).

188

189

190

191

192

193

194

195

196

197

198

199

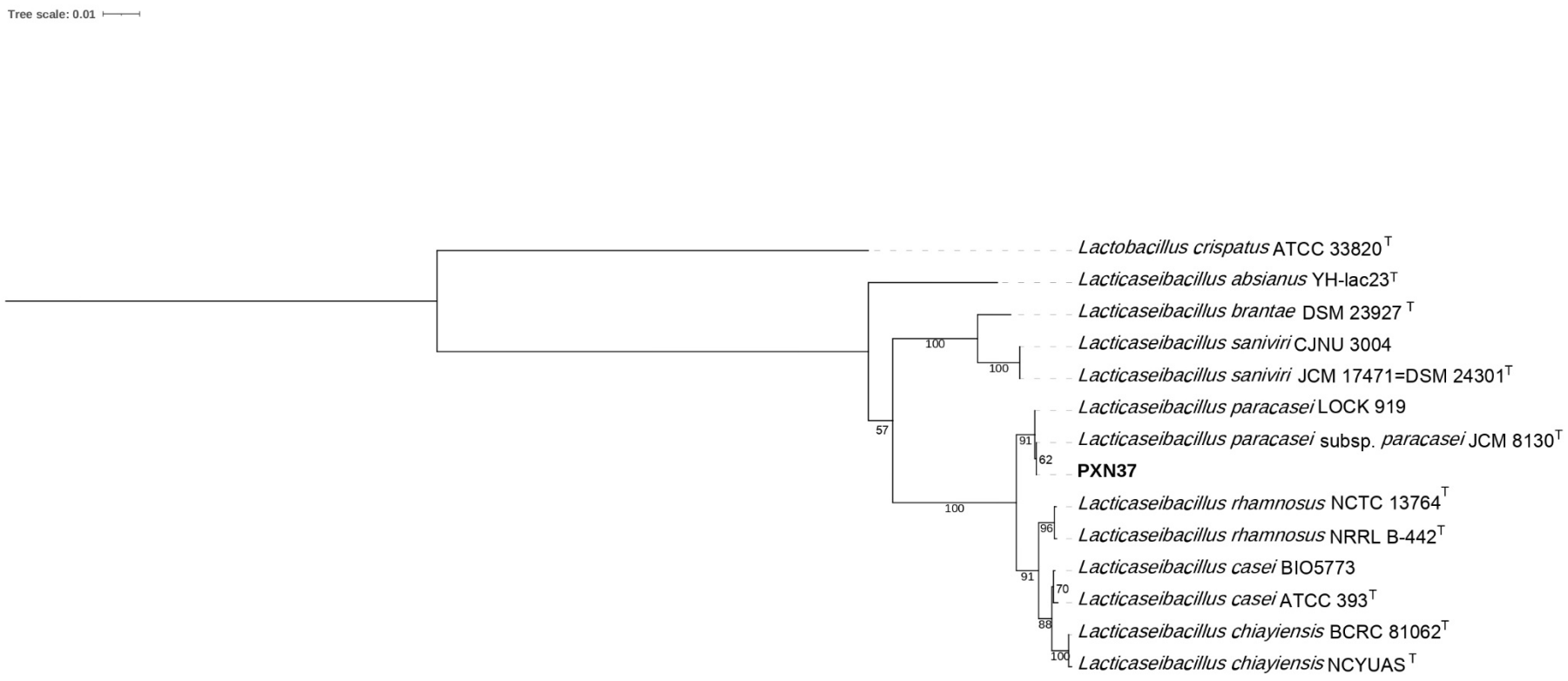


Figure 4. Phylogenetic tree constructed using the neighbour-joining method based on 23S rRNA gene sequences. Strain PXN37 sequence is close to *L. paracasei*. Bar, 10% nucleotide substitution.

Table 8. PXN37 16s rRNA gene % homology.

	L. crispatus ATCC33820T	*L. absianus* YH-lac23	*L. paracasei subsp paracasei* JCM8130^{T}	*L. paracasei* LOCK919	*L. rhamnosus* NCTC13764^{T}	*L. rhamnosus* NRRL B-442	*L. casei* ATCC393^{T}	*L. casei* BIO5773	*L. chiayiensis* BCRC81062	*L. chiayiensis* NCYUAS	*L. brantae* DSM23927^{T}	*L. saniviri* CJNU3004	*L. saniviri* DSM24301^{T}	*PXN37*
L. crispatus ATCC33820^{T}	100.00													
L. absianus YH-lac23	83.85	100.00												
L. paracasei subsp paracasei JCM8130^{T}	83.85	93.48	100.00											
L. paracasei LOCK919	83.85	93.44	99.96	100.00										
L. rhamnosus NCTC13764^{T}	83.57	93.44	98.47	98.51	100.00									
L. rhamnosus NRRL B-442	83.65	93.48	98.51	98.55	99.88	100.00								
L. casei ATCC393^{T}	83.49	93.20	98.59	98.59	99.15	99.11	100.00							
L. casei BIO5773	83.49	93.24	98.59	98.59	99.15	99.11	99.84	100.00						
L. chiayiensis BCRC81062	83.68	93.15	98.51	98.51	98.87	98.83	99.40	99.48	100.00					
L. chiayiensis NCYUAS	83.57	93.04	98.39	98.39	98.79	98.75	99.36	99.44	99.92	100.00				
L. brantae DSM23927^{T}	84.01	93.92	94.28	94.24	94.12	94.12	93.88	94.04	94.07	93.96	100.00			
L. saniviri CJNU3004	84.30	93.60	94.32	94.28	93.92	93.96	93.72	93.88	93.75	93.64	98.07	100.00		
L. saniviri DSM24301^{T}	84.30	93.60	94.32	94.28	93.92	93.96	93.72	93.88	93.75	93.64	98.07	100.00	100.00	
PXN37	83.85	93.48	100.00	99.96	98.47	98.51	98.59	98.59	98.51	98.39	94.28	94.32	94.32	100.00

205 The strain PXN37 was isolated from human origin as a gram-positive, catalase negative
206 strain and deposited in NCIMB collection under reference NCIMB 30185. Cells are
207 nonmotile and non-spore-forming, rod-shaped. Strain is obligately homofermentative
208 and grows well in commercially available media for lactic acid bacteria (MRS, Mann
209 Rogosa and Sharpe) at 37°C. The strain PXN37 has been shown to inhibit growth of
210 *Salmonella Typhimurium*, *Clostridium difficile*, *Staphylococcus aureus*, *Escherichia coli*
211 and *Enterococcus faecalis*. Strain PXN37 produces organic acids and significantly reduces
212 pH [21,22]. Originally designated as *L. casei*, the present study demonstrates its
213 unequivocal identification as *Lacticaseibacillus paracasei*.

214

215

216

217

218

219

220

221

222

223

224

225

ACKNOWLEDGEMENTS

We thank Janice Rueda and Marta Tortajada for critical revision.

COMPETING INTERESTS

A.R., B.C., B.A., J.F.M-B. and E.C. are employed by Biopolis ADM. V.V. and R.D. are employed by Protexin ADM. G.S. is employed by ADM Brazil.

REFERENCES

1. Orla-Jensen, S. *The lactic acid bacteria*; Fred, A., Son, H.a., Eds.; Copenhagen:, 1919.
2. Schleifer, K.H.; Stackebrandt, E. Molecular systematics of prokaryotes. *Annu Rev Microbiol* **1983**, *37*, 143-187, doi:10.1146/annurev.mi.37.100183.001043.
3. Zheng, J.; Ruan, L.; Sun, M.; Gänzle, M. A Genomic View of Lactobacilli and Pediococci Demonstrates that Phylogeny Matches Ecology and Physiology. *Appl Environ Microbiol* **2015**, *81*, 7233-7243, doi:10.1128/aem.02116-15.
4. Sun, Z.; Harris, H.M.; McCann, A.; Guo, C.; Argimón, S.; Zhang, W.; Yang, X.; Jeffery, I.B.; Cooney, J.C.; Kagawa, T.F., et al. Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. *Nat Commun* **2015**, *6*, 8322, doi:10.1038/ncomms9322.
5. Vancanneyt, M.; Naser, S.M.; Engelbeen, K.; De Wachter, M.; Van der Meulen, R.; Cleenwerck, I.; Hoste, B.; De Vuyst, L.; Swings, J. Reclassification of Lactobacillus brevis strains LMG 11494 and LMG 11984 as Lactobacillus parabrevis sp. nov. *Int J Syst Evol Microbiol* **2006**, *56*, 1553-1557, doi:10.1099/ijs.0.64215-0.
6. Naser, S.M.; Vancanneyt, M.; Snauwaert, C.; Vrancken, G.; Hoste, B.; De Vuyst, L.; Swings, J. Reclassification of Lactobacillus amylophilus LMG 11400 and NRRL B-4435 as Lactobacillus amylotrophicus sp. nov. *Int J Syst Evol Microbiol* **2006**, *56*, 2523-2527, doi:10.1099/ijs.0.64463-0.
7. Zheng, J.; Wittouck, S.; Salvetti, E.; Franz, C.; Harris, H.M.B.; Mattarelli, P.; O'Toole, P.W.; Pot, B.; Vandamme, P.; Walter, J., et al. A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *Int J Syst Evol Microbiol* **2020**, *70*, 2782-2858, doi:10.1099/ijsem.0.004107.
8. Chapman, C.M.; Gibson, G.R.; Todd, S.; Rowland, I. Comparative in vitro inhibition of urinary tract pathogens by single- and multi-strain probiotics. *Eur J Nutr* **2013**, *52*, 1669-1677, doi:10.1007/s00394-013-0501-2.
9. Koradia, P.; Kapadia, S.; Trivedi, Y.; Chanchu, G.; Harper, A. Probiotic and cranberry supplementation for preventing recurrent uncomplicated urinary tract infections in premenopausal women: a controlled pilot study. *Expert Rev Anti Infect Ther* **2019**, *17*, 733-740, doi:10.1080/14787210.2019.1664287.

264 10. Pitcher, D.G.; Saunders, N.A.; Owen, R.J. Rapid extraction of bacterial genomic DNA with
265 guanidium thiocyanate. *Letters in Applied Microbiology* **1989**, *8*, 151-156,
266 doi:https://doi.org/10.1111/j.1472-765X.1989.tb00262.x.
267 11. Chenoll, E.; Macián, M.C.; Aznar, R. Identification of Carnobacterium, Lactobacillus,
268 Leuconostoc and Pediococcus by rDNA-based techniques. *Syst Appl Microbiol* **2003**, *26*,
269 546-556, doi:10.1078/072320203770865855.
270 12. Elizaquível, P.; Chenoll, E.; Aznar, R. A TaqMan-based real-time PCR assay for the specific
271 detection and quantification of Leuconostoc mesenteroides in meat products. *FEMS*
272 *Microbiol Lett* **2008**, *278*, 62-71, doi:10.1111/j.1574-6968.2007.00974.x.
273 13. Ibrahim, A.; Hofman-Bang, J.; Ahring, B.K. Amplification and direct sequence analysis of
274 the 23S rRNA gene from thermophilic bacteria. *Biotechniques* **2001**, *30*, 414-416, 418,
275 420, doi:10.2144/01302rr05.
276 14. Di Tommaso, P.; Moretti, S.; Xenarios, I.; Orobitg, M.; Montanyola, A.; Chang, J.M.; Taly,
277 J.F.; Notredame, C. T-Coffee: a web server for the multiple sequence alignment of
278 protein and RNA sequences using structural information and homology extension.
279 *Nucleic Acids Res* **2011**, *39*, W13-17, doi:10.1093/nar/gkr245.
280 15. Darriba, D.; Taboada, G.L.; Doallo, R.; Posada, D. jModelTest 2: more models, new
281 heuristics and parallel computing. *Nat Methods* **2012**, *9*, 772, doi:10.1038/nmeth.2109.
282 16. Guindon, S.; Gascuel, O. A simple, fast, and accurate algorithm to estimate large
283 phylogenies by maximum likelihood. *Syst Biol* **2003**, *52*, 696-704,
284 doi:10.1080/10635150390235520.
285 17. Guindon, S.; Dufayard, J.F.; Lefort, V.; Anisimova, M.; Hordijk, W.; Gascuel, O. New
286 algorithms and methods to estimate maximum-likelihood phylogenies: assessing the
287 performance of PhyML 3.0. *Syst Biol* **2010**, *59*, 307-321, doi:10.1093/sysbio/syq010.
288 18. Letunic, I.; Bork, P. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree
289 display and annotation. *Nucleic Acids Res* **2021**, *49*, W293-w296,
290 doi:10.1093/nar/gkab301.
291 19. Sievers, F.; Higgins, D.G. The Clustal Omega Multiple Alignment Package. *Methods Mol*
292 *Biol* **2021**, *2231*, 3-16, doi:10.1007/978-1-0716-1036-7_1.
293 20. Ludwig, W.; Schleifer, K.H. Bacterial phylogeny based on 16S and 23S rRNA sequence
294 analysis. *FEMS Microbiology Reviews* **1994**, *15*, 155-173, doi:10.1111/j.1574-
295 6976.1994.tb00132.x.
296 21. Tejero-Sariñena, S.; Barlow, J.; Costabile, A.; Gibson, G.R.; Rowland, I. In vitro evaluation
297 of the antimicrobial activity of a range of probiotics against pathogens: evidence for the
298 effects of organic acids. *Anaerobe* **2012**, *18*, 530-538,
299 doi:10.1016/j.anaerobe.2012.08.004.

300 22. Tejero-Sariñena, S.; Barlow, J.; Costabile, A.; Gibson, G.R.; Rowland, I. Antipathogenic
301 activity of probiotics against Salmonella Typhimurium and Clostridium difficile in
302 anaerobic batch culture systems: is it due to synergies in probiotic mixtures or the
303 specificity of single strains?. *Anaerobe* **2013**, *24*, 60-65, doi:
304 10.1016/j.anaerobe.2013.09.011.

305