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Lactobacillus curvatus subsp. *melibiosus* is a later synonym of *Lactobacillus sakei* subsp. *carnosus*

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On the basis of phenotypic and DNA–DNA reassociation studies, strain CCUG 34545^T has been considered to represent a distinct *Lactobacillus curvatus* subspecies, *Lactobacillus curvatus* subsp. *melibiosus*. However, in several independent studies dealing with *Lactobacillus sakei* and *L. curvatus* strains, the subspecies division of *L. curvatus* has been found to be controversial. The original study distinguishing the two subspecies within both *L. curvatus* and *L. sakei* also lacked 16S rRNA gene sequence analyses. Therefore, the taxonomic position of *L. curvatus* subsp. *melibiosus* CCUG 34545^T was re-evaluated in a polyphasic taxonomy study that included 16S rRNA gene sequence analysis, DNA–DNA reassociation, DNA G+C content determination, numerical analysis of ribotypes and whole-cell protein patterns and the examination of some fundamental phenotypic properties. The results obtained indicate that strain CCUG 34545^T and its duplicate, CCUG 41580^T, are *Lactobacillus sakei* subsp. *carnosus* strains and that *L. curvatus* subsp. *melibiosus* is a later synonym of *L. sakei* subsp. *carnosus*.

Determining the taxonomy of *Lactobacillus sakei* and *Lactobacillus curvatus* has been an objective of various studies (Reuter, 1970; Klein *et al.*, 1996; Torriani *et al.*, 1996; Berthier & Ehrlich, 1999; Champomier-Vergès *et al.*, 2002). Early classification relied heavily on phenotypic properties, distinguishing these species mostly by the type of sugar-fermentation pattern and whether ammonia was produced from arginine (Reuter, 1970). Identification of these organisms was hampered not only because of the similar phenotypic reactions possessed by them but apparently also because of the heterogeneity (Berthier & Ehrlich, 1999) within the species. The need for correct identification of *L. curvatus* and *L. sakei* species led to the use of molecular methods. On the basis of phenotypic and genotypic properties, both species were divided into two subspecies in 1996 (Klein *et al.*, 1996; Torriani *et al.*, 1996). In the case of *L. curvatus*, high (81–101 %) DNA–DNA reassociation levels were detected between a group of melibiose-utilizing strains and the melibiose-negative *L. curvatus* type strain (DSM 20019^T), whereas low levels (46–50 %) were detected with the *L. sakei* type strain (DSM 20017^T). Differentiation between the two *L. curvatus* subspecies was further established on the basis of ability to use melibiose and clustering in whole-cell protein and random amplified polymorphic DNA (RAPD)-PCR pattern analyses (Klein *et al.*, 1996; Torriani *et al.*, 1996). The melibiose-utilizing strains were assigned to the subspecies *melibiosus* with CCUG 34545^T as the type strain, whereas *L. curvatus* DSM 20019^T and other melibiose-negative strains were assigned to the subspecies *curvatus*. Subspecies division of *L. sakei* was based mainly on the results from numerical analyses of whole-cell protein and RAPD patterns (Klein *et al.*, 1996; Torriani *et al.*, 1996).

Several studies (Mäkelä *et al.*, 1992; Björkroth & Korkeala, 1996b; Berthier & Ehrlich, 1999; Lyhs *et al.*, 1999, 2002) dealing with DNA-based *L. sakei* and *L. curvatus* identification have shown results contradictory to the subspecies division of Torriani *et al.* (1996). In a study of meat-associated, ropy-slime-producing *L. sakei* strains (Björkroth & Korkeala, 1996b), strain A210 was reported to possess

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Abbreviations: CCUG, Culture Collection of the University of Göteborg; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen; MAP, modified-atmosphere-packaged; RAPD, random amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences obtained in this study are AY204889–AY204898.

Dendrograms and banding patterns associated with *Eco*RI and *Hin*dIII ribotypes and a dendrogram obtained by combining the equally weighted pattern information of both *Eco*RI and *Hin*dIII ribotypes into one numerical analysis are available, together with the complete DNA–DNA reassociation results, as supplementary material in IJSEM Online.

exactly the same *Eco*RI and *Hin*dIII ribotypes as the *Lactobacillus curvatus* subsp. *melibiosus* type strain (Lyhs *et al.*, 1999). This finding was unexpected because strain A210 had shown 84 % DNA–DNA reassociation with the *Lactobacillus sakei* subsp. *sakei* type strain (Mäkelä *et al.*, 1992). Clustering of the *L. curvatus* subsp. *melibiosus* type strain together with the two *L. sakei* subspecies in the numerical RAPD fingerprinting analysis can already be seen in the study of Torriani *et al.* (1996); even the authors used this data for delineating the four subspecies. Controversial clustering results were later reported by Berthier & Ehrlich (1999), who employed RAPD, and in three studies that employed ribotyping (Lyhs *et al.*, 1999, 2002; Susiluoto *et al.*, 2002). Because of these inconsistencies, a duplicate strain of *L. curvatus* subsp. *melibiosus* strain CCUG 34545^{T} was requested by the curator of the Culture Collection of the University of Göteborg (CCUG), Göteborg, Sweden (E. Falsen, personal communication) from the original depositors; this was designated CCUG 41580^{T}.

The inability to repeat the subspecies-level classification within *L. curvatus* and the high degree of similarity between the *L. curvatus* subsp. *melibiosus* type strain and *L. sakei* strains prompted the present study. Our work was designed to resolve the controversy associated with *L. curvatus* subsp. *melibiosus* by means of a polyphasic approach including 16S rRNA gene sequence analysis, DNA–DNA reassociation, DNA G+C content determination, numerical analysis of ribotypes and whole-cell protein patterns and the examination of some fundamental phenotypic properties.

The type strains used in this study were *Lactobacillus curvatus* subsp. *curvatus* DSM 20019^{T} [DSM refers to Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany], *L. curvatus* subsp. *melibiosus* CCUG 34545^{T} and its duplicate, CCUG 41580^{T}, *L. sakei* subsp. *sakei* DSM 20017^{T} and *Lactobacillus sakei* subsp. *carnosus* CCUG 31331^{T}. Seven additional reference strains, used also in the studies in which the subspecies division had been described (Klein *et al.*, 1996; Torriani *et al.*, 1996), were included in the numerical analyses of protein and ribotype patterns to allow comparison between the studies. Five of these were as follows: *L. sakei* strain LMG 7941 (=DSM 20198), isolated from a starter culture; and LMG 17301, LMG 17304, LMG 17305 and LMG 17306 (=CCUG 8045, CCUG 30939, CCUG 32077 and CCUG 32584, respectively), all of which were isolated from human blood. The two *L. curvatus* strains were *L. curvatus* LMG 17299 (=CCUG 31333) and LMG 17303 (=CCUG 31332), both of which were isolated from raw sausages. In addition to the culture-collection strains, six strains originating from modified-atmosphere-packaged (MAP), raw, poultry-meat products were included. These strains were selected on the basis of the dendrogram deduced from *Hin*dIII ribopatterns by Susiluoto *et al.* (2002). Two of the strains (YMRS3a and PSTJA3a) had clustered together with the *L. curvatus* subsp. *curvatus* type strain and four (HNMRS2c, HNSL5a, HNSL5c and ITSL2c) had clustered with the type strains of the two *L. sakei* subspecies and *L. curvatus* subsp. *melibiosus*. All strains were maintained at −70 °C in MRS broth (Difco) and routinely cultured at 30 °C either overnight in MRS broth or for 3 days on MRS agar plates (Oxoid) in an anaerobic CO_2 atmosphere [Anaerogen; 9–13 % CO_2 according to the manufacturer (Oxoid)].

Phenotypic reactions of the six strains originating from MAP sources were determined; the reactions of the four type strains were re-determined. Gram staining of all the strains revealed morphology typical of either *L. curvatus* or *L. sakei* species. The strains were tested for their sugar-fermentation abilities using the API 50 CHL *Lactobacillus* identification system (bioMérieux) according to the manufacturer's instructions. All strains fermented ribose, D-glucose, D-fructose, D-mannose and *N*-acetylglucosamine within 24–48 h. None of the strains fermented any of the sugar alcohols or complex polysaccharides tested. All of the strains were also negative for D-arabinose, D- and L-xylose, methyl β-xyloside, lactose, D-tagatose, L-sorbose, rhamnose, methyl α-D-mannoside, melezitose, D-raffinose, D- and L-fucose, 2-ketogluconate and 5-ketogluconate, D-turanose and D-lyxose. Production of ammonia from arginine was determined by the method of Briggs (1953); production of acetoin from glucose was tested as described by Reuter (1970). Growth at 4, 37 and 45 °C or in the presence of 10 % (w/v) NaCl was tested in MRS broth (Difco) incubated until growth was observed or, alternatively, for at least 21 days. All of the strains grew in MRS broth at 4 and 37 °C but none of them grew at 45 °C. None of the strains grew in MRS broth containing 10 % (w/v) NaCl. Differential carbohydrate patterns and the results of other biochemical and physiological tests are shown in Table 1. All of the reactions of the type strains are in accordance with the results of previous studies (Klein *et al.*, 1996; Berthier & Ehrlich, 1999). *L. curvatus* does not contain melibiose-positive strains, apart from CCUG 34545^{T} and CCUG 41580^{T} (the two subcultures of the *L. curvatus* subsp. *melibiosus* type strain). The type strain CCUG 34545^{T} showed results typical of the majority of *L. sakei* strains, giving positive results for the utilization of arginine and melibiose. The strains originating from MAP broiler-meat products showed results typical of either *L. curvatus* or *L. sakei* species with respect to arginine and melibiose utilization (Table 1). These results are also in harmony with the results from the numerical analyses made by Susiluoto *et al.* (2002) and the other analyses performed in the present study.

The whole-cell protein profiles were determined from the type and reference strains mentioned and five of the MAP strains. All strains were grown for 24 h on MRS agar (Oxoid) at 24 °C in a microaerobic atmosphere (in $O_2/CO_2/N_2$ at approx. 5 : 10 : 85). Preparation of cellular protein extracts and PAGE were performed as described previously (Pot *et al.*, 1994). The densitometric analysis, normalization and interpolation of the scanned (LKB 2202 UltroScan

Table 1. Phenotypic characteristics of strains studied

Strains: 1, *L. sakei* subsp. *sakei* DSM 20017^{T}; 2, *L. sakei* subsp. *carnosus* CCUG 31331^{T}; 3, *L. curvatus* subsp. *curvatus* DSM 20019^{T}; 4, *L. curvatus* subsp. *melibiosus* CCUG 34545^{T}; 5, *L. curvatus* subsp. *melibiosus* HNSL5a; 6, *L. curvatus* subsp. *melibiosus* HNSL5c; 7, *L. curvatus* subsp. *melibiosus* HNMRS2c; 8, *L. curvatus* subsp. *melibiosus* PSTJA3a; 9, *L. curvatus* subsp. *melibiosus* YMRS3a; 10, *L. curvatus* subsp. *melibiosus* ITSL2c. +, Positive test result; −, negative test result; w, weakly positive test result.

Characteristic	1	2	3	4	5	6	7	8	9	10
L-Arabinose	+	+	−	−	−	−	+	−	−	−
Galactose	+	+	+	+	+	+	+	+	−	+
Methyl α-D-glucoside	−	−	−	−	−	−	+	−	+	−
Amygdalin	−	−	w	−	+	w	−	−	+	−
Arbutin	−	−	−	−	+	+	−	−	+	−
Salicin	−	w	−	−	+	+	+	+	+	−
Cellobiose	−	−	−	+	w	w	−	−	−	−
Maltose	+	+	+	−	−	−	−	+	+	−
Melibiose	+	+	−	+	+	+	+	−	−	−
Sucrose	+	+	−	+	+	+	+	+	+	+
Trehalose	−	+	−	+	+	+	+	−	+	+
β-Gentiobiose	−	w	−	+	−	−	−	w	+	−
Gluconate	w	w	−	w	w	w	w	−	−	w
Production of:										
NH_3 from arginine	−	+	−	+	+	+	+	−	−	+
Acetoin from glucose	−	−	−	−	+	−	−	−	−	−

Laser Densitometer; LKB) protein profiles and the numerical analysis were performed using the GelCompar 4.2 software package (Applied Maths). Similarity between all pairs of traces was expressed by using the Pearson product moment correlation coefficient converted, for convenience, to a percentage value. The results from the numerical analysis of the whole-cell protein patterns are shown in Fig. 1. Three distinct clusters could be delineated after numerical analysis and a visual examination of the protein profiles. Cluster I comprises the three *L. curvatus* subsp. *curvatus* reference strains and one of the MAP strains grouping above a similarity level of 84 %. Cluster II comprises the two *L. sakei* subsp. *sakei* reference strains grouping above a similarity level of 86 %. Cluster III comprises the five *L. sakei* subsp. *carnosus* reference strains, both subcultures of the *L. curvatus* subsp. *melibiosus* type strain and four of the MAP strains grouping above a similarity level of 82 %. These results only partially confirmed the results from previous taxonomic studies based on cellular protein electrophoresis (Klein *et al.*, 1996): when the variability among the protein patterns of *L. sakei* subsp. *carnosus* reference strains is considered, the *L. curvatus* subsp. *melibiosus* type strain could not be distinguished from *L. sakei* subsp. *carnosus.* This similarity in whole-cell protein profiles was not reported by Klein *et al.* (1996), although the five *L. sakei* subsp. *carnosus* reference strains and the *L. curvatus* subsp. *melibiosus* type strain were included in both studies. Klein *et al.* (1996), however, decided to publish the crude, native whole-cell protein profiles in a one dendrogram, whereas more sophisticated dendrograms derived from silver diamine and Coomassie brilliant blue-stained polyacrylamide gels were both separated into two figures, one comprising the presumed *L. curvatus* subspecies and the other the *L. sakei* subspecies. Therefore, the high degree of similarity of the patterns of *L. curvatus* subsp. *melibiosus* type strain and the *L. sakei* strains may have been overlooked.

DNA for all DNA-based analyses was isolated by using the guanidium thiocyanate method of Pitcher *et al.* (1989), as modified by Björkroth & Korkeala (1996a). *Hin*dIII and *Eco*RI enzymes were used for restriction endonuclease treatment of DNA as specified by the manufacturer (New England Biolabs) and restriction endonuclease analysis was performed as described previously (Björkroth & Korkeala, 1996a). Southern blotting was performed using a vacuum device (Vacugene; Pharmacia) and the 16 and 23S rRNA gene probe for ribotyping (Grimont & Grimont, 1986) was labelled by reverse transcription [AMV-RT (Promega) and the Dig Labelling Kit (Roche Molecular Biochemicals)] as described previously by Blumberg *et al.* (1991). Membranes were hybridized at 58 °C overnight and the detection of the digoxigenin label was performed as recommended by

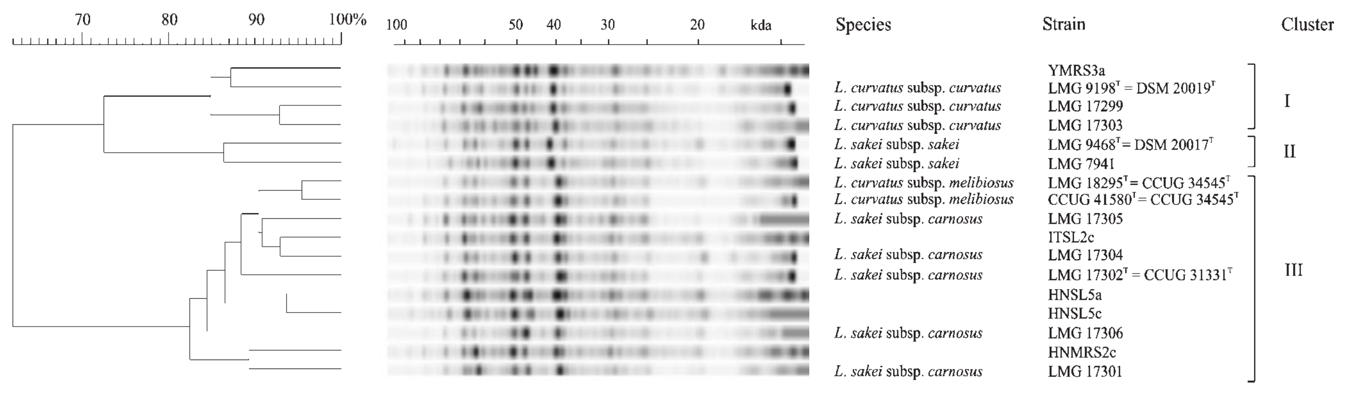

Fig. 1. Dendrogram based on numerical analysis of the whole-cell protein profiles of all strains examined.

Roche Molecular Biochemicals. The *Eco*RI and *Hin*dIII ribopatterns were compared with the corresponding patterns in the previously established Lactic Acid Bacteria Database at the Department of Food and Environmental Hygiene. Scanned (ScanJet 4c/T; Hewlett Packard) ribopatterns were analysed using the BioNumerics 3.0 software package (Applied Maths). The similarity between all pairs was expressed by using Dice coefficient correlation, and UPGMA clustering was used for the construction of the dendrogram. On the basis of the use of internal controls, position tolerance of 1·5 % was allowed for the bands. The dendrograms and banding patterns associated with *Eco*RI and *Hin*dIII ribotypes and a dendrogram obtained by combining the equally weighted pattern information of both *Eco*RI and *Hin*dIII ribotypes into one numerical analysis are available as supplementary material in IJSEM Online. As in the case of numerical analysis of the whole-cell protein patterns, both subcultures of the *L. curvatus* subsp. *melibiosus* type strain clustered clearly together with the *L. sakei* type and reference strains. Moreover, they shared identical ribotypes. The clustering of the *L. curvatus* subsp. *melibiosus* type strain together with *L. sakei* was also reported previously (Lyhs *et al.*, 1999, 2002; Susiluoto *et al.*, 2002). The similarity levels between *L. curvatus* subsp. *melibiosus* type strain CCUG 34545^T and other strains in the *L. sakei* cluster varied from 80 to 85 % in different ribopattern analyses, whereas the values between CCUG 34545^T and the strains in the *L. curvatus* cluster varied from 33 to 72 %.

One salient difference in the dendrograms derived from whole-cell protein profiles and ribotyping profiles was noted. Whereas the former allowed a clear separation between the *L. sakei* subspecies *sakei* and *carnosus* (Fig. 1), confirming data reported by Klein *et al.* (1996), the latter did not (see supplementary material in IJSEM Online).

The nearly complete (at least 1400 bases sequenced) 16S rRNA gene was amplified by using a PCR with a universal primer pair, F19-38 (5′-CTGGCTCAGGAYGAACGCTG-3′) and R1541-1522 (5′-AAGGAGGTGATCCAGCCGCA-3′). Sequencing of the purified (QIAquick PCR purification kit; Qiagen) PCR product was performed by using Sanger's dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) with primers F19-38, R1541-1522, F908-926 (5′-AACTCAAAGGAATTGACGG-3′) and R536-519 (5′-GTATTACCGCGGCTGCTG-3′). Samples were run in a Global IR^2 sequencing device with e-Seq 1.1 software (LiCor) according to the manufacturer's instructions. Overlapping complementary sequences were joined by the Align IR 1.2 program (LiCor). The consensus sequences of strains belonging to the *L. sakei*, *L. curvatus* and *Lactobacillus fuchuensis* (outgroup) species (retrieved from/deposited in the NCBI GenBank, http://www.ncbi.nlm.nih.gov, using BLASTN 2.2.6; Altschul *et al.*, 1997) were aligned and a phylogenetic tree was constructed from the global alignment by the neighbour-joining algorithm using the BioNumerics 3.0 software package (Applied Maths). Bootstrap probability values were calculated from 1000 resampled trees. Fig. 2 shows the distance matrix tree based on 16S rRNA gene sequences and the accession numbers of the 16S rRNA gene sequences used/deposited. Two main branches, possessing bootstrap values of 100 %, separated *L. curvatus* subsp. *curvatus* type and reference strains YMRS3a and PSTJA3a from the *L. sakei* group (*L. curvatus* subsp. *melibiosus* included). The strains branching together with *L. curvatus* subsp. *curvatus* DSM 20019^T shared 16S rRNA gene sequence similarity from 99·5 to 100 %. The other branch, containing the type and reference strains of two *L. sakei* subspecies and *L. curvatus* subsp. *melibiosus* and the meat-originated strains HNMRS2c, HNSL5a, HNSL5c and ITSL2c, possessed 16S rRNA gene sequence similarity of 99·3–100 %. Similarities ranging from 98·2 to 99 % were obtained between the strains in the *L. sakei* and *L. curvatus* branches. The 16S rRNA gene sequence similarity levels between *L. fuchuensis* JCM 11249^T and the *L. curvatus*/*sakei* strains varied from 96·6 to 97·2 %.

The DNA G+C content (mol%) was estimated using LightCycler (Roche Molecular Diagnostics) and Formula A

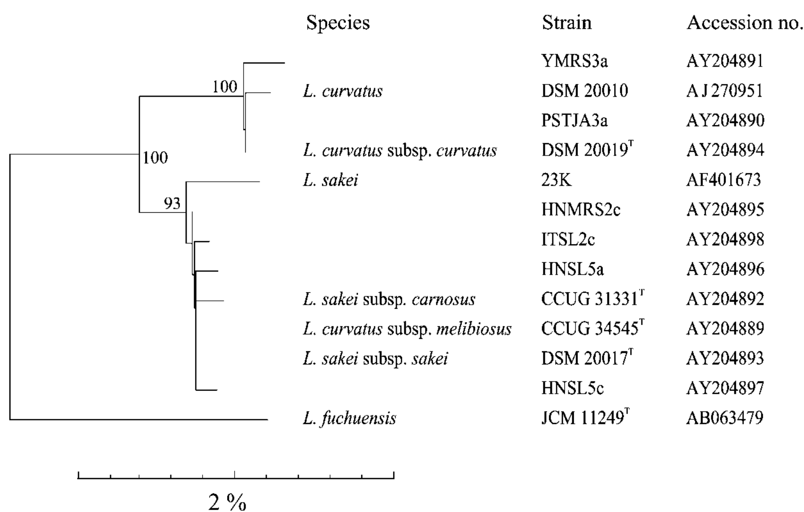

Fig. 2. Phylogenetic tree based on similarities of almost-complete 16S rRNA gene sequences (at least 1400 bases). Bootstrap probability values from 1000 resampled trees are given at the branch points.

as described by Xu *et al.* (2000). The reassociation values were determined spectrophotometrically (Gilford Response spectrophotometer; Giba Corning Diagnostics) from renaturation rates according to De Ley *et al.* (1970). The G+C content of all strains varied around the value 42·5±0·3 mol%, but strain YMRS3a gave a value of 40·5 mol%. These values are in agreement with the previously described values, generally ranging from 42 to 44 mol% (Hammes & Vogel, 1995). The complete DNA–DNA reassociation results are available as supplementary material in IJSEM Online. The DNA of *L. curvatus* subsp. *melibiosus* CCUG 34545^T hybridized with the DNA of *L. sakei* subsp. *sakei* DSM 20017^T and *L. sakei* subsp. *carnosus* CCUG 31331^T at a level of 82 and 87 %, respectively, whereas the hybridization level with *L. curvatus* subsp. *curvatus* DSM 20019^T was as low as 30 %. The corresponding values with *L. curvatus* subsp. *melibiosus* CCUG 41580^T (duplicate of strain CCUG 34545^T) were 87, 88 and 39 %, respectively. These values are not in agreement with earlier studies of Klein *et al.* (1996). However, the reassociation values obtained for *L. curvatus* subsp. *melibiosus* CCUG 34545^T and its duplicate, CCUG 41580^T, show that both strains belong to *L. sakei* species. These values also are in harmony with the findings obtained in all other analyses performed in this study. Our reassociation results confirm that *L. sakei* is a heterogeneous species, as stated by Berthier & Ehrlich (1999). Subspecies division of *L. sakei* was not clearly seen within the reassociation values.

The classification of strain CCUG 34545^T in *L. curvatus* subgroup II (Klein *et al.*, 1996) and later into a separate subspecies, *melibiosus* (Torriani *et al.*, 1996), was based on DNA–DNA hybridization results, protein and RAPD fingerprints and the ability to ferment melibiose. In the present report, DNA–DNA hybridization values unambiguously indicate that strain CCUG 34545^T and its duplicate, CCUG 41580^T, both belong to *L. sakei*. This species-level conclusion was supported by the numerical analyses of protein and RFLP patterns and also by 16S rRNA gene sequence analysis. According to our study, only the analyses of *Eco*RI and *Hin*dIII ribotypes and 16S rRNA genes cannot be used for the subspecies-level identification of *L. sakei*. Of the original criteria (Klein *et al.*, 1996; Torriani *et al.*, 1996) used for distinguishing the subspecies *melibiosus*, only the ability to ferment melibiose is not useful, since it does not subdivide the strains within *L. sakei* species. According to the present study and the study of Klein *et al.* (1996), protein fingerprints clearly divide *L. sakei* into the two subspecies, *sakei* and *carnosus* (includes *L. curvatus* subsp. *melibiosus* strains in the present study). When Torriani *et al.* (1996) compared the RAPD profiles of *L. curvatus* and *L. sakei*, the *L. curvatus* subsp. *melibiosus* strains clustered also with (but not among) the *L. sakei* subsp. *carnosus* strains. On the basis of their reassociation data, the authors considered that this subcluster represents *L. curvatus* subsp. *melibiosus* even though the fingerprints showed greater similarity to the fingerprints of the two *L. sakei* subspecies.

Of all the previously published data of Klein *et al.* (1996) and Torriani *et al.* (1996), only the DNA–DNA hybridization values show a clear discrepancy with our conclusion. Our study demonstrates that *L. curvatus* subsp. *melibiosus* is a later synonym of *L. sakei* subsp. *carnosus* and, as a consequence, the subspecies division within *L. curvatus* should be abandoned.

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