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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 34545T 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 34545T 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 34545T and its duplicate, CCUG 41580T, 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 20019T), whereas low levels (46–50%) were detected with the L. sakei type strain (DSM 20017T). 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 34545T as the type strain, whereas L. curvatus DSM 20019T 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 & Korceala, 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 & Korceala, 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 EcoRI and HindIII ribotypes and a dendrogram obtained by combining the equally weighted pattern information of both EcoRI and HindIII ribotypes into one numerical analysis are available, together with the complete DNA–DNA reassociation results, as supplementary material in IJSEM Online.
CCUG 34545T was requested by the curator of the Culture Collection of the University of Göteborg (CCUG), Göteborg, Sweden (E. Falsen, personal communication) from the original depositories; this was designated CCUG 41580T.

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 20019T [DSM refers to Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany], L. curvatus subsp. melibiosus CCUG 34545T and its duplicate, CCUG 41580T, L. sakei subsp. sakei DSM 20017T and Lactobacillus sakei subsp. carnosus CCUG 31331T. 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 HindIII 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 (HNMR52c, 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 CO₂ atmosphere [Anaerogen; 9–13 % CO₂ 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-xylene, methyl β-xylode, lactose, D-tagatose, L-sorbose, rhamnose, methyl α-D-mannoside, melezitose, D-raffinose 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 melibioso-positive strains, apart from CCUG 34545T and CCUG 41580T (the two subcultures of the L. curvatus subsp. melibiosus type strain). The type strain CCUG 34545T 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 O₂/CO₂/N₂ 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 Ultradensitometry System).
profiles. Cluster I comprises the three numerical analysis and a visual examination of the protein
Fig. 1. Three distinct clusters could be delineated after analysis of the whole-cell protein patterns are shown in to a percentage value. The results from 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
Laser Densitometer; LKB) protein profiles and the numerical
L. sakei 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). HindIII and EcoRI 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 (Grömberg & Grömberg, 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

Table 1. Phenotypic characteristics of strains studied

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L. curvatus subsp. melibiosus is L. sakei subsp. carnosus

Fig. 1. Dendrogram based on numerical analysis of the whole-cell protein profiles of all strains examined.
Roche Molecular Biochemicals. The EcoRI and HindIII 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 EcoRI and HindIII ribotypes and a dendrogram obtained by combining the equally weighted pattern information of both EcoRI and HindIII 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 34545T and other strains in the L. sakei cluster varied from 80 to 85% in different ribopattern analyses, whereas the values between CCUG 34545T 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’-GTATTACCAGCGGCTGCTG-3’). Samples were run in a Global IR² 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 20019T 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 HNMR52c, HNSL5a, HNSL5c and ITS1L2c, possessed 16S rRNA gene sequence similarity of 99.3 to 100%. Similarities ranging from 98.2 to 99.9% were obtained between the strains in the L. sakei and L. curvatus branches. The 16S rRNA gene sequence similarity levels between L. fuchuensis JCM 11249T 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
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 34545T hybridized with the DNA of L. sakei subsp. sakei DSM 20017T and L. sakei subsp. carnosus CCUG 31331T at a level of 82 and 87%, respectively, whereas the hybridization level with L. curvatus subsp. curvatus DSM 20019T was as low as 30%. The corresponding values with L. curvatus subsp. melibiosus CCUG 41580T (duplicate of strain CCUG 34545T) 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 34545T and its duplicate, CCUG 41580T, 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 34545T 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 34545T and its duplicate, CCUG 41580T, 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 EcoRI and HindIll 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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References


