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

Joanna Koort,1 Peter Vandamme,2 Ulrich Schillinger,3 Wilhelm Holzapfel2 and Johanna Björkroth1

1Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine, University of Helsinki, 00014 Helsinki, Finland

2Laboratory of Microbiology, University of Ghent, 9000 Ghent, Belgium

3Institute for Hygiene and Toxicology, Federal Research Centre for Nutrition, 76131 Karlsruhe, Germany

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 carnosus. 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...
exactly the same EcoRI and HindIII 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 (Makela 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 34545T was requested by the curator of the Culture Collection (CCUG), Göteborg, Sweden (E. Falsen, personal communication) from the original depositors; 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 PSTJ3a) had clustered together with the *L. curvatus* subsp. *curvatus* type strain and four (HNMS2c, 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-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 and CCUG 41580T (the two subcultures of the *L. curvatus* subsp. *melibiosus* type strain). The type strain CCUG 34545 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 UltraScan
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’-CTGGCTCAGGAYGACGCTG-3’) and R1541-1522 (5’-AAGGAGGTGATCCAGCGCA-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’-AATCTAAAGGAATTGACGG-3’) and R536-519 (5’-GTATTACCGCGGCTGCTG-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 YMR35 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, HNS5a, HNS15c 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 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

![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.](image-url)
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 *EcoRI* and *HindIII* 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**


