Lactobacillus sobrius Konstantinov *et al.* 2006 is a later synonym of *Lactobacillus amylovorus* Nakamura 1981

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While studying the taxonomy of six lactic acid bacterium isolates from Finnish porcine intestine and faeces, the taxonomic positions of *Lactobacillus sobrius* type strain DSM 16698^T and strain AD5 based on comparative 16S rRNA sequence analysis were found to be controversial, as they showed high similarity to *Lactobacillus amylovorus* strains. Therefore, the taxonomy of these species was addressed in a polyphasic taxonomy study that included, in addition to re-evaluating the 16S rRNA gene sequence and DNA–DNA reassociation results, multilocus sequence analysis (MLSA) of the housekeeping genes encoding the phenylalanyl-tRNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*) as well as numerical analysis of *Hind*III and *Eco*RI ribotypes. 16S rRNA gene sequence analysis demonstrated a very high similarity between the *L. sobrius* and *L. amylovorus* type and reference strains and representative Finnish porcine isolates (99.6–99.9%). The MLSA data showed the close phylogenetic relationship of these strains; *pheS* and *rpoA* gene sequence similarities were 98.5–100% and 99.6–99.8%, respectively. Numerical analyses of *Hind*III/*Eco*RI ribotypes placed these strains in a single cluster by both enzymes. Finally, the DNA–DNA reassociation experiments revealed high reassociation levels (higher than 79%) between the strains. These results indicate that DSM 16698^T, AD5 and the related porcine lactobacilli strains from Finland constitute a single species, *Lactobacillus amylovorus*, and that the name *Lactobacillus sobrius* should be considered as a later synonym of *Lactobacillus amylovorus*.

Twelve starch-hydrolysing strains isolated from cattle waste-corn fermentations in the USA were included in a study by Nakamura (1981), resulting in the description of *Lactobacillus amylovorus*. These strains were Gram-positive, non-spore-forming, facultatively anaerobic, catalase-negative and produced D- and L-lactic acid homofermentatively. There was no growth at 15 °C, but the bacterium grew at 45 °C. The DNA G + C content was reported as 40.4 mol% in the original description (Nakamura, 1981). Based on

DNA–DNA hybridization studies, the large *Lactobacillus acidophilus* group is divided into six groups, A1–A4 and B1–B2, that correspond to the previously assigned species *L. acidophilus* (A1), *Lactobacillus crispatus* (A2), *L. amylovorus* (A3), *Lactobacillus gallinarum* (A4), *Lactobacillus gasseri* (B1) and *Lactobacillus johnsonii* (B2) (Johnson *et al.*, 1980; Lauer *et al.*, 1980; Fujisawa *et al.*, 1992). Although these genome clusters have been designated separate species, they are difficult to distinguish solely on the basis of phenotypic characteristics. Recent *Eco*RI ribotyping data further suggest that the group should be divided into 14 genotypes, A1–A11 and B1–B3, and gives evidence that some of the *Lactobacillus* strains identified previously would require reclassification in different species (Ryu *et al.*, 2001). In 2006, *Lactobacillus sobrius* was described by Konstantinov *et al.* (2006) in a study dealing with six strains isolated from piglet intestine and faeces. The classification of *L. sobrius* DSM 16698^T (Konstantinov *et al.*, 2006) was based on DNA–DNA

Abbreviations: FOS, fructo-oligosaccharides; MLSA, multilocus sequence analysis.

The GenBank/EMBL/DDBJ accession numbers for the *pheS* and *rpoA* gene sequences of strains DSM 20531^T, DSM 20532, DSM 16698^T, AD5, LAB7, LAB8, LAB13 and LAB52 are EU000474–EU000489, respectively.

Supplementary phylogenetic trees and a table showing DNA–DNA relatedness among *Lactobacillus* strains are available with the online version of this paper.

hybridization values, the ability to ferment raffinose and fructo-oligosaccharides (FOS) and the ability to grow at 45 °C. Cell morphology, growth temperature and fermentation abilities seem to be very similar to those of the species *L. amylovorus*. Based on 16S rRNA gene sequence similarity, *Lactobacillus kitasatonis* (99 %), *L. crispatus* (98 %) and *L. amylovorus* (97 %) were the nearest relatives of the novel species, but their DNA–DNA relatedness was found to be lower than 49 %. However, the 16S rRNA gene sequence published in the study distinguishing the two species *L. sobrius* and *L. amylovorus* was later revised. On 25 September 2006, the 16S rRNA sequence of *L. sobrius* was updated by the submitter. This revised sequence possesses much higher similarity (99.9 %) to the corresponding sequence of the *L. amylovorus* type strain.

The high degree of similarity based on our preliminary results between *L. sobrius*, *L. amylovorus* and *L. sobrius/amylovorus*-like strains (Jakava-Viljanen & Palva, 2007) prompted the present study. Our work was designed to resolve the controversy associated with *L. sobrius* by means of a polyphasic approach including phylogenetic analyses of 16S rRNA and two other housekeeping genes (*pheS* and *rpoA*), numerical analysis of ribotypes and DNA–DNA reassociation experiments.

Three strains of *L. amylovorus*, DSM 20531^T, DSM 20532 and DSM 20552, two strains of *L. sobrius*, DSM 16698^T and AD5 (supplied by S. Konstantinov, Wageningen University, The Netherlands), and six *L. amylovorus/sobrius*-like strains, LAB2, LAB7, LAB8, LAB13, LAB16 and LAB52 (Jakava-Viljanen & Palva, 2007), were selected for the present study. All strains were cultivated and maintained on MRS medium (Oxoid) and incubated anaerobically at 37 °C, unless otherwise indicated. DNA from *Lactobacillus* strains was extracted for all analyses by the guanidine thiocyanate method of Pitcher *et al.* (1989) with the modifications described by Björkroth & Korkeala (1996).

The similarity between the six Finnish LAB isolates, *L. amylovorus* DSM 20531^T and *L. sobrius* DSM 16698^T was investigated by comparing the available 16S rRNA gene sequences. The latter sequences and sequences from *L. sobrius/amylovorus*-like strains (GenBank accession nos EF120368–EF120371; Jakava-Viljanen & Palva, 2007) and those of related species were retrieved and aligned. A tree was constructed from the global alignment by the neighbour-joining algorithm using the BioNumerics 4.6 software package (Applied Maths). Comparison of the sequences of the type strains of *L. amylovorus* and *L. sobrius* and *L. sobrius/amylovorus*-like strains revealed 99.6–99.9 % 16S rRNA gene sequence similarity. Supplementary Fig. S1, available in IJSEM Online, presents a neighbour-joining tree deduced using the latest sequences available.

Recently, the application of multilocus sequence analysis (MLSA) using the housekeeping genes that code for the phenylalanyl-tRNA synthase alpha subunit (*pheS*) and RNA polymerase alpha subunit (*rpoA*) has been developed

for the identification of *Lactobacillus* species (Naser *et al.*, 2006). The combined use of *pheS* and *rpoA* gene sequences offered a reliable identification system for nearly all *Lactobacillus* species (Naser, 2006). These housekeeping gene sequences were determined for LAB7, LAB8, LAB13, LAB52, DSM 20531^T, DSM 20532, DSM 16698^T and AD5. Amplification and sequencing reactions were performed as described by Naser *et al.* (2005). Fragments of 390 and 513 bp were used for aligning the *pheS* and *rpoA* genes sequences, respectively. Neighbour-joining and maximum-parsimony trees were constructed using the BioNumerics 4.6 software package (Applied Maths) (Fig. 1 and Supplementary Figs S2–S4). To create the maximum-parsimony tree, positions with gaps were ignored and cost settings of 100 % for each possible conversion were applied. The number of bootstrap simulations for both tree types was 500. Regardless of the tree type and the gene studied, all the strains were located in the same branch, but distinct from the other related *Lactobacillus* sequences retrieved from GenBank.

Ribotyping was determined for LAB2, LAB7, LAB8, LAB13, LAB16, LAB52, DSM 20531^T, DSM 20552, DSM 16698^T and AD5. *Hind*III and *Eco*RI ribotyping procedures were performed as described previously (Björkroth & Korkeala, 1996). Numerical analysis of the scanned (Hewlett Packard ScanJet 4c/T) ribopatterns was performed using the BioNumerics 4.6 software package (Applied Maths). The similarity between all pairs was expressed by Dice coefficient correlation, and the unweighted pair-group method with arithmetic averages (UPGMA) was used for the construction of the dendrogram. Based on internal controls, 1.6 % position tolerance and 0.6 % optimization were allowed for the bands/patterns. The ribopatterns were compared with the corresponding patterns in the LAB database and identification of the isolates was made on the basis of the locations of the type strains in the clusters, taking into account pattern similarity levels detected in the previous studies utilizing this database. The *L. sobrius* type strain DSM 16698^T and strain AD5 and *L. sobrius/amylovorus*-like strains clustered clearly together with the *L. amylovorus* type and reference strains (Supplementary Fig. S5). Moreover, they shared identical ribotypes. The similarity levels between *L. sobrius* DSM 16698^T, *L. amylovorus* DSM 20531^T and other strains varied from 60.0 to 100.0% and 68.7% to 100% in *Eco*RI and *Hind*III ribopattern analyses, respectively.

DNA–DNA reassociation was determined for LAB7, LAB8, LAB13, DSM 20531^T, DSM 20552, DSM 16698^T and AD5. For the DNA–DNA reassociation experiments, the DNA was purified and fragmented according to Huß *et al.* (1983). In this procedure, DNA was dialysed using Cellu•Sep cellular tubular membranes with a 12 000–14 000 Da pore size (Orange Scientific). The first dialysis was against 1 × SSC (0.15 M sodium chloride and 0.015 M sodium citrate; Amresco) containing 10 mM EDTA and the second against 1 × SSC. The DNA was fragmented three times in a mini French pressure cell (Spectronic

Fig. 1. Maximum-parsimony tree based on the *rpoA* gene sequences of strains of the *Lactobacillus acidophilus* group. Bootstrap percentages after 500 simulations are shown. *Lactobacillus sakei* subsp. *carnosus* LMG 17302^T was included as an outgroup.

Unicam) at 900 p.s.i. (approx. 6210 kPa) with a French pressure cell press (SLM-Aminco Spectronic). The fragmented DNA solution was dialysed again against $2 \times$ SSC. Each dialysis was done overnight at 5 °C. The quantity and purity of DNA solutions were determined with an Eppendorf BioPhotometer spectrophotometer from absorbance at 230, 260 and 280 nm. Finally, reassociation measurements were done in 20 µl $2 \times$ SSC containing 30 % formamide. The sample solutions contained DNA of strain A, the same amount of DNA of strain B or a mixture of equal amounts of DNA of A and B ($A/2 + B/2$). The concentration of DNA in the solutions was 60 ng µl⁻¹. A fluorescent dye, SYBR green I (Roche Diagnostics), was used to stain double-stranded DNA. A 10^4 final dilution was used for the dye. Three replicates of each sample were made. Fluorescence levels were measured with the LightCycler instrument (Roche Diagnostics) and the LightCycler software was used to visualize the renaturation curves. In the LightCycler protocol, DNA was first denatured at 95 °C for 10 min. The solution was then cooled to the renaturation temperature of 47 °C in a few seconds. The fluorescence intensity was measured continuously over 30 min and the fluorescence data were plotted against time. The renaturation temperature was calculated using the following formulae by Gillis *et al.* (1970) and Hutton (1977). Formamide has been shown to decrease the melting temperature and thus the renaturation temperature by 0.60 °C per % formamide (Hutton, 1977). To calculate the renaturation temperature the formula $T = (0.51 \times GC\% + 47) - 0.60 \times \text{formamide}$ was used. Renaturation rates were calculated from the renaturation curves as increase in fluorescence/time ($\Delta F/\Delta t$).

Reassociation values were calculated according to the formula of De Ley *et al.* (1970) as $100 \times (4V'_M - V'_A - V'_B) / (2(V'_A \times V'_B))$, where V'_M is the renaturation rate of the DNA mixture of two strains and V'_A and V'_B the renaturation rates of DNA from individual strains. Hybridizations were performed between *L. sobrius*, *L. amylovorus* and *L. amylovorus/sobrius*-like strains. All strains studied showed high DNA–DNA relatedness values, in the range of 79 to 121 %, indicating clearly that the two taxa represent the same species. A DNA–DNA hybridization value of 85 % was found between the type strains of the two species (Supplementary Table S1). Previous data by Konstantinov *et al.* (2006) reported a DNA–DNA relatedness value for *L. sobrius* DSM 16698^T and *L. amylovorus* DSM 20531^T of 49 %, which is much lower than the value determined in our study. The DNA–DNA hybridization experiments confirmed the results of our other investigation methods (16S rRNA and housekeeping gene sequencing and ribotyping).

In the present report, DNA–DNA hybridization values indicate unambiguously that strain DSM 16698^T and related *L. sobrius/amylovorus*-like strains belong to *L. amylovorus*. This species-level conclusion was supported by numerical analysis of ribotypes, sequence comparison of two housekeeping genes (*pheS* and *rpoA*) and also by 16S rRNA gene sequence analysis. On the basis of these results, it is proposed that the two species, *Lactobacillus sobrius* and *Lactobacillus amylovorus*, be united under the same name; according to the rules of priority (Rules 38 and 42 of the Bacteriological Code; Lapage *et al.*, 1992), the name *Lactobacillus amylovorus* should be retained, strains of

Lactobacillus sobrius should be reclassified as such and the name *Lactobacillus sobrius* should be considered a later heterotypic synonym.

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References

- Björkroth, K. J. & Korkeala, H. J. (1996). Evaluation of *Lactobacillus sakei* contamination in vacuum-packaged sliced cooked meat products by ribotyping. *J Food Prot* **59**, 398–401.
- De Ley, J., Cattoir, H. & Reynaerts, A. (1970). Quantitative measurement of DNA hybridization from renaturation rates. *Eur J Biochem* **12**, 133–142.
- Fujisawa, T., Benno, Y., Yaeshima, T. & Mitsuoka, T. (1992). Taxonomic study of the *Lactobacillus acidophilus* group, with recognition of *Lactobacillus gallinarum* sp. nov. and *Lactobacillus johnsonii* sp. nov. and synonymy of *Lactobacillus acidophilus* group A3 (Johnson et al. 1980) with the type strain of *Lactobacillus amylovorus* (Nakamura 1981). *Int J Syst Bacteriol* **42**, 487–491.
- Gillis, M., De Ley, J. & De Cleene, M. (1970). The determination of molecular weight of bacterial genome DNA from renaturation rates. *Eur J Biochem* **12**, 143–153.
- Huß, V. A. R., Festl, H. & Schleifer, K. H. (1983). Studies on the spectrophotometric determination of DNA hybridization from renaturation rates. *Syst Appl Microbiol* **4**, 184–192.
- Hutton, J. R. (1977). Renaturation kinetics and thermal stability of DNA in aqueous solutions of formamide and urea. *Nucleic Acids Res* **4**, 3537–3555.
- Jakava-Viljanen, M. & Palva, A. (2007). Characterization of *Lactobacillus* species carrying surface (S) layer protein isolated from porcine intestine and faeces and their adhesion to different host tissues. *Vet Microbiol* **124**, 264–273.
- Johnson, J. L., Phelps, C. F., Cummins, C. S., London, J. & Gasser, F. (1980). Taxonomy of the *Lactobacillus acidophilus* group. *Int J Syst Bacteriol* **30**, 53–68.
- Konstantinov, S. R., Poznanski, E., Fuentes, S., Akkermans, A. D., Smidt, H. & de Vos, W. M. (2006). *Lactobacillus sobrius* sp. nov., abundant in the intestine of weaning piglets. *Int J Syst Evol Microbiol* **56**, 29–32.
- Lapage, S. P., Sneath, P. H. A., Lessel, E. F., Skerman, V. B. D., Seelinger, H. P. R. & Clark, W. A. (editors) (1992). *International Code of Nomenclature of Bacteria (1990 Revision). Bacteriological Code*. Washington, DC: American Society for Microbiology.
- Lauer, E., Helming, C. & Kandler, O. (1980). Heterogeneity of the species *Lactobacillus acidophilus* (Moro) Hansen and Mocquot as revealed by biochemical characteristics and DNA-DNA hybridization. *Zentralbl Bakteriol Mikrobiol Hyg 1 Abt Orig C* **1**, 150–168.
- Nakamura, L. K. (1981). *Lactobacillus amylovorus*, a new starch-hydrolyzing species from cattle waste-corn fermentations. *Int J Syst Bacteriol* **31**, 56–63.
- Naser, S. M. (2006). *A novel robust identification system of the lactic acid bacteria Enterococcus, Lactobacillus and Streptococcus based on pheS, rpoA and atpA multilocus sequence analysis*. PhD thesis, Ghent University, Belgium.
- Naser, S. M., Thompson, F. L., Hoste, B., Gevers, D., Dawyndt, P., Vancanneyt, M. & Swings, J. (2005). Application of multilocus sequence analysis (MLSA) for rapid identification of *Enterococcus* species based on *rpoA* and *pheS* genes. *Microbiology* **151**, 2141–2150.
- Naser, S. M., Vancanneyt, M., Snauwaert, C., Vrancken, G., Hoste, B., De Vuyst, L. & Swings, J. (2006). Reclassification of *Lactobacillus amylophilus* LMG 11400 and NRRL B-4435 as *Lactobacillus amylophilus* sp. nov. *Int J Syst Evol Microbiol* **56**, 2523–2527.
- Pitcher, D. G., Saunders, N. A. & Owen, R. J. (1989). Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* **8**, 151–156.
- Ryu, C. S., Czajka, J. W., Sakamoto, M. & Benno, Y. (2001). Characterization of the *Lactobacillus casei* group and the *Lactobacillus acidophilus* group by automated ribotyping. *Microbiol Immunol* **45**, 271–275.