Polyphasic taxonomic analysis of *Bifidobacterium animalis* and *Bifidobacterium lactis* reveals relatedness at the subspecies level: reclassification of *Bifidobacterium animalis* as *Bifidobacterium animalis* subsp. *animalis* subsp. nov. and *Bifidobacterium lactis* as *Bifidobacterium animalis* subsp. *lactis* subsp. nov.

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The taxonomic standing of *Bifidobacterium lactis* and *Bifidobacterium animalis* was investigated using a polyphasic approach. Sixteen representatives of both taxa were found to be phenotypically similar and shared more than 70 % DNA–DNA relatedness (76–100 %), which reinforces the conclusions of previous studies in which *B. lactis* and *B. animalis* were considered to be one single species. However, the results of protein profiling, BOX-PCR fingerprinting, Fluorescent Amplified Fragment Length Polymorphism (FAFLP), and *atpD* and *groEL* gene sequence analysis demonstrate that representatives of *B. animalis* and *B. lactis* constitute two clearly separated subgroups; this subdivision was also phenotypically supported based on the ability to grow in milk. Given the fact that *B. lactis* Meile *et al.* 1997 has to be considered as a junior synonym of *B. animalis* (Mitsuoka 1969) Scardovi and Trovatelli 1974, our data indicate that the latter species should be split into two new subspecies, i.e. *Bifidobacterium animalis* subsp. *animalis* subsp. nov. (type strain R101-8^T = LMG 10508^T = ATCC 25527^T = DSM 20104^T = JCM 1190^T) and *Bifidobacterium animalis* subsp. *lactis* subsp. nov. (type strain UR1^T = LMG 18314^T = DSM 10140^T = JCM 10602^T).

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Abbreviations: ERIC, Enterobacterial Repetitive Intergenic Consensus; FAFLP, Fluorescent Amplified Fragment Length Polymorphism; rep, repetitive DNA element.

The GenBank/EMBL/DDBJ accession numbers for the *atpD* and *groEL* gene sequences determined in this study are given in Fig. 3.

Tables of additional descriptive data of the strains used in this study, and DNA base compositions and levels of DNA relatedness of *B. lactis* and *B. animalis* are available as supplementary material in IJSEM Online.

The taxonomic standing of the species *Bifidobacterium lactis* has been much debated since its description by Meile *et al.* (1997), and several studies have investigated its affiliation with the closely related but earlier described *Bifidobacterium animalis* (Scardovi & Trovatelli, 1974). Based on phenotypic characteristics, 16S rDNA sequence analysis and DNA–DNA hybridization, Cai *et al.* (2000) proposed that *B. lactis* should be considered as a junior synonym of *B. animalis*. However, new genotypic evidence, recently reported by Ventura & Zink (2002, 2003) and Zhu *et al.* (2003), suggested that *B. lactis* and *B. animalis* should still be considered to be two separate taxonomic entities, not at the species level but at the subspecies level.

Compared to *B. animalis*, strains of *B. lactis* exhibit elevated

oxygen tolerance, which is a remarkable trait within the bifidobacteria that allows them to reach high numbers in commercial products under non-anaerobic conditions. Because of this, *B. lactis* strains are frequently applied in probiotic dairy products, food supplements and pharmaceutical preparations (Prasad *et al.*, 1998). To guarantee the quality and the correct labelling of such products, it is thus very important that the taxonomic position of this industrially applied micro-organism is clear.

The aim of this polyphasic study was to investigate the taxonomic relationship between *B. animalis* and *B. lactis* on the basis of DNA–DNA hybridization, mol% G+C determination, sugar fermentation patterns, the ability to grow in milk, protein profiling, BOX-PCR and Fluorescent Amplified Fragment Length Polymorphism (FAFLP) fingerprinting and *atpD* and *groEL* gene sequence analysis.

The 16 *Bifidobacterium* strains used in this study, namely *B. animalis* LMG 10508^T, LMG 18900 and NCC 273, and *B. lactis* LMG 18314^T, LMG 11615, LMG 18906, LMG 11580, NCC 239, NCC 282, NCC 311, NCC 330, NCC 362 (=Bb12, Chr. Hansen, Denmark), NCC 363, NCC 383, NCC 387 and NCC 402 were obtained from the BCCM/LMG Bacteria Collection, Ghent University, Belgium (http://www.belspo.be/bccm/lmg.htm) or from the Nestlé Culture Collection (NCC), Nestlé Research Centre, Lausanne, Switzerland (additional descriptive data are available as supplementary Table 1 in IJSEM Online). All strains were grown overnight at 37 °C under anaerobic conditions (80 % N_2, 10 % H_2, 10 % CO_2) on modified Columbia agar comprising 23 g special peptone (Oxoid), 1 g soluble starch, 5 g NaCl, 0·3 g cysteine-HCl.H_2O (Sigma), 5 g glucose and 15 g agar dissolved in 1 l distilled water (BCCM/LMG, Medium 144).

Strains were phenotypically characterized using the AN MicroPlate system (Biolog) according to the instructions of the manufacturer. Cells were subcultured twice on modified Columbia agar, after which the MicroPlates were incubated under a hydrogen-free anaerobic atmosphere (100 % CO_2) for 24 h. The MicroPlates were spectrophotometrically read using the Biolog Micro Station-reader. The ability to ferment starch was tested separately by inoculation of the strains on modified Columbia agar depleted of the usual carbon sources and subsequently supplemented with an equal amount (w/v) of soluble starch. After incubation under anaerobic conditions at 37 °C for 72 h, Lugol's solution (0·5 % I_2 + 1 % KI in distilled water) was poured on the growth zone and visually checked for a hydrolysis halo.

High-molecular-mass DNA for DNA–DNA hybridizations and mol% G+C determination was prepared using a combination of the protocols of Marmur (1961) and Pitcher *et al.* (1989), as described by Goris *et al.* (1998). DNA base compositions were determined by the method of Mesbah *et al.* (1989). DNA was enzymically digested into deoxyribonucleosides and separated by HPLC using a Waters Symmetry Shield C8 column thermostatted at 37 °C. The solvent used was 0·02 M $NH_4H_2PO_4$, pH 4·0, with 1·5 % acetonitrile. Unmethylated λ phage DNA (Sigma) was used as the calibration reference. DNA–DNA hybridizations were performed with biotin-labelled probes in microplate wells (Ezaki *et al.*, 1989), using a HTS7000 Bio Assay Reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 45 °C in the presence of 50 % formamide. Reciprocal experiments were performed for every pair of strains.

SDS-PAGE analysis of whole-cell proteins, using standardized conditions for comparison with the laboratory-based protein-pattern database, was performed according to the methods of Pot *et al.* (1994).

Micro-scale DNA extraction was based on the method of Pitcher *et al.* (1989) with slight modifications as described previously (Masco *et al.*, 2003). Micro-scale DNA extracts were used for BOX-PCR and FAFLP fingerprinting. Repetitive DNA element (rep-) PCR fingerprinting using the BOXA1R primer was carried out as described previously (Masco *et al.*, 2003). FAFLP template preparation was carried out essentially as described by Thompson *et al.* (2001) with slight modifications. High-molecular-mass DNA was digested with *Taq*I (Westburg) and *Eco*RI (Amersham Pharmacia Biotech). For the pre-selective PCR the E00 primer (5′-GACTGCGTACCAATTC-3′, 1 μM) and T00 primer (5′-CGATGAGTCCTGACCGA-3′, 5 μM) (Sigma Genosys) were used. The initial denaturation step was performed at 94 °C. In the selective PCR, the E01-6FAM primer (5′-6FAM-GACTGCGTACCAATTCA-3′, 1 μM) and T01 primer (5′-CGATGAGTCCTGACC-GAA-3′, 5 μM) (Sigma Genosys) were used. The selective PCR products were separated on a denaturing polyacrylamide gel (10·6 %, v/v, acrylamide; 36 %, w/v, urea; 1 %, w/v, resin; and 10 %, v/v, 1× TBE in HPLC water) in 1× TBE buffer. Numerical analysis was performed with BioNumerics V2.5 software (Applied Maths).

For sequencing of the *atpD* and *groEL* genes, DNA was prepared as described previously (Ventura *et al.*, 2001). A 1133 bp fragment of *atpD* and a 1158 bp fragment of *groEL* were amplified using oligonucleotide primers atp-1 (5′-CACCCTCGAGGTCGAAC-3′, position 180 of *Bifidobacterium longum* NCC 2705) and atp-2 (5′-CTGCATCTT-GTGCCACTTC-3′, position 1313 of *B. longum* NCC 2705), and gro-1 (5′-GACCATCACCAACGATG-3′, position 138 of *B. longum* NCC 2705) and gro-2 (5′-GCTCCGGC-TTGTTGGC-3′, position 1296 of *B. longum* NCC 2705), respectively. Each PCR mixture (50 μl) contained 20 mM Tris/HCl, 50 mM KCl, 200 μM each dNTP, 50 pmol each primer, 1·5 mM $MgCl_2$ and 1 U *Taq* DNA polymerase (Gibco-BRL). The PCR cycling profile consisted of an initial denaturation step of 3 min at 95 °C, followed by amplification for 30 cycles as follows: denaturation (30 s at 95 °C), annealing (30 s at 50 °C) and extension (2 min at 72 °C), and completed with an elongation phase (10 min at 72 °C). The resulting amplicons were separated on a 1 %

agarose gel followed by ethidium bromide staining. PCR fragments were purified using the PCR purification kit (Qiagen) and were subsequently cloned in the pGEM-T Easy plasmid vector (Promega) following supplier's instructions. Nucleotide sequencing of both strands from cloned DNA was performed using the fluorescent-labelled primer cycle-sequencing kit (Amersham Buchler) following supplier's instructions. The primers used were atp-1, atp-2 and gro-1, gro-2 labelled with IRD800 (MWG Biotech). Sequence alignment was done using the MULTIALIGN program and the CLUSTAL W program. Dendrograms from gene sequences were drawn using the CLUSTAL X program. All *atpD* and *groEL* gene sequences reported in this study have been deposited at GenBank and their accession numbers are indicated in Fig. 3.

The ability to grow in milk was checked by measuring changes in the impedance of the milk medium using the Rapid Automated Bacterial Impedance Technique (Don Whitley System). This system measures the transformation of polar uncharged lactose into charged lactic acid via changes in the electric conductivity. The changes versus incubation time curve is proportional to the acidification of the medium that was measured with a pH electrode. The bacterial growth was measured as c.f.u. ml^{-1}. To determine the cell yield, fermentations were performed using skimmed milk (Difco) medium. Anaerobic fermentations were conducted in duplicate and samples were taken periodically during fermentation and analysed for viable counts using duplicate MRS-cysteine agar plates.

According to the DSMZ Bacterial Nomenclature Up-to-date website (http://www.dsmz.de/bactnom/bactname.htm), *B. lactis* is considered to be a heterotypic synonym of *B. animalis* based on the proposal of Cai *et al.* (2000). In spite of this proposal, both names are still regularly used. In the period from January 2001 to August 2003, following the proposal of Cai *et al.* (2000) to unify *B. lactis* and *B. animalis*, the species name *B. lactis* was cited in at least 37 papers. A recent genotypic study of Ventura & Zink (2002) supported this unification, but also concluded from their Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR fingerprinting and 16S–23S internally transcribed spacer analysis that strains formerly classified as *B. lactis* should be allocated in a subspecies of *B. animalis*.

The current study was initiated to collect more polyphasic evidence in support of the subdivision of *B. animalis* at the subspecies level. DNA G+C content ranged from 60·3 to 61·4 mol%, with means of 61·3 and 61·0 mol% for representatives of *B. animalis* and *B. lactis*, respectively (additional data are available as supplementary Table 2 in IJSEM Online). DNA–DNA hybridizations were performed using seven strains of which some were also included in the study of Cai *et al.* (2000). Consistent with their findings, all DNA–DNA reassociation values were above 70 %, ranging from 76 to 100 %, and the type strains of *B. lactis* and *B. animalis* displayed at least 90 % DNA relatedness. This is in contrast to the findings of Meile *et al.* (1997) who found only 27 % DNA homology between the type strain of *B. lactis* and *B. animalis* using a rather unusual technique based on hybridization of uniformly labelled *Eco*RI-restricted chromosomal DNA of the *B. lactis* type strain followed by Southern hybridization with the same amounts of *Eco*RI-restricted DNA of other *Bifidobacterium* strains. Based on the narrow G+C content range and the high DNA reassociation values, our data reinforce the proposal of Cai *et al.* (2000) to join *B. lactis* and *B. animalis* in one single species for which the name of the oldest description, i.e. *B. animalis*, should be maintained according to Rule 42 of the *Bacteriological Code* (1990 Revision) (Lapage *et al.*, 1992).

Following a polyphasic approach, all *B. animalis* and *B. lactis* strains were subjected to a number of techniques that have the potential to unravel relationships at the subspecific level which included protein and DNA (BOX-PCR and FAFLP) fingerprinting as well as *atpD* and *groEL* gene sequence typing. Furthermore, the ability of some strains to grow in milk was determined. As further discussed below, the overall result of this approach showed that each of these methods allowed the unambiguous separation of *B. animalis* from *B. lactis*. In case of SDS-PAGE protein profiling, BOX-PCR and FAFLP, the resulting *B. lactis* and *B. animalis* clusters exhibited similarity levels that were comparable to those between clusters of other *Bifidobacterium* species (data not shown). The results of the numerical analysis of the SDS-PAGE protein patterns are shown in Fig. 1. After numerical comparison of the digitized protein electrophoretic fingerprints, two well delineated clusters were observed which corresponded to strains previously assigned to *B. animalis* and *B. lactis*, respectively. Given the fact that protein profiling displays a lower taxonomic resolution compared to genotypic techniques such as rep-PCR and FAFLP, these findings indicate that both species are distinct from each other on a phenotypic basis. As shown previously by BOX-PCR fingerprinting (Masco *et al.*, 2003), reference strains of *B. lactis* and *B. animalis* group in two separate clusters indicating their pronounced genotypic heterogeneity. In the present study, BOX-PCR was performed on additional strains from the NCC, which demonstrated the robustness of these genotypic subgroups (data not shown). Recently, Ventura & Zink (2002) reported that rep-PCR targeting the ERIC element also allowed differentiation between type and reference strains of *B. animalis* and *B. lactis*, respectively. FAFLP exhibits a slightly higher resolution than BOX-PCR fingerprinting and is considered, along with PFGE, as the most discriminating genotypic technique. Clustering of the FAFLP banding patterns of 14 strains studied resulted in two clusters representing *B. animalis* and *B. lactis* at a cut-off level of 59 % (Fig. 2).

The partial nucleotide sequences of the *atpD* and *groEL* genes from *Bifidobacterium* strains belonging to *B. lactis* and *B. animalis* species were determined and phylogenetic trees based on these data were constructed. The topology

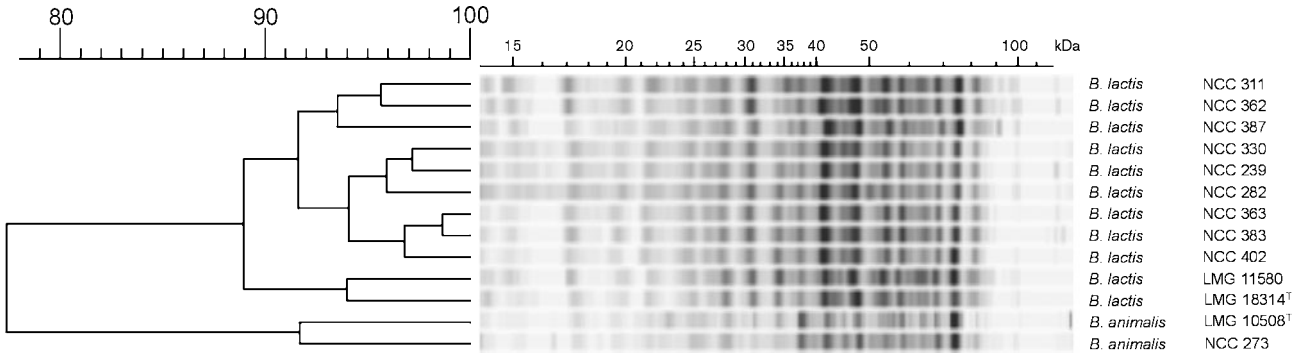

Fig. 1. Dendrogram of protein profiles calculated by the unweighted pair-group method using arithmetic averages (UPGMA) for 13 strains investigated. Correlation levels are expressed as percentage values of the Pearson correlation coefficient.

of the *atpD*- and *groEL*-based trees was highly comparable (Fig. 3). In these trees, *Bifidobacterium* strains were grouped into two clusters. Cluster I contained only the type strain of *B. animalis* and the reference strain ATCC 27672, whereas cluster II contained six representatives of *B. lactis* including its type strain. Twenty-eight nucleotide substitutions were observed between the *atpD* gene sequences of *B. lactis* DSM 10140^T and *B. animalis* ATCC 25527^T. Likewise, 31 synonymous nucleotide substitutions were noticed between the *groEL* gene sequences of the two type strains. The phylogenetic distances calculated from the nucleotide substitution ratios at synonymous positions in the *atpD* and *groEL* genes were examined for all possible combinations of these *Bifidobacterium* genes. A significant correlation between the phylogenetic distances in the *atpD* genes and those in the *groEL* genes was observed. This result was not unexpected, because it has been demonstrated that a synonymous substitution rate is constant for many chromosomal genes in many organisms, and can thus serve as a molecular clock of their evolution (Lawrence *et al.*, 1991). It is noteworthy that a clear separation of *B. animalis* ATCC 25527^T and *B. lactis* DSM 10140^T was not possible based on 16S rDNA sequence analysis since their sequences displayed at least 98·8 % homology (Cai *et al.*, 2000).

Twelve *B. lactis* and *B. animalis* strains used in this work

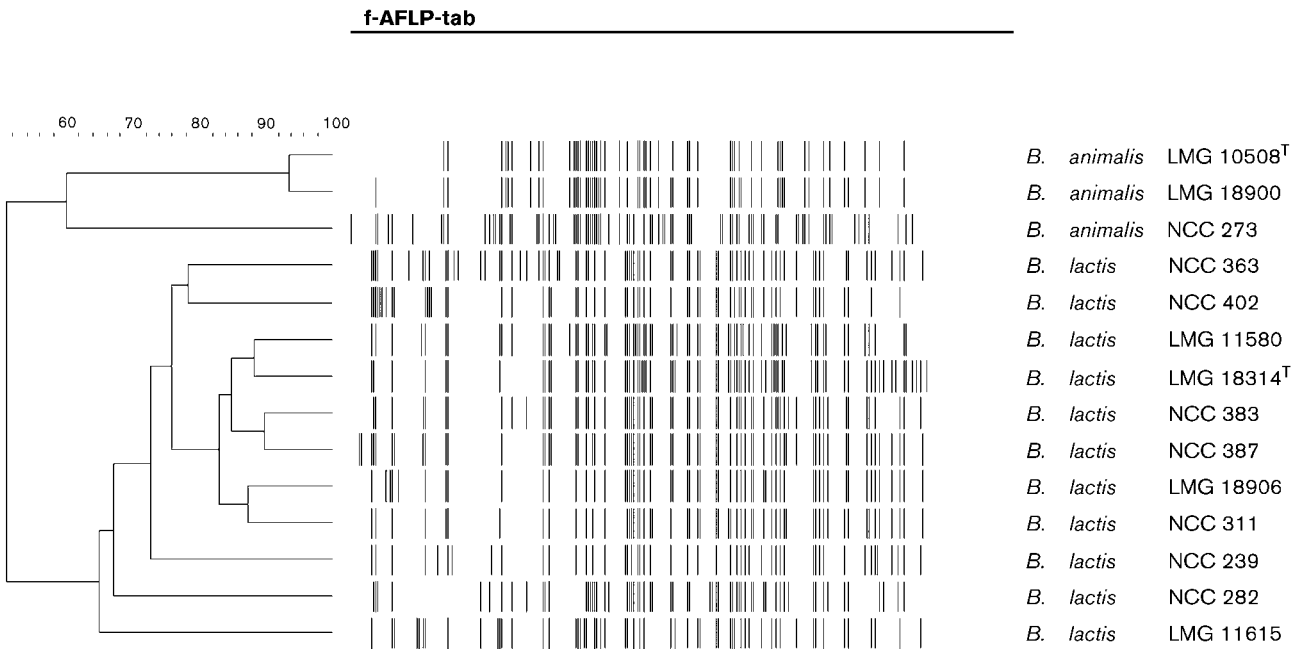

Fig. 2. Dendrogram generated after cluster analysis of digitized FAFLP fingerprints of *B. animalis* and *B. lactis* strains. A band-based (Dice) cluster analysis (UPGMA) was used. The threshold for cluster delineation was 59 %.

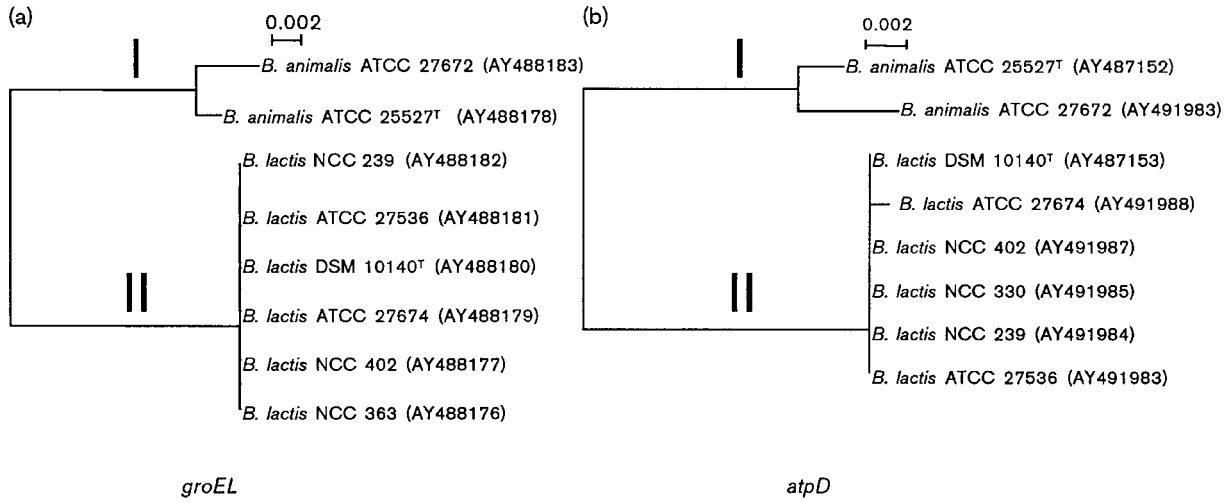

Fig. 3. Phylogenetic trees of *B. animalis* and *B. lactis* strains based on (a) partial *groEL* sequences (1158 base positions) and (b) partial *atpD* sequences (1133 base positions). Drawn using the CLUSTAL X program. Accession numbers are given in parentheses. Bars indicate evolutionary distance.

were tested for their ability to grow on a milk-based medium during which growth was monitored by measuring changes in conductance. When the milk medium was inoculated with 10^6 c.f.u. ml^{-1}, only *B. lactis* strains DSM 10140^T, NCC 363, NCC 383, NCC 311, NCC 387, NCC 402, NCC 239, ATCC 27673, ATCC 27674 and ATCC 27536 showed an increase in conductivity, whereas *B. animalis* ATCC 25527^T and ATCC 27672 did not reveal any changes in the impedance values of the milk medium. All *B. lactis* strains maintained viable counts greater than 2×10^8 c.f.u. ml^{-1} throughout the 24 h fermentation and displayed differences in growth of 1·5 log (Fig. 4a). On the other hand, *B. animalis* ATCC 25527^T and ATCC 27672 did not reveal any significant growth and their viable counts dropped steadily to below the value of 5×10^7 c.f.u. ml^{-1} with small differences (below 0·5 log) in relative growth (Fig. 4b). Collectively, these findings indicate that only *B. lactis* has the potential to grow in milk or milk-based media.

As could be expected from the results of Cai *et al.* (2000), the carbohydrate-fermentation patterns of *B. animalis* and *B. lactis* were very similar based on the examination of 95 different carbon sources. As some characters varied from strain to strain, it was not possible to define a species-specific pattern for representatives of *B. animalis* and *B. lactis*. This is clearly illustrated by the fact that, of all tested carbon sources, only dextrin, α-D-glucose, maltose, maltotriose, D-raffinose and sucrose were fermented by all tested strains. At the individual strain level, only strains NCC 311 and NCC 362 displayed an identical fermentation behaviour. In addition to the AN MicroPlate characterization, the ability to ferment starch was verified based on the formation of a hydrolysis halo on M144 medium depleted of the usual sugars and supplemented with 0·6 % soluble starch. Meile *et al.* (1997) asserted that the non-utilization of starch by *B. lactis* was a major difference between both species. However, consistent with the findings of Cai *et al.* (2000) and Lauer & Kandler (1983), we observed that neither species was able to use this carbon source.

In support of the proposal of Cai *et al.* (2000), the DNA–DNA hybridization data and phenotypic results reported in this study are evidence that *B. animalis* and *B. lactis* belong to one single species. However, results of protein profiling, genotypic analyses and growth evaluation in milk indicate that both these taxa are clearly different. Based on the fact that members of both species share more than 70 % DNA–DNA relatedness, *B. lactis* should be reclassified as *B. animalis*, as required by Rule 42 of the *Bacteriological Code* (1990 Revision) (Lapage *et al.*, 1992). Taking into account that strains formerly assigned to *B. animalis* and *B. lactis* can be clearly distinguished at the intraspecific level, we propose two subspecies in *B. animalis*, for which the names *B. animalis* subsp. *animalis* and *B. animalis* subsp. *lactis* are suggested, respectively.

The following descriptions are based on data obtained from the present study and on previously reported data (Scardovi & Trovatelli, 1974; Meile *et al.*, 1997).

Description of *Bifidobacterium animalis* emend.

Strains display the following characteristics typical for the genus *Bifidobacterium*: Gram-positive, non-motile, non-spore forming, irregular rod-shaped anaerobes. Glucose is fermented using the characteristic enzyme fructose-6-phosphate phosphoketolase in the so-called Bifidus-shunt. Dextrin, α-D-glucose, maltose, maltotriose, D-raffinose and sucrose are fermented; starch is not fermented.

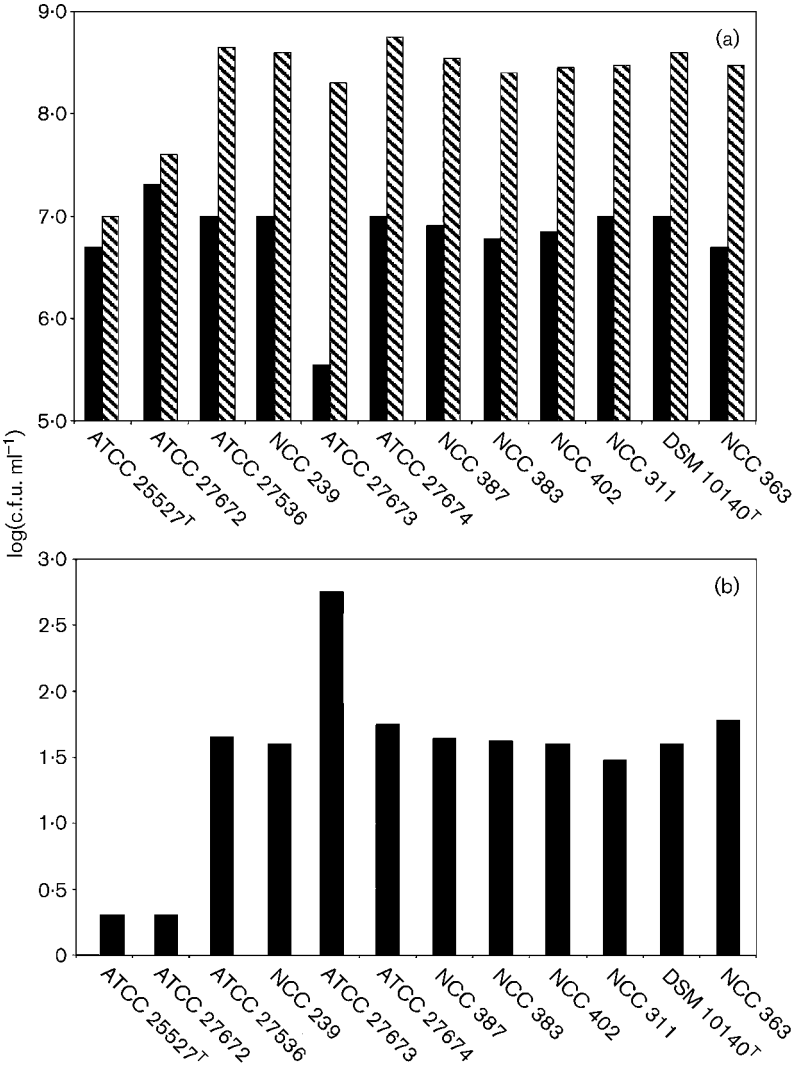

Fig. 4. Bar diagrams showing (a) viable counts at $t=0$ (black) and 24 h (hatched) and (b) difference in viable counts after 24 h incubation.

Description of *Bifidobacterium animalis* subsp. *animalis* subsp. nov.

Strains display characteristics typical for the species *B. animalis* as described above. The optimum growth temperature is 39–41 °C. No growth occurs in slants incubated in air or in air enriched with carbon dioxide. No growth occurs in milk or milk-based media. Lactate and acidic acids are produced in a molar ratio of $1:3{\cdot}6\pm0{\cdot}3$. Strains originate from the faeces of rats. The DNA G+C content is $61{\cdot}3\pm0{\cdot}0$ mol%. Type strain: *Bifidobacterium animalis* subsp. *animalis* R101-8^T (LMG 10508^T = ATCC 25527^T = DSM 20104^T = JCM 1190^T).

Description of *Bifidobacterium animalis* subsp. *lactis* subsp. nov.

Strains display characteristics typical for the species *B. animalis* as described above. The optimum growth temperature is 39–42 °C. No growth occurs on agar plates exposed to air, but 10 % oxygen in the headspace atmosphere above liquid media is tolerated. Growth occurs in milk or milk-based media. The molar ratio of acetate to lactate from glucose metabolism is about 10 to 1 under anaerobic conditions, e.g. lactate production is replaced by formate production. Strains have been isolated from fermented milk samples, human and infant faeces, rabbit and chicken faeces and from sewage. The DNA G+C content is $61{\cdot}0\pm0{\cdot}5$ mol%. Type strain: *Bifidobacterium animalis* subsp. *lactis* UR1^T (LMG 18314^T = DSM 10140^T = JCM 10602^T).

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