

IDENTIFICATION OF LACTOBACILLUS UFV H2B20 (PROBIOTIC STRAIN) USING DNA-DNA HYBRIDIZATION

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ABSTRACT

Sequence analyses of the 16S rDNA gene and DNA-DNA hybridization tests were performed for identification of the species of the probiotic *Lactobacillus* UFV H2b20 strain. Using these two tests, we concluded that this strain, originally considered *Lact. acidophilus*, should be classified as *Lact. delbrueckii*.

Key-words: Sequencing, probiotic, acid lactic bacteria, hibridization DNA-DNA, *Lactobacillus*

Lactobacillus species are widely distributed in nature, and some are known for their importance in the food industry and their benefits as probiotic agents in human and animal health (5). To be considered a probiotic agent, a strain must first be characterized and properly identified. The name of the species and strain are generally indicated by the manufacturer, but in many cases, they are not in agreement with the most recent taxonomy of acid lactic bacteria (12,32). This occurs when the bacterial classification is based only on phenotypic characteristics, many times without reproducibility tests and phylogenetic analyses (12). Acid lactic bacteria have low morphological diversity and very similar nutritional and growth requirements, which makes a polyphasic approach very important for species discrimination (29).

Numerous revisions of the acid lactic bacteria nomenclature have shown the wide gap between results obtained by traditional phenotypic tests, such as carbohydrate fermentation, and molecular taxonomy, such as rRNA sequencing (11,29). However, because of low resolution of image sequences, rRNA sequence analysis may not be able to discriminate species of highly related organisms, especially those phenotypically similar (8,13,22,29). Under these conditions, DNA-DNA hybridization, which determines the similarity between two DNA samples, became a reference method to discriminate species and heterogeneous taxa. With the consolidation of molecular techniques, different studies proposed new classifications of

various microorganisms (1,10,14) including acid lactic bacteria and *lactobacilli* (25). The current phylogenetic classification of acid lactic bacteria was determined by rRNA sequencing and DNA-DNA hybridization (29). However, the subdivision of *lactobacilli* in fermentation groups is still maintained, since fermentation is a main characteristic for the identification and classification of these organisms.

The *Lactobacillus* UFV H2b20 strain has been the subject of research for its probiotic properties (19,20,23). It was first identified as *Lactobacillus acidophilus* on account of its sugar fermentation profile (23) and its origin in the human digestive tract. However, a polyphasic approach was required to avoid an equivocal identification of this organism. In the present study, we investigated the identity of the *Lactobacillus* UFV H2b20 strain, through rDNA sequencing and DNA-DNA hybridization, and confirmed it as *Lactobacillus delbrueckii*.

Bacterial strains used in this study and their sources are listed in Table 1. *Lactobacillus*. Cells were harvested by centrifugation, resuspended in TE (10 mM TRIS-HCl and 1 mM EDTA, pH 8.0) and lysed with 1% final concentration of SDS after cell wall hydrolysis using 40 µg ml⁻¹ lysozyme and 1 µg ml⁻¹ mutanolisin (Sigma). RNase A was added to a final concentration of 50 µg ml⁻¹ and samples were incubated with 100 µg ml⁻¹ proteinase K, for 15 min at 55°C. The DNA was extracted with phenol-chloroform: isoamyl alcohol (25:24:1), precipitated with ethanol (24) and resuspended in TE solution.

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Table 1. Bacterial strains used in this work.

Species	Strain (s)
<i>Lact. delbrueckii</i> subsp. <i>delbrueckii</i>	ATCC 9649 ^T (type strain)
<i>Lact. delbrueckii</i> subsp. <i>lactis</i>	ATCC 12315
<i>Lact. acidophilus</i>	ATCC 4356 ^T (type strain)
<i>Lactobacillus</i> UFV H2b20	Isolated from breast feeding infant feces*

ATCC - American Type Culture Collection, Manassas, VA, USA;
*Industrial Microbiology Laboratory, BIOAGRO –Universidade Federal de Viçosa, Minas Gerais, Brazil. All bacterial strains were grown on MRS broth (Merck), pH6,5 at 37°C for 12h and preserved at -80°C in fresh MRS broth with 20% glycerol.

DNA from all the samples was quantified on agarose gel with ethidium bromide. The 16S rRNA gene of the H2b20 strain was amplified by PCR with the primers FD 5'-CCGAATTCGTCGACAACAGAGTTTGGATCCTGGCTCAG-3' and RD 5'-CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC-3' corresponding to conserved regions of eubacterial rDNA extremities (31). Amplification of 16S rDNA was performed in a Perkin Elmer Thermal Cycler in a total volume of 25 µl containing 20 ng DNA, 1 mM of each primer, 100 µM dNTPs, 2 mM MgCl₂, 1 µg ml⁻¹ bovine serum albumin - BSA (Sigma) and 2.5U *Taq* DNA polymerase (Promega). The PCR was performed under the following conditions: 25 cycles of 2 min at 95°C, 50 s at 50°C and 2 min at 72°C and one final 2-min cycle at 92°C, 50 s at 50°C and 4 min at 72°C. The amplified 16S rDNA was cloned into pGEM[®]-T Easy Vector (Promega). Plasmids bearing the insert were purified with kit Wizard[™] Minipreps DNA Purification System (Promega) and were subject to sequencing reactions. Sequencing was done with two kits: Termo Sequenase Dye Terminator Cycle Sequencing Pre-mix Kit (Amersham) and MicroSeq[™] 16S rRNA Gene (PE Applied Biosystems) on the ABI Prism[™] 310 Genetic Analyzer (Perkin Elmer). Data analyses were done using ABI Prism DNA Sequencing Analysis Software. The rDNA sequence was compared to those available in the GenBank databanks, EMBL Nucleotide Sequence Database (<http://www.ebi.ac.uk/embl>), Ribosomal Database Project (15) and BLAST, NCBI (National Center of Biotechnology Information) tools (<http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/nph-newblast>) and CSM Molecular Biology Resource (<http://vega.igh.cnrs.fr/bin/lalign-guess.cgi>). The sequences were aligned using the CLUSTALW program (28). About 120 ng of total DNA of each strain was applied on Nylon membrane using the Hybri Slot Blot Manifold as described by the manufacturer (Bio-Rad) (5). The PCR-amplified 16S rDNA, and total DNA of

Lactobacillus UFV H2b20 were labeled with digoxigenin, using the DIG DNA labeling kit (Boehringer Mannheim). The hybridization buffer containing 50% formamide, 5X SSC (75 mM sodium citrate; 0.75 M NaCl, pH 7.0), 0.1% N-laurylsarcosine, 0.02% SDS and 2% blocking reagent was used in the hybridization experiment performed overnight at 42°C. The membranes were washed twice at room temperature for 5 min in a solution containing 2X SSC (0.03M sodium citrate and 0.3M NaCl) and 0.1% SDS, then twice at 68°C for 15 min in 0.1X SSC, 0.1% SDS. The detection reaction was carried out using the chemiluminescent substrate CSPD [disodium 3 - (4 methoxyspiro {1,2 - dioxetane - 3, 2'' - (5'' - chloro) tricyclo [3.3.1.1^{3,7}] decan} - 4 - yl) phenyl phosphate], as recommended by the manufacturer (Boehringer Mannheim). The signal, detected on X-ray film (Kodak X-OTMAT K) exposed at 37°C for one hour, was measured using an Ultrascan XL-Enhancer laser densitometer (LKB Bromma) (4).

Analysis by PCR amplification of a 16S rRNA gene copy of H2b20 strain with a set of primers designed for Eubacteria (31) yielded a 1519 pb fragment (accession number EF015468). In the sequence analysis, the identity of this DNA, with 99% identity to 16S rDNA, was detached from all four *Lact. delbrueckii* subspecies from NCBI: *Lact. delbrueckii* subsp. *bulgaricus* (accession number AY675257.1), *Lact. delbrueckii* subsp. *lactis* (accession number AY050173.1), *Lact. delbrueckii* subsp. *delbrueckii* (accession number AY773949.1) and *Lact. delbrueckii* subsp. *indicus* (accession number AY421720.1). It bears only 91% identity with partial sequence of the 16S rRNA gene of *Lact. acidophilus* (accession numbers AY590776, AY186042 and AF375937).

The amount of each DNA in the membranes was verified by prior hybridization using the gene UFV H2b20 16S rDNA as a probe; it was amplified by PCR against total DNAs of *Lact. delbrueckii* (ATCC 9649 and ATCC 12315) and *Lact. acidophilus* (ATCC 4356) (Fig. 1A). The signal was 1.5 times weaker in sample 3 because of the different number of copies of *rrn* operon between the species rather than because of gene similarity. *Lact. acidophilus* has 4 operons (2) while *Lact. delbrueckii* has at least 6 operons (18). The identity of 16S rDNA from H2b20 from the public databanks is similar to the sequences of this same gene in related bacteria.

In the hybridization experiments shown in figure 1B, total DNA from UFV H2b20 was used as a probe against total DNA of all the samples. The experiment control considered the 100ng total DNA from UFV H2b20 in the membrane. This DNA was 100% homologous to the probe, i.e., to its own total DNA. All the other hybridization readings were fitted to this pattern (Table 2).

The phylogenetic structure of the acid lactic bacteria was deduced from rRNA sequencing and DNA-DNA hybridization and differs from the physiological and chemotaxonomic classifications used for almost a century (29). The sugar fermentation pattern of the probiotic *Lactobacillus* placed it

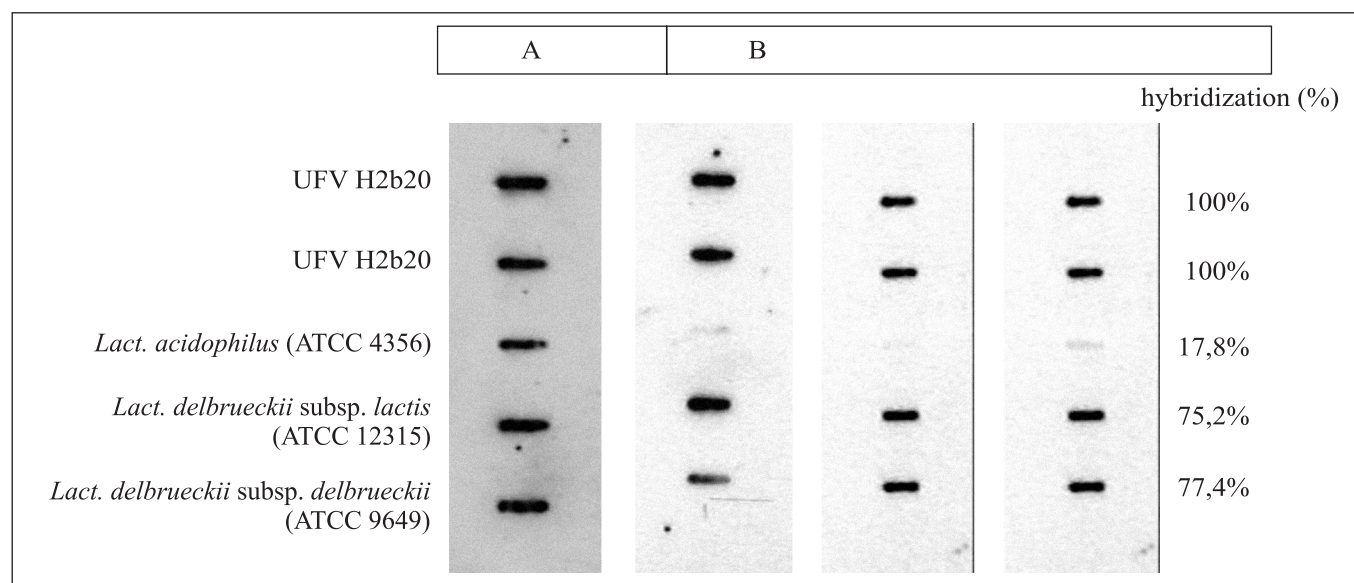


Figure 1. Total DNA hybridization of the studied strains. (A) UFV H2b20 16S rDNA as probe; (B) UFV H2b20 total DNA as probe (three repetitions).

Table 2. Homology (%) in the hybridizations measured by densitometer and correction according to the amount of DNA in sample membranes.

Sample	Concentration of DNA on the membrane (ng)	Relative surface of hybridization			Corrected value			Homology (%)			Homology average
		1	2	3	1	2	3	1	2	3	
UFVH2B20	100	26.02	27.97	26.08	26.02	27.97	26.08	100.0	100.0	100.0	100.0
<i>Lact. acidophilus</i>	64	3.3	2.82	3.01	5.15	4.4	4.7	19.8	15.73	18.0	17.8
<i>Lact. delbrueckii</i> subsp. <i>lactis</i>	125	25.75	23.58	25.84	20.6	18.8	20.67	79.1	67.21	79.3	75.2
<i>Lact. delbrueckii</i> subsp. <i>delbrueckii</i>	89	16.93	18.85	19.44	19.0	21.17	21.84	73.0	75.6	83.7	77.4

closest to *Lact. acidophilus* (23), an identification that was accepted at the time because of the strain's probiotic properties and also because it was originally isolated from a breastfeeding infant's fecal matter. There is a close phylogenetic relationship between *Lact. delbrueckii* subsp. *bulgaricus* and *Lact. acidophilus*, and confusion between their nomenclature is common (9). Hence, the need for unequivocal identification motivated PCR amplification of a 16S rRNA gene of H2b20 strain.

Many times, the analysis of rRNA sequence may not differentiate closely related organisms, especially those with similar phenotypes, because of the low quality of sequence resolution (22,29). The correlation between DNA-DNA

hybridization and homology to rDNA 16S is, in many cases, non-linear, i.e., distinct species may have high similarity in rDNA 16S sequences. Likewise, low DNA-DNA homology does not necessarily indicate low similarity of rDNA 16S among the strains, since this gene contains sequences that are preserved in the different bacteria groups (6,16,26).

Studies using DNA-DNA hybridization have been used to describe new species and have contributed to the reduction of former heterogeneous taxa. Hence, the use of this technique is indispensable to clear up taxonomic uncertainties over acid lactic bacteria, especially in genus *Lactobacillus* (25). Although some authors currently consider that the resolution power of

this technique is high, it is suggested that rRNA sequencing may be carried out as well to increase confidence in the results (21,27). A classic example is the bacteria *Lact. Casei* (17). In the present study, to guarantee a strain's classification, both 16S rDNA homology analysis and DNA-DNA hybridization were used and the results show agreement in classifying the UFV H2b20 strain as *Lact. delbrueckii*.

A re-association above 70% between two strains indicates that they belong to a same species, given that organisms with 70-100% DNA similarity have at least 96% similarity in sequence identity (3,27,30). These values depend strongly on experimental conditions and reflect only an indirect measure of homologies of DNA sequences. The stringency of the reaction is determined by salt and formamide concentrations, temperature and molar G+C content of the DNA used. The hybridizations are usually carried out under standard conditions that are not necessarily optimal or stringent for all the bacteria. As a rule, it was determined that a temperature of 25°C under sample T_m is required to achieve optimal conditions for renaturation or hybridization. The temperature value decreases about 0.7°C per percentage point of formamide added to the hybridization solution, and the formamide's concentration is associated to the C+G content of the DNA. Hence, hybridizations are usually carried out with 5 or 6X SSC, 40-50% formamide at around 42°C. These conditions would be acceptable for DNAs with about 45 mol% G+C and suboptimal for DNAs with higher or lower amounts of these bases (7). Although a range of protocols have described this technique, in many cases it is not clear if the hybridization used was optimal, suboptimal or stringent for the organism studied. Bacteria from acid lactic belong to a branch of the *Clostridium* group that contains low G+C amounts (from 32 to 50%). The molar percent of G+C in *Lact. acidophilus* is 36%, while in *Lact. delbrueckii*, which is considered the group with the highest G+C percent, this value is 50% (5,29). We can thus consider that the hybridization conditions used were optimal for the species studied.

When bacteria species share common phenotypical properties, between 30 and 65% homology would support the idea that they belong to a same genus (3). Therefore, because the hybridization value of H2b20 was 30% lower than that of the reference strain of *Lact. acidophilus*, it could not indicate that H2b20 belonged to another genus. The *Lactobacillus* species have different genotypes showing low degree of DNA-DNA homology to each other. *Lact. helveticus* is similar to *Lact. acidophilus* in its G+C content and in various biochemical characteristics. Nevertheless, *Lact. helveticus* has only 13 to 44% of DNA-DNA homology with the different genotypes of *Lact. acidophilus* (*lato sensu*), which contain *Lact. acidophilus* (*stricto sensu*), *Lact. gasseri*, *Lact. crispatus*, *Lact. amylovorus*, *Lact. gallinarum* and *Lact. johnsonii* species. This discrepancy in homology values could also be found among the members themselves of this group, with values varying from 20 to 50% (10,29).

Therefore, the results presented here support that the previous classification of the *Lact. delbrueckii* strain studied should be abolished.

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RESUMO

Identificação de *Lactobacillus* UFV H2b20 (linhagem probiótica) através de hibridização DNA-DNA

Análise da seqüência do gene 16S rDNA e ensaios de hibridização DNA-DNA foram empregados para identificar a espécie da cepa probiótica *Lactobacillus* UFV H2b20. Empregando-se estes dois testes, concluímos que esta cepa, originalmente considerada *Lact. acidophilus*, deve ser classificada como *Lact. delbrueckii*.

Palavras-chaves: Sequenciamento, probiótico, bactéria do ácido láctico, hibridização DNA-DNA, *Lactobacillus*

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