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BIOCHEMICAL AND MOLECULAR-GENETIC IDENTIFICATION OF *LACTOBACILLUS* STRAINS OF HUMAN ORIGIN

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Biochemical and molecular-genetic identification of Lactobacillus strains of human origin: *The morphological and physiological characteristics of two newly isolated Lactobacillus strains (Lactobacillus Pr9 and Lactobacillus Pr10) of human origin were determined. The strains were identified as representatives of the species Lactobacillus acidophilus by the application of biochemical (API 50 CHL) and molecular-genetic methods (ARDRA-analysis and sequencing of the 16S rRNA gene). After software processing with CLC Sequence Viewer it has been found that Lactobacillus acidophilus Pr9 and Lactobacillus acidophilus Pr10 were identical.*

Key words: *Lactobacillus, API 50 CHL, ARDRA, sequencing, 16S rRNA, CLC sequence viewer.*

INTRODUCTION

Lactobacilli are similar in phenotypic and physiological characteristics, probably due to their coevolution in the same ecological niche. The *Lactobacillus* genus includes more than 140 species. Horizontal transfer of plasmid - associated traits is characteristic for *Lactobacillus* species. Therefore, molecular-genetic methods are applied along with biochemical methods for their species differentiation. Molecular-genetic methods allow for the more accurate, rapid, and reproducible differentiation between closely related species, that are difficult to differentiate only on the basis of their phenotypic characteristics [4]. The similarity in the biochemical profiles of phylogenetically closely related species, as well as the influence of some factors on the metabolic activities of lactic acid bacteria require additional characterization by applying molecular-genetic methods [5]. In fact, many species of the genus *Lactobacillus* have been reclassified based on new information from molecular-genetic analyses and their taxonomic status has been determined, for example *L. cellobiosus, L. pastorianus, L. arizonensis* are allocated appropriately to the species *L. fermentum* [1], *L. paracollinoides* [2], and *L. plantarum*, respectively.

The purpose of the present work was to identify two newly isolated *Lactobacillus* strains using biochemical (API 50 CHL) and molecular-genetic methods (ARDRA-analysis and sequencing of the 16S rRNA gene).

MATERIALS AND METHODS

Microorganisms

Lactobacillus Pr9, *Lactobacillus* Pr10 of human origin.

Reference microorganisms: *Lactobacillus acidophilus* DSM 20079, *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081, *Lactobacillus casei* ssp. *casei* DSM 20011, *Lactobacillus helveticus* DSM 20075, *Lactobacillus plantarum* ssp. *plantarum* DSM 20174.

Determination of the biochemical profile - API 50 CHL (BioMerieux SA, France) according to manufacturer's instructions.

Molecular-genetic methods

Isolation of total DNA - E.Z.N.A.® kit according to manufacturer's instructions

PCR reactions and visualization. All PCR reactions were performed using PCR kit - Ready To Go™ PCR beads (Amersham Biosciences), in a volume of 25 μl in Progene cycler (Techne, UK). The resulting products were visualized on a 2% agarose gel, stained with ethidium bromide solution (0.5 μg/ml), using a UVP Documentation System (U.K.).

16S rDNA amplification and ARDRA-analysis (Amplified Ribosomal DNA Restriction Analysis). All PCR reactions were performed using PCR kit - PCR VWR, in a volume of 25 μl in Progene cycler (Techne, UK) according to the manufacturer's instructions. 50 ng of total DNA of the studied strain and 10 pmol primers were used in each reaction. The DNA of the studied strain was amplified using universal primers for 16S rDNA - 27f (5'AGAGTTTGATCMTGGCTCAG3') [3] and 1492r (5'ACCTTGTTACGACTT3') [3]. The amplification program included: denaturation - 95 °C for 3 min, 40 cycles - 93 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, final elongation - 72 °C for 7 min.

The PCR product obtained was subjected to restriction with FastDigest endonucleases *Eco* RI, *Hae* III and *Alu* I (ThermoFisher Scientific) at a concentration of 10 units/μl.

The products were visualized on a 2% agarose gel stained with ethidium bromide solution (0.5 μg/ml) using a UVP Documentation System (U.K.).

Purification of the PCR product (16S rDNA) from TAE-agarose gel

The purification of the 16S rDNA was performed with a kit for DNA purification (GFX Microspin™) according to the manufacturer's instructions.

Sequencing of the 16S rDNA

The sequencing of the 16S rDNA was performed by the Sanger method by "Macrogen Europe Laboratory", the Netherlands.

The sequencing results for the forward and reverse partial sequences of each strain were assembled using software *CLC Sequence Viewer*. The assembled sequences of the 16S rRNA gene were compared with the sequences available in the online GenBank database through online software BLASTn and the species identification of the strains with the corresponding percentage of similarity between the sequence of the studied strain and the reference strain from the online database was determined.

RESULTS AND DISCUSSION

Phenotypic characteristics of the newly isolated lactobacilli strains

In cultivation on MRS-agar *Lactobacillus* Pr9 and *Lactobacillus* Pr10 formed small, milky white colonies with star shape and uneven edges, which could easily be separated from the medium. The cells were long and rod-shaped, with rounded edges, arranged singly and in short chains.

Biochemical characteristics of the newly isolated Lactobacillus strains

The biochemical profiles of *Lactobacillus* Pr9 and *Lactobacillus* Pr10 were examined using the system for rapid lactobacilli identification API 50 CHL (Biomerieux, France). Both strains utilized galactose, D-glucose, D-fructose, D-mannose, manitol, sorbitol, N-acetyl-glucosamine, amigdalin, arbutin, esculin, salicin, cellobiose, maltose, lactose, melibiose, saccharose, trehalose, melezitose, D-raffinose, β-gentiobiose, D-turanose, gluconate. *Lactobacillus* Pr9 utilized D-xylose as well, while *Lactobacillus* Pr10 utilized amidon. The results from the API 50 CHL for the strains' ability to utilize the 49 carbon sources included in the system API 50 CHL were processed with software apiweb® and the two strains were identified as representatives of the species *Lactobacillus plantarum* with the corresponding percentage of reliability - for *Lactobacillus* Pr9 - 87,9%, for *Lactobacillus* Pr10 - 99,9%.

The analyses using the API 50 CH systems provide rapid and reproducible identification of certain lactobacilli types, but sometimes the percentage of discrimination is not high enough. The use of classic phenotypic and biochemical characteristics alone does not always allow to reliably distinguish between lactobacilli types, especially considering that in the *Lactobacillus* genus there is phenotypic variability. Therefore, in accordance with modern concepts of taxonomic lactobacilli identification are applied both phenotypic and molecular-genetic methods, especially in cases with a relatively low percentage of discrimination by the use of classical methods alone [5].

Molecular - taxonomic characterization

ARDRA-analysis of *Lactobacillus* Pr9 and *Lactobacillus* Pr10 with the FastDigest endonucleases *Hae* III, *Alu* I and *Eco* RI was performed to confirm the results for their species identification obtained by the conventional identification methods. The results of the molecular-genetic experiments are shown in Fig. 1, Fig. 2 and Fig. 3.

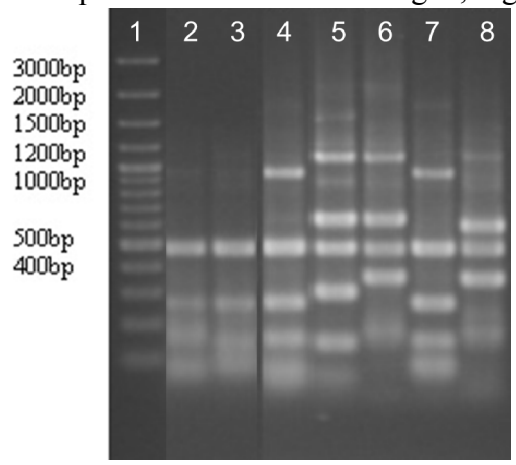


Fig. 1. Restriction profile with *Hae* III

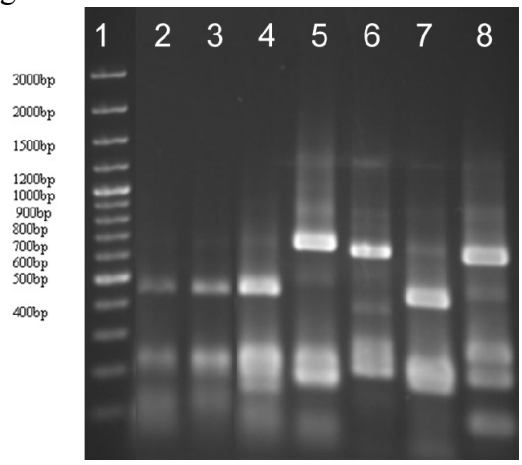


Fig. 2. Restriction profile with *Alu* I

1. M 100 bp plus DNA Ladder
2. *Lactobacillus* Pr9
3. *Lactobacillus* Pr10
4. *Lactobacillus acidophilus* DSM 20079
5. *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081
6. *Lactobacillus casei* ssp. *casei* DSM 20011
7. *Lactobacillus helveticus* DSM 20075
8. *Lactobacillus plantarum* ssp. *plantarum* DSM 20174

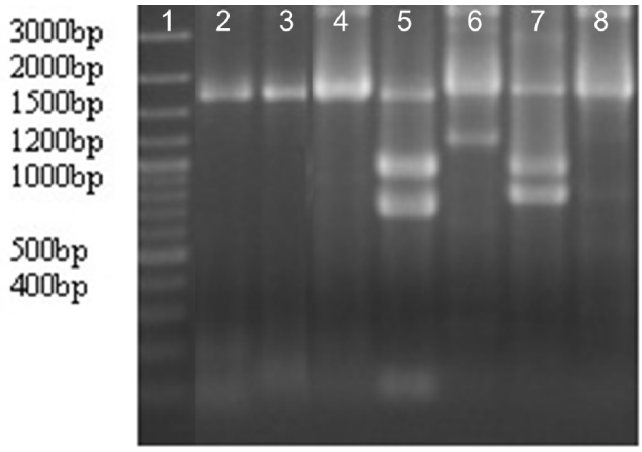


Fig. 3. Restriction profile with *Eco* RI

When comparing the restriction profiles obtained with *Hae* III it was found that the profiles of *Lactobacillus* Pr9 and *Lactobacillus* Pr10 were similar to these of *Lactobacillus acidophilus* and *Lactobacillus helveticus*, but in order to determine the species identification of *Lactobacillus* Pr9 and *Lactobacillus* Pr10 it was necessary to conduct ARDRA-analysis with two more restriction enzymes - *Alu* I and *Eco* RI.

The ARDRA results obtained with *Alu* I (Fig. 2) and *Eco* RI (FIG. 3) identified *Lactobacillus* Pr9 and *Lactobacillus* Pr10 as belonging to the species *Lactobacillus acidophilus*.

1.3.2. Sequencing of the 16S rRNA gene

For the complete species identification of the studied strains a second molecular-genetic method was used - sequencing of the 16S rRNA gene. The results of the sequence analysis of the 16S rDNA identified *Lactobacillus* Pr9 and *Lactobacillus* Pr10 as representatives of the species *Lactobacillus acidophilus* with 98% of similarity between the sequence of the 16S rDNA of *Lactobacillus* Pr9 and the partial sequence of the 16S rDNA of *Lactobacillus acidophilus* VPI 6032 (Fig. 4); and 98% of similarity between the sequence of the 16S rDNA of *Lactobacillus* Pr10 and the partial sequence of the 16S rDNA of *Lactobacillus acidophilus* NBRC 13951 (Fig. 5).

The results of the conducted comparative sequence analysis of the 16S rRNA genes of the strains *Lactobacillus acidophilus* Pr9 and *Lactobacillus acidophilus* Pr10 with the software *CLC Sequence Viewer* showed that the two strains were identical (Fig. 6).

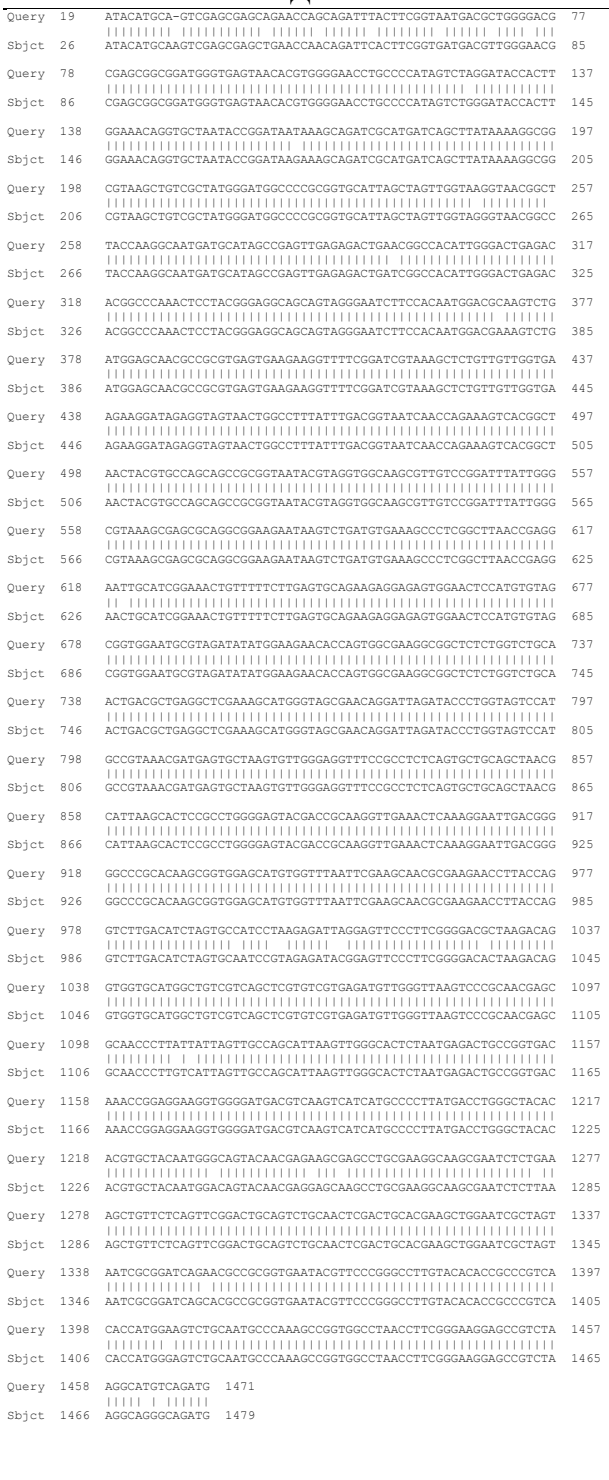


Fig. 4. Comparison between the nucleotide sequence of the 16S rDNA of *Lactobacillus* Pr9 and the partial sequence of the 16S rDNA of *Lactobacillus acidophilus* VPI 6032

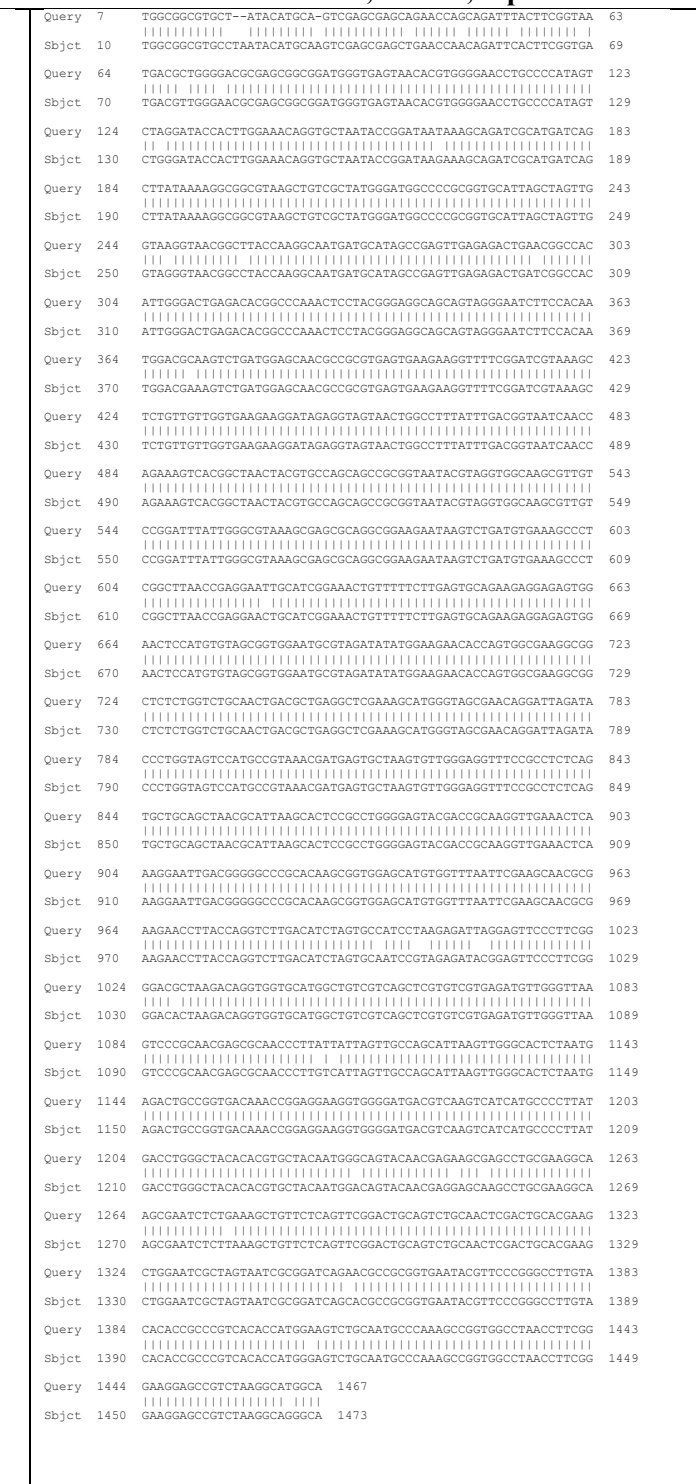
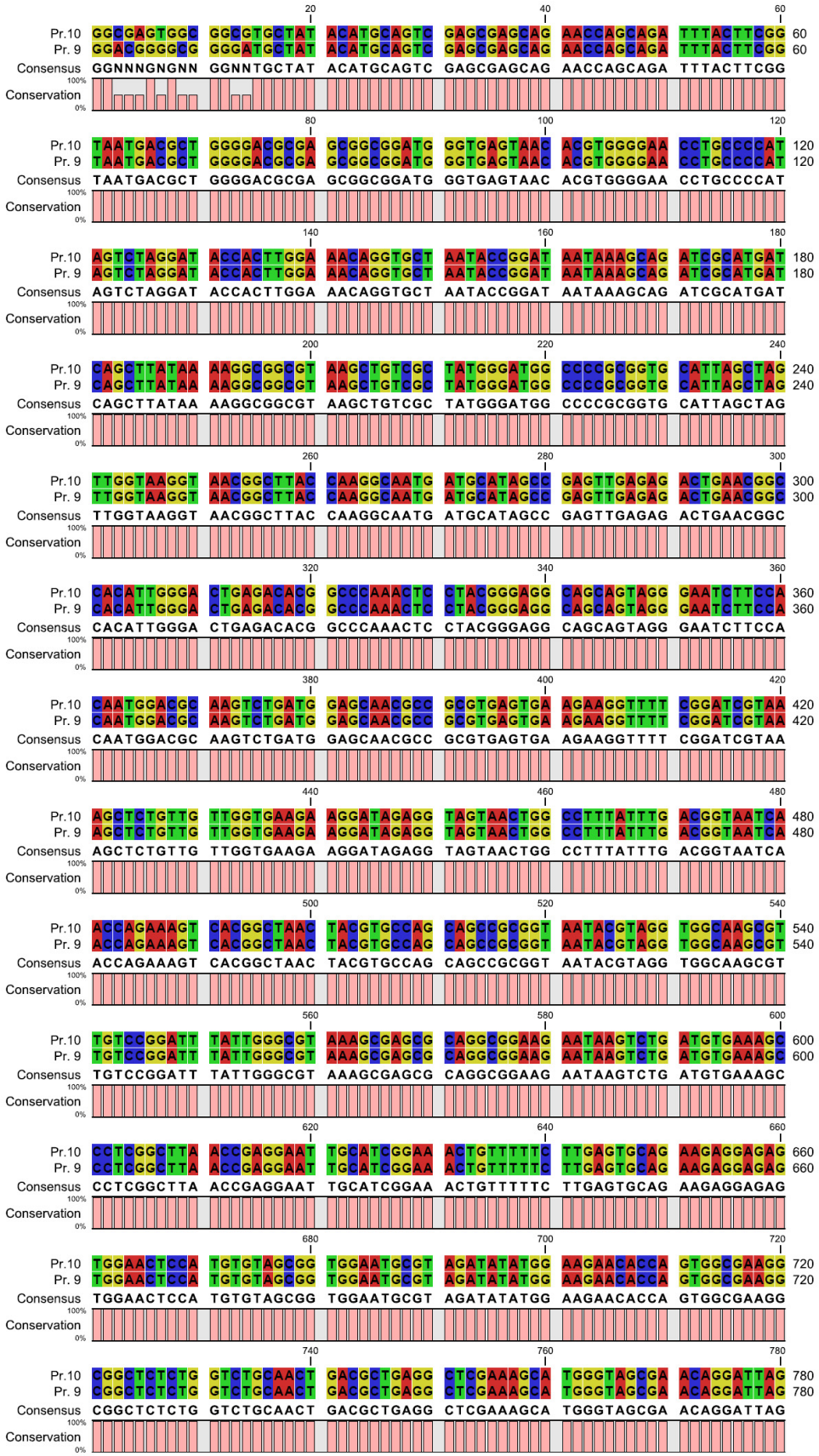


Fig. 5. Comparison between the nucleotide sequence of the 16S rDNA of *Lactobacillus* Pr10 and the partial sequence of the 16S rDNA of *Lactobacillus acidophilus* NBRC 13951


Pr.10 GGCGAGTGGC GGCGTGCTAT ACATGCAGTC GAGCGAGCAG AACCAGCAGA TTTACTTCGG 60
Pr. 9 GGACGGGGCG GGGATGCTAT ACATGCAGTC GAGCGAGCAG AACCAGCAGA TTTACTTCGG 60
Consensus GGNNNGNGNN GGNNTGCTAT ACATGCAGTC GAGCGAGCAG AACCAGCAGA TTTACTTCGG
Conservation
Pr.10 TAATGACGCT GGGGACGCGA GCGGCGGATG GGTGAGTAAC ACGTGGGGAA CCTGCCCCAT 120
Pr. 9 TAATGACGCT GGGGACGCGA GCGGCGGATG GGTGAGTAAC ACGTGGGGAA CCTGCCCCAT 120
Consensus TAATGACGCT GGGGACGCGA GCGGCGGATG GGTGAGTAAC ACGTGGGGAA CCTGCCCCAT
Conservation
Pr.10 AGTCTAGGAT ACCACTTGGA AACAGGTGCT AATACCGGAT AATAAAGCAG ATCGCATGAT 180
Pr. 9 AGTCTAGGAT ACCACTTGGA AACAGGTGCT AATACCGGAT AATAAAGCAG ATCGCATGAT 180
Consensus AGTCTAGGAT ACCACTTGGA AACAGGTGCT AATACCGGAT AATAAAGCAG ATCGCATGAT
Conservation
Pr.10 CAGCTTATAA AAGGCGGCGT AAGCTGTCGC TATGGGATGG CCCCGCGGTG CATTAGCTAG 240
Pr. 9 CAGCTTATAA AAGGCGGCGT AAGCTGTCGC TATGGGATGG CCCCGCGGTG CATTAGCTAG 240
Consensus CAGCTTATAA AAGGCGGCGT AAGCTGTCGC TATGGGATGG CCCCGCGGTG CATTAGCTAG
Conservation
Pr.10 TTGGTAAGGT AACGGCTTAC CAAGGCAATG ATGCATAGCC GAGTTGAGAG ACTGAACGGC 300
Pr. 9 TTGGTAAGGT AACGGCTTAC CAAGGCAATG ATGCATAGCC GAGTTGAGAG ACTGAACGGC 300
Consensus TTGGTAAGGT AACGGCTTAC CAAGGCAATG ATGCATAGCC GAGTTGAGAG ACTGAACGGC
Conservation
Pr.10 CACATTGGGA CTGAGACACG GCCCAAACTC CTACGGGAGG CAGCAGTAGG GAATCTTCCA 360
Pr. 9 CACATTGGGA CTGAGACACG GCCCAAACTC CTACGGGAGG CAGCAGTAGG GAATCTTCCA 360
Consensus CACATTGGGA CTGAGACACG GCCCAAACTC CTACGGGAGG CAGCAGTAGG GAATCTTCCA
Conservation
Pr.10 CAATGGACGC AAGTCTGATG GAGCAACGCC GCGTGAGTGA AGAAGGTTTT CGGATCGTAA 420
Pr. 9 CAATGGACGC AAGTCTGATG GAGCAACGCC GCGTGAGTGA AGAAGGTTTT CGGATCGTAA 420
Consensus CAATGGACGC AAGTCTGATG GAGCAACGCC GCGTGAGTGA AGAAGGTTTT CGGATCGTAA
Conservation
Pr.10 AGCTCTGTTG TTGGTGAAGA AGGATAGAGG TAGTAACTGG CCTTTATTTG ACGGTAATCA 480
Pr. 9 AGCTCTGTTG TTGGTGAAGA AGGATAGAGG TAGTAACTGG CCTTTATTTG ACGGTAATCA 480
Consensus AGCTCTGTTG TTGGTGAAGA AGGATAGAGG TAGTAACTGG CCTTTATTTG ACGGTAATCA
Conservation
Pr.10 ACCAGAAAGT CACGGCTAAC TACGTGCCAG CAGCCGCGGT AATACGTAGG TGGCAAGCGT 540
Pr. 9 ACCAGAAAGT CACGGCTAAC TACGTGCCAG CAGCCGCGGT AATACGTAGG TGGCAAGCGT 540
Consensus ACCAGAAAGT CACGGCTAAC TACGTGCCAG CAGCCGCGGT AATACGTAGG TGGCAAGCGT
Conservation
Pr.10 TGTCCGGATT TATTGGGCGT AAAGCGAGCG CAGGCGGAAG AATAAGTCTG ATGTGAAAGC 600
Pr. 9 TGTCCGGATT TATTGGGCGT AAAGCGAGCG CAGGCGGAAG AATAAGTCTG ATGTGAAAGC 600
Consensus TGTCCGGATT TATTGGGCGT AAAGCGAGCG CAGGCGGAAG AATAAGTCTG ATGTGAAAGC
Conservation
Pr.10 CCTCGGCTTA ACCGAGGAAT TGCATCGGAA ACTGTTTTTC TTGAGTGCAG AAGAGGAGAG 660
Pr. 9 CCTCGGCTTA ACCGAGGAAT TGCATCGGAA ACTGTTTTTC TTGAGTGCAG AAGAGGAGAG 660
Consensus CCTCGGCTTA ACCGAGGAAT TGCATCGGAA ACTGTTTTTC TTGAGTGCAG AAGAGGAGAG
Conservation
Pr.10 TGGAACTCCA TGTGTAGCGG TGGAATGCGT AGATATATGG AAGAACACCA GTGGCGAAGG 720
Pr. 9 TGGAACTCCA TGTGTAGCGG TGGAATGCGT AGATATATGG AAGAACACCA GTGGCGAAGG 720
Consensus TGGAACTCCA TGTGTAGCGG TGGAATGCGT AGATATATGG AAGAACACCA GTGGCGAAGG
Conservation
Pr.10 CGGCTCTCTG GTCTGCAACT GACGCTGAGG CTCGAAAGCA TGGGTAGCGA ACAGGATTAG 780
Pr. 9 CGGCTCTCTG GTCTGCAACT GACGCTGAGG CTCGAAAGCA TGGGTAGCGA ACAGGATTAG 780
Consensus CGGCTCTCTG GTCTGCAACT GACGCTGAGG CTCGAAAGCA TGGGTAGCGA ACAGGATTAG
Conservation

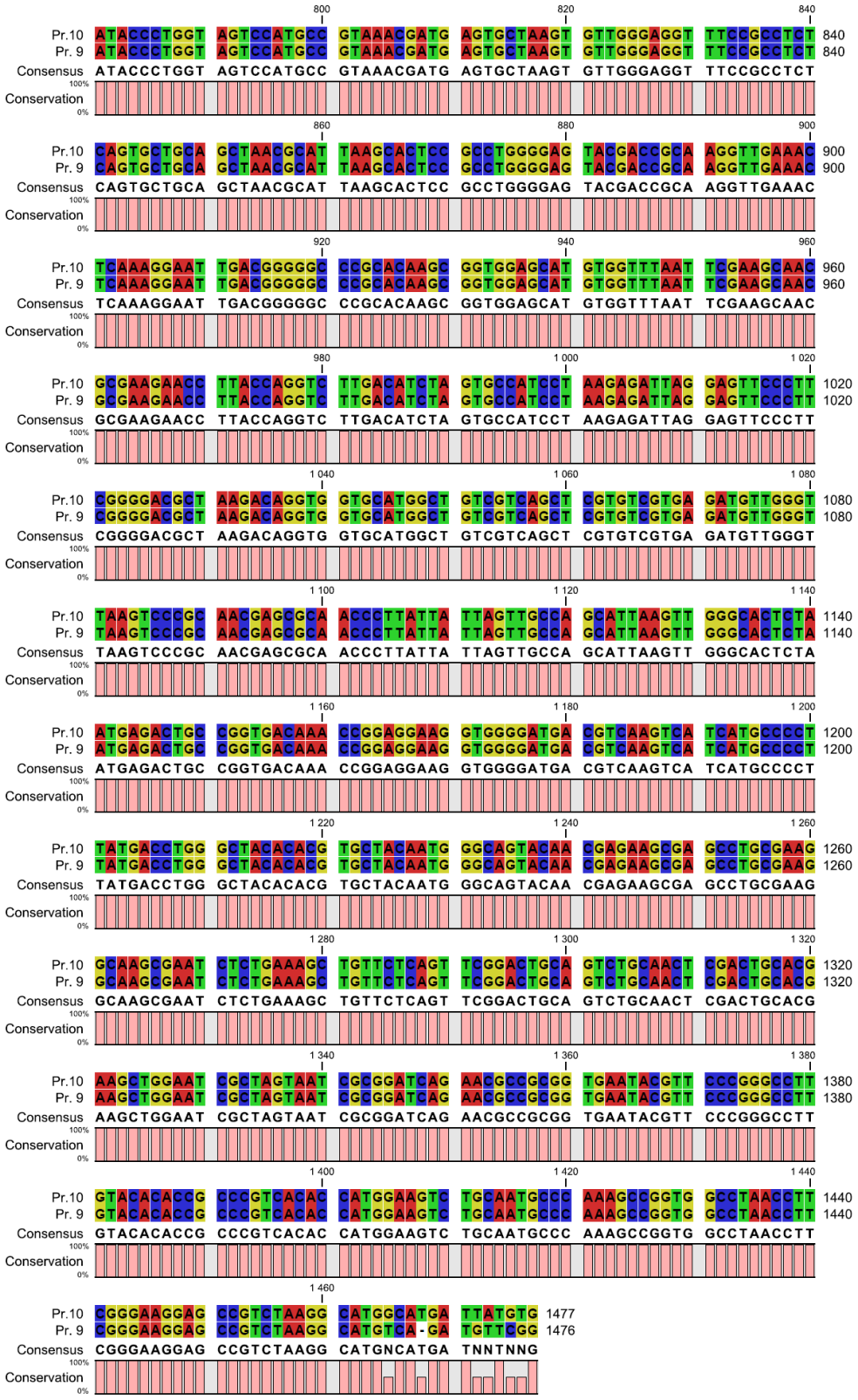


Fig. 6. Comparison between the sequences of the 16S rRNA genes of *Lactobacillus acidophilus* Pr9 and *Lactobacillus acidophilus* Pr10 with software *CLC Sequence Viewer*

CONCLUSION

The newly isolated strains *Lactobacillus* Pr9 and *Lactobacillus* Pr10 were identified using biochemical (API 50 CHL) and molecular-genetic methods (ARDRA-analysis and 16S rDNA sequencing). The results of the analysis with API 50 CHL and the consequtive software processing with apiweb® related the two strains to the species *Lactobacillus plantarum*. The conducted molecular-genetic (ARDRA-analysis and sequencing of the 16S rRNA gene) identified

Lactobacillus Pr9 and *Lactobacillus* Pr10 as representatives of the species *Lactobacillus acidophilus*. When comparing the nucleotide sequences of the 16S rDNA of the two newly isolated strains with the software *CLC Sequence Viewer* it was found that both strains were identical.

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