Biochemical and molecular-genetic identification of *Lactobacillus* strains, isolated from salad dressings

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To develop starters for functional foods the newly isolated strains should be identified and examined for desirable and beneficial properties. The strains Lactobacillus B1 and Lactobacillus TAB2 were isolated from salad dressings. The biochemical profiles of the studied strains were determined using the kit system for rapid identification of lactobacilli API 50 CHL and after the subsequent software processing with apiweb® the strains were identified as representatives of the species Lactobacillus delbrueckii ssp. bulgaricus. The applied molecular-genetic methods – ARDRA-analysis with the restriction enzymes Eco RI, Hae II and Alu I and sequencing of the 16S rDNA confirmed that Lactobacillus B1 and Lactobacillus TAB2 belong to the species Lactobacillus delbrueckii ssp. bulgaricus.

Key words: *Lactobacillus, Identification, ARDRA, Sequencing, Salad dressings*

INTRODUCTION

Functional foods are taking an increasingly prominent part in human nutrition. The importance of functional foods for the preservation of the health of contemporary man is unquestionable. That`s why the requirements towards probiotic bacteria are the working field for many research teams. The inclusion of probiotic strains in the compositions of food products transforms them into functional foods.

Probiotic lactobacilli and bifidobacteria are also included in the composition of probiotics and they benefit the human organism through their metabolites. Lactic acid bacteria have GRAS-status; thus they have great potential for use in biopreservation [1].

Today, consumers' demand for "natural" and "minimally processed" foods is increasing [3]. The popularity of salad dressings among salad consumers requires the production of dressings and sauces, including low-fat dressings and sauces, in order to satisfy users' needs [2].

The purpose of the present article was the biochemical and molecular genetic identification of strains of the genus Lactobacillus, isolated from salad dressings.

MATERIALS AND METHODS

Microorganisms

Two strains of lactic acid bacteria isolated from salad dressings were used in the present work - *Lactobacillus* TAB2, *Lactobacillus* B1.

Methods

Biochemical methods.

Determination of biochemical profile of the studied cultures.

The system API 50 CHL (BioMerieux SA, France) was used for the identification of the species of the genus *Lactobacillus* based on their ability to utilize 49 carbon sources. Fresh 24-hour culture of the studied strain was centrifuged for 15 min at 5000xg. The obtained sludge, containing biomass, was washed twice with PBS-buffer and resuspended in API 50 CHL medium, an integral part of the used kit. The API strips were placed in the incubation boxes, the microtubules were inoculated with the prepared cell suspension and sealed with sterile liquid paraffin. The results were reported on the 24th and the 48th hour of incubation at 37±1°C. Reporting was done, based on the colour change of each microtubule, compared to the colour of the control microtubule (microtubule 0). Positive results were recorded in the cases of color change from blue to green or bright yellow. The obtained results were processed with apiweb® identification software.

Molecular-genetic methods

Identification

Isolation of total DNA

The isolation of DNA was performed by the method of Delley et al. [4].

PCR reactions and visualization

All PCR reactions were performed using PCR VWR in a volume of 25 µl in a Progene cycler (Techne, UK). The resulting products were visualized on a 2% agarose gel stained with ethidium bromide solution (0.5 µg/ml), using an UVP Documentation System (UK).

16S rDNA amplification and 16S rDNA ARDRA (Amplified Ribosomal DNA Restriction Analysis)

The method ARDRA involves enzymatic multiplication of the gene encoding the 16S rRNA, using primers complementary to the conservative regions at both ends of the 16S rRNA gene and the product of the multiplication is then restricted with restriction enzymes. The resulting profile is highly specific for the particular studied species. DNA of the studied strain was amplified using universal primers for the 16S rDNA gene – 27F and 1492R [5]. The amplification program included: denaturation - 94°C for 3 minutes, 40 cycles - 94°C for 30 s, 50°C for 30 s, 72°C for 2 min, final elongation - 72°C for 7 min. The resulting PCR product from the 16S rDNA amplification of the tested strain was treated with the endonucleases *Eco* RI, *Hae* III and *Alu* I (Boehringer Mannhem GmbH, Germany). Reactions were carried out according to the following quantities: PCR products – 10 µl, enzyme solution - 10 µl (1 µl of the respective enzyme, 2 µl buffer, 7 µl dH2O). Incubation for 1 night at 37°C was performed. The resulting restriction products were visualized on a 2% agarose gel.

Purification of the product of the PCR-reaction – 16S rDNA – from TAEagarose Gel

The purification of 16S rDNA was conducted using DNA-purification kit (GFX MicrospinTM) according to the manufacturer's instructions:

1) Sample capture.

After visualizing the product of the 16S PCR-amplification reaction on a 2% agarose gel with UV light with wavelength 302 nm, the gel was visualized with UV light with wavelength 365 nm. The 16S PCR product was cut from the gel and placed in a DNA-free microcentrofuge tube. Through weighing the microcentrofuge tube before and after the gel fragments were put in them, the weight of the fragments was calculated and 10µl Capture buffer was added to every 10mg of the gel. The microcentrofuge tube were mixed gently and incubated at 60°C for about 20 minutes until the full dissolution of the gel fragments.

2) Sample binding

A GFX MicrospinTM column was labelled and placed in a collection tube and the centrofuged (shortspin) samples in the eppendorf tubes from 1) were poured in the GFX MicrospinTM columns (no more than 600µl). The GFX MicrospinTM columns were allowed to wet for about 60 seconds and centrofuged until the whole volume passes through the column. The liquid from the column was disposed and the GFX MicrospinTM column was placed in the same collection tube. If a sample was more than 600µl, all the steps from the sample binding were repeated until the whole sample was eluated.

3) Wash and dry

500 µl of wash buffer type 1 were poured in each GFX MicrospinTM column, the columns were centrofuged (shortspin), the collection tubes were disposed and each GFX MicrospinTM column was placed in a new 1,5 ml DNAase free microcentrofuge tube.

4) Elution

10-50µl Elution buffer type 4 or type 6 were poured in each GFX MicrospinTM column. The column was allowed to wet at room temperature for 60 seconds and the microcentrofuge tubes with the GFX MicrospinTM columns were centrofuged for about 60 seconds. The eluate (containing purified 16S rDNA) was collected and freezed at -20°C.

DNA-sequencing

The sequencing of the 16S rRNA gene using the forward and reverse primer was performed by „Macrogen Europe Laboratory", the Netherlands using the Sanger method for DNA-sequencing. The obtained whole sequence of the 16S rDNA gene, using CLC Sequence Viewer Software, was compared with the sequences of the strains registered in

the online database using the algorithm BLASTn and the strain was identified to species level with the corresponding percentage of reliability.

RESULTS AND DISCUSSION

Two lactic acid bacteria strains, *Lactobacillus* TAB2 and *Lactobacillus* B1, were isolated from salad dressings. The ability of the two strains of lactic acid bacteria to absorb the 49 carbon sources included in the kit system for rapid identification of lactobacilli API 50 CHL (BioMerieux SA, France) was examined (Table 1).

After processing of the obtained results with the software apiweb® the strains *Lactobacillus* TAB2 and *Lactobacillus* B1 were identified as *Lactobacillus delbrueckii* ssp. *bulgaricus* strains with high percentage of reliability - 99.9% (Table 2).

Table 2

Ability of the tested strains to utilize the 49 carbon sources included in the identification system API 50 CHL

#	Carbohydrates	B1	TAB2
1	Glycerol	-	-
2	Erythriol	-	-
3	D-arabinose	-	-
4	L-arabinose	+ (90%-100%)	-
5	Ribose	+ (90%-100%)	+ (90%-100%)
6	D-xylose	-	-
7	L-xylose	-	-
8	Adonitol	-	-
9	β-metil-D-xyloside	-	-
10	Galactose	+ (90%-100%)	+ (90%-100%)
11	D-glucose	+ (90%-100%)	+ (90%-100%)
12	D-fructose	+ (90%-100%)	+ (90%-100%)
13	D-mannose	+ (90%-100%)	+ (90%-100%)
14	L-sorbose	-	-
15	Rhamnose	-	-
16	Dulcitol	-	-
17	Inositol	-	-
18	Manitol	+ (90%-100%)	+ (90%-100%)
19	Sorbitol	+ (90%-100%)	+ (90%-100%)
20	α-methyl-D-mannoside	+ (90%-100%)	+ (90%-100%)
21	α-methyl-D-glucoside	-	-
22	N-acetyl-glucosamine	+ (90%-100%)	+ (90%-100%)
23	Amigdalin	+ (90%-100%)	+ (90%-100%)
24	Arbutin	+ (90%-100%)	+ (90%-100%)
25	Esculin	+ (90%-100%)	+ (90%-100%)
26	Salicin	+ (90%-100%)	+ (90%-100%)
27	Cellobiose	+ (90%-100%)	+ (90%-100%)
28	Maltose	+ (90%-100%)	+ (90%-100%)
29	Lactose	+ (90%-100%)	+ (90%-100%)
30	Melibiose	+ (90%-100%)	+ (90%-100%)
31	Saccharose	+ (90%-100%)	+ (90%-100%)
32	Trehalose	+ (90%-100%)	+ (90%-100%)
33	Inulin	-	-
34	Melezitose	+ (90%-100%)	+ (90%-100%)
35	D-raffinose	+ (90%-100%)	+ (90%-100%)
36	Amidon	-	-
37	Glycogen	-	-
38	Xylitol	-	-
39	β-gentiobiose	+ (90%-100%)	+ (90%-100%)
40	D-turanose	-	-
41	D-lyxose	-	-
42	D-tagarose	-	-
43	D-fuccose	-	-
44	L-fuccose	-	-
45	D-arabitol	-	-
46	L-arabitol	-	-
47	Gluconate	-	+ (90%-100%)
48	2-keto-gluconate	-	-
49	5-keto-gluconate	-	-

Table 3

Identification of the newly isolated lactobacilli strains after processing of the results from the API 50 CHL kit with apiweb®

Strain	Species	Reliability, %
Lactobacillus B1	*Lactobacillus delbruieckii* ssp. *bulgaricus*	99.9
Lactobacillus TAB2	*Lactobacillus delbruieckii* ssp. *bulgaricus*	99.9

To confirm the results from the biochemical methods for identification for the two studied strains, *Lactobacillus* B1 and *Lactobacillus* TAB2, ARDRA analysis followed by sequencing of the gene encoding the 16S rRNA were performed.

ARDRA analysis. As a result of the ARDRA analysis with the enzymes *Eco* RI (Fig.1), *Hae* III (Fig. 2) and *Alu* I (Fig. 3) the studied strains were confirmed to be representatives of the species *Lactobacillus delbrueckii* ssp. *bulgaricus*.

The sequencing of the 16S rDNA of *Lactobacillus* B1 and *Lactobacillus* TAB2 was conducted by Macrogen Europe Laboratory, the Netherlands by the method of chain termination (method of Sanger). After careful comparison of the obtained sequence with the public online nucleotide BLAST database, the strains *Lactobacillus* B1 and *Lactobacillus* TAB2 were confirmed to be Lactobacillus delbrueckii ssp. bulgaricus strains (Fig. 4, Fig. 5).

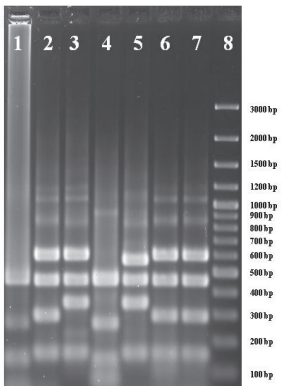

Fig. 1. Restriction profile of the 16S rDNA with *Hae* III:

1. *Lactobacillus acidophilus* DSM 20079;
2. *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081;
3. *Lactobacillus casei* ssp. *casei* DSM 20011;
4. *Lactobacillus helveticus* DSM 20075;
5. *Lactobacillus plantarum* DSM 20174;
6. *Lactobacillus* B1;
7. *Lactobacillus* TAB2;
8. 100 bp Plus DNA Ladder

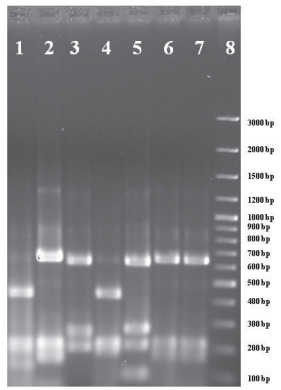

Fig. 2. Restriction profile of the 16S rDNA with *Alu* I:

1. *Lactobacillus acidophilus* DSM 20079;
2. *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081;
3. *Lactobacillus casei* ssp. *casei* DSM 20011;
4. *Lactobacillus helveticus* DSM 20075;
5. *Lactobacillus plantarum* DSM 20174;
6. *Lactobacillus* B1;
7. *Lactobacillus* TAB2;
8. 100 bp Plus DNA Ladder

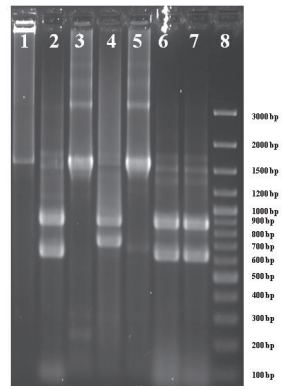

Fig. 3. Restriction profile of the 16S rDNA with *Eco* RI:

1. *Lactobacillus acidophilus* DSM 20079;
2. *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081;
3. *Lactobacillus casei* ssp. *casei* DSM 20011;
4. *Lactobacillus helveticus* DSM 20075;
5. *Lactobacillus plantarum* DSM 20174;
6. *Lactobacillus* B1;
7. *Lactobacillus* TAB2;
8. 100 bp Plus DNA Ladder

Lactobacillus delbrueckii ssp. *bulgaricus* strain NBRC 13953 16S ribosomal RNA gene, partial sequence

Alignment statistics

Score	Expect	Identities	Gaps	Strand
1535 bits(1702)	0.0	878/889(99%)	5/889(0%)	Plus/Minus

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Query  1     CCCACGTCTCCTGCCATGGCATTAGGCGGCTGACTCCTATAAAGGTTATCCCACCGACTT  60
             |||| |||   ||||| || | ||||||||||||||||||||||||||||||||||||
Sbjct  1483  CCCA-GTCATCTGCCCTGCC-TTAGGCGGCTGACTCCTATAAAGGTTATCCCACCGACTT  1426

Query  61    TGGGCATTGCAGACTTCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTC  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1425  TGGGCATTGCAGACTTCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTC  1366

Query  121   ACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCAGCTTCGTGCAGGCGAGTTGCAGC  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1365  ACCGCGGCGTGCTGATCCGCGATTACTAGCGATTCCAGCTTCGTGCAGGCGAGTTGCAGC  1306

Query  181   CTGCAGTCCGAACTGAGAACAGCTTTAAGAGATCCGCTTACCCTCGCGGGTTCGCTTCTC  240
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Sbjct  1305  CTGCAGTCCGAACTGAGAACAGCTTTAAGAGATCCGCTTACCCTCGCGGGTTCGCTTCTC  1246

Query  241   GTTGTACTGCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGACTTGAC  300
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Sbjct  1245  GTTGTACTGCCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGACTTGAC  1186

Query  301   GTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCTTTAGAGTGCCCAACTTAATG  360
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Sbjct  1185  GTCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCTTTAGAGTGCCCAACTTAATG  1126

Query  361   ATGGCAACTAAAGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1125  ATGGCAACTAAAGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACAC  1066

Query  421   GAGCTGACGACAGCCATGCACCACCTGTCTCTGCGTCCCCGAAGGGAACCACCTATCTCT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1065  GAGCTGACGACAGCCATGCACCACCTGTCTCTGCGTCCCCGAAGGGAACCACCTATCTCT  1006

Query  481   AGGTGTAGCGCAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCA  540
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Sbjct  1005  AGGTGTAGCACAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCA  946

Query  541   CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGT  600
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Sbjct  945   CATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAACCTTGCGGTCGT  886

Query  601   ACTCCCCAGGCGGAGCGCTTAATGCGTTTGCTGCGGCACTGAGGACCGGAAAGTCCCCAA  660
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Sbjct  885   ACTCCCCAGGCGGAGCGCTTAATGCGTTTGCTGCGGCACTGAGGACCGGAAAGTCCCCAA  826

Query  661   CACCTAGCGCTCATCGTTTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTACCC  720
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Sbjct  825   CACCTAGCGCTCATCGTTTACGGCATGGACTACCAGGGTATCTAATCCTGTTCGCTACCC  766

Query  721   ATGCTTTCGAGCCTCAGCGTCAGTTGCAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCT  780
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Sbjct  765   ATGCTTTCGAGCCTCAGCGTCAGTTGCAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCT  706

Query  781   TCCATATATCTACGCATTCCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCA  840
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Sbjct  705   TCCATATATCTACGCATTCCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCA  646

Query  841   AGAATGACAGTTTTCCGATGCAGTTCCACGGGTTGAGCCCGTGGGGttt  889
             |||||||||| |||||||||||||||||| ||||||| ||||||| |||
Sbjct  645   AGAATGACAG-TTTCCGATGCAGTTCCAC-GGTTGAG-CCGTGGGCTTT  600
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Fig. 4. Comparison of the partial nucleotide sequence of the 16S rDNA of *Lactobacillus* B1 and the partial sequence of the 16S rDNA of *Lactobacillus delbrueckii* ssp. *bulgaricus* NBRC 13953

Lactobacillus delbrueckii ssp. *bulgaricus* strain ATCC 11842 16S ribosomal RNA gene, partial sequence

Alignment statistics

Score	Expect	Identities	Gaps	Strand
1548 bits(1716)	0.0	921/947(97%)	18/947(1%)	Plus/Plus

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Query  17    GCATTAGGCGGCTGACTCCTATAAAGGTTATCCCACCGACTTTGGGCATTGCAGACTTCC  76
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Sbjct  1488  GCCTTAGGCGGCTGACTCCTATAAAGGTTATCCCACCGACTTTGGGCATTGCAGACTTCC  1429
Query  77    ATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCGTGCTGATCC  136
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Sbjct  1428  ATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCGTGCTGATCC  1369
Query  137   GCGATTACTAGCGATTCCAGCTTCGTGCAGGCGAGTTGCAGCCTGCAGTCCGAACTGAGA  196
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Sbjct  1368  GCGATTACTAGCGATTCCAGCTTCGTGCAGGCGAGTTGCAGCCTGCAGTCCGAACTGAGA  1309
Query  197   ACAGCTTTAAGAGATCCGCTTACCCTCGCGGGTTCGCTTCTCGTTGTACTGCCCATTGTA  256
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Sbjct  1308  ACAGCTTTAAGAGATCCGCTTACCCTCGCGGGTTCGCTTCTCGTTGTACTGCCCATTGTA  1249
Query  257   GCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGACTTGACGTCATCCCCACCTTCCTC  316
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Sbjct  1248  GCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGACTTGACGTCATCCCCACCTTCCTC  1189
Query  317   CGGTTTGTCACCGGCAGTCTCTTTAGAGTGCCCAACTTAATGATGGCAACTAAAGACAAG  376
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Sbjct  1188  CGGTTTGTCACCGGCAGTCTCTTTAGAGTGCCCAACTTAATGATGGCAACTAAAGACAAG  1129
Query  377   GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATG  436
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Sbjct  1128  GGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATG  1069
Query  437   CACCACCTGTCTCTGCGTCCCCGAAGGGAACCACCTATCTCTAGGTGTAGCACAGGATGT  496
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Query  497   CAAGACCTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTG  556
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Query  617   TTAATGCGTTTGCTGCGGCACTGAGGACCGGAAAGTCCCCAACACCTAGCGCTCATCGTT  676
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Query  737   GTCAGTTGCAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCATT  796
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Sbjct  768   GTCAGTTGCAGACCAGAGAGCCGCCTTCGCCACTGGTGTTCTTCCATATATCTACGCATT  709
Query  797   CCACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCAAGAATGACAGTTTTCCGA  856
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Sbjct  708   ACACCGCTACACATGGAATTCCACTCTCCTCTTCTGCACTCAAGAATGACAG-TTTCCGA  650
Query  857   TGCAGTTTCCACGGGTTGAGCCCGTGGGGCTTTTCCCATCAAAACTTTATCATTTCCGGC  916
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Sbjct  649   TGCAG-TTCCAC-GGTTGAG-CCGTGGG--CTTTCACATCAGA--CTTATCATT--CCGC  599
Query  917   CTGCGCCTCGCTTTTACCCCCCAAATAAAATCCCGGGACAACCGCTT  963
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Sbjct  598   CTGCG-CTCGC-TTTACGCCC--AAT-AAATCC--GGACAA-CGCTT  560
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Fig. 5. Comparison of the partial nucleotide sequence of the 16S rDNA of *Lactobacillus* TAB2 and the partial sequence of the 16S rDNA of *Lactobacillus delbrueckii* ssp. *bulgaricus* ATCC 11842

CONCLUSION

As a result of the experimental studies the following important conclusions can be resumed:

1. The two newly isolated *Lactobacillus* strains were identified by biochemical tests (API 50 CHL) and the subsequent processing of the results with apiweb® as representatives of the species *Lactobacillus delbrueckii* ssp. *bulgaricus*.

2. The strains *Lactobacillus* B1 and *Lactobacillus* TAB2, isolated from salad dressings, were confirmed as belonging to the species *Lactobacillus delbrueckii* ssp. *bulgaricus* by application of molecular-genetic methods – ARDRA-analysis and sequencing of the 16S rDNA.

3. After investigation of the functional properties of two newly isolated *Lactobacillus* strains they can be incorporated in the composition of starters for functional foods.

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This paper has been reviewed