MOLECULAR STUDY OF THE USEFUL AND THE CONTAMINANT MICROFLORA IN FERMENTED DAIRY PRODUCTS

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ABSTRACT
Lactic acid bacteria have been used as starter strains in the production of fermented dairy products for centuries. Most of the dairy products contain lactic acid bacteria, but also other bacteria involved as contaminant microflora. We explored the microbial content of home-made dairy products and those purchased from the market. In our study twenty-six pure cultures were isolated. The isolated strains were investigated by a set of physiological and molecular-genetic methods for their accurate species identification and genotyping. From the microorganisms, involved in fermentation and ripening of dairy products with proven health benefits to human, in studied foods predominated Lactobacillus delbrueckii, Lactobacillus helveticus and Lactobacillus plantarum. Another part of the isolated strains, representatives of the genus Kluyveromyces, Rhodotorula and Candida were contaminant microflora, as a result of poor hygiene in the manufacture and storage of the dairy products. Some of these strains were isolated from commercially available dairy products. The obtained results raise again the question about the efficacy of microbiological quality control and food safety.

Keyword: contaminant microflora, Lactobacillus, probiotics

Introduction
The dairy products have been a part of the human diet for millennia. They play an important role in a healthy diet, both for nutritional value and personal enjoyment. The dairy industry progress, the development of foods with different taste qualities and the increasing interest of consumers to probiotic preparations justify need to search for new strains of microorganisms with valuable properties. The differences in the flavour and aroma of the various dairy products are due to different microbial starter cultures, additional fermentation products (that may be present in very low concentrations) and the inclusion or omission of secondary microbial species late in the process. Each potential probiotic strain should be documented and assessed independently. Only well defined strains should be used according Establishing Standards for Probiotic Products of International Scientific Association for Probiotics and Prebiotics (ISAPP). The aim of our work was to identify new strains with potential probiotic properties of traditional dairy products.

Materials and methods
Bacterial strains and growth conditions
The following strains, from various collections (ATCC—American Type Culture Collection; NBIMCC—National Bank of Industrial Microorganisms and Cell Cultures, Bulgaria) were used in this study: Lactobacillus acidophilus (ATCC 4356, ATCC 4357), Lactobacillus casei subsp. casei (ATCC 27139, NBIMCC 1014), Lactobacillus delbrueckii subsp. bulgaricus (ATCC 11842), Lactobacillus fermentum (ATCC 14931T), Lactobacillus helveticus (ATCC 15009, ATCC 27558), Lactobacillus plantarum (ATCC 14917T), Lactobacillus reuteri (ATCC 23272), and Lactobacillus rhamnosus (ATCC 7469). They were grown in MRS (Difco) agar or broth anaerobically for 24 h at 37°C. The 21 isolates were identified as lactobacilli based on growth on selective media, Gram-staining, cell morphology and negative catalase reaction. The isolated Lactobacillus strains were cultivated on MRS agar and/ or broth for 24–48 h at 37°C, under anaerobic conditions (BBL® Gas Pak® Anaerobic System Envelopes, USA). For the yeast isolates, one loop of each sample was plated on YPD agar media and cultured aerobically at 30°C. Individual colonies appearing after 48 h were re-streaked on YPD agar plates for pure cultures isolation. We isolated five yeast strains.

DNA extraction
For DNA studies the Lactobacillus strains were grown in MRS broth for 18h and the chromosomal DNA was isolated.
according to Delley et al., 1990 (3) and by using the GFX Genomic Blood DNA Purification kit (Amersham, Biosciences) following the manufacturer’s instructions for Gram positive bacteria and yeast.

**Amplified ribosomal DNA restriction analysis (ARDRA)**
The universal primers used for PCR amplification of *rrn* fragments are: 16-01 5’-AGAGTTTGAT(CT)(AC)TGGCTCAG-3’ and 16-02 5’-AAAGGAGGTGAATCC-3’ (1). The primers were obtained from MWG Biotech (Munich, Germany). The PCR reactions were performed in a Progene cycler (Techne, Cambridge, UK) in 25 μl volume and contained: 0.5 U *Taq* polymerase (Boehringer) with the corresponding buffer, 5 mmol l⁻¹ MgCl₂, 0.100 mmol l⁻¹ of each of dATP, dTTP, dGTP and dCTP, 10 pmol of each primer and 50ng DNA. The PCR products were digested with the following restriction enzymes recognizing four bp sites: *Alu* I, *Hae* III, and with enzyme recognizing six bp sites: *Eco* RI. The restriction patterns were analyzed in 2% Sigma Agarose (Type II) in 0.5×TBE. Lambda phage DNA, digested with Hind III (Boehringer, Mannheim, Germany), was used as a molecular weight marker.

**PCR amplification with species-specific primers**
The PCR reactions were done in a Progene cycler (Techne, UK) in 25 μl volume, using Ready To Go™ PCR beads (Amersham Biosciences). The species-specific primer pairs based on 16S rRNA gene sequences or the 16S–23S rRNA intergenic spacer region are: for *L. acidophilus* (9), *L. helveticus* (8), *L. fermentum* (9), *L. plantarum* (2) and *L. casei* (9).

**Dot blot hybridization**
The probe pY85 for *Lactobacillus delbrueckii* (3) was labelled non-radioactively by random priming with the DIG Labelling and Detection System (Boehringer). This experiment was performed according Miteva et al., 2001 (6).

**Numerical analysis**
Numerical analysis of the ARDRA profiles was done using the cluster program MVSP 3.0 Package (UK). The program grouped the isolates by the UPGMA (outweighed pair group method with arithmetic averages) cluster analysis of the distribution of the DNA bands.

**Sequencing analysis**
The yeast isolates were identified by D1-D2 region of 26S rDNA sequencing according Kurtzman et al., 1997 (5). The BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) was used for sequencing. The sequences obtained were compared with the NCBI database.

**Results and Discussion**
Twenty-one *Lactobacillus* pure cultures, isolated from homemade yoghurt, cheeses and yellow cheeses were included in this study. In the first stage of the study by ARDRA method we grouped the isolated strains according restriction patterns type. The same method was applied to the referent strains from the genus *Lactobacillus*. ARDRA analysis was carried out with different restriction enzymes (*Alu* I, *Hae* III and *Eco* R I), aiming at more reliable grouping of the strains, based on summarized data from several experiments (Fig. 1).

![ARDRA profiles](image1.png)

**Fig. 1** 16S rDNA ARDRA of isolates and reference *Lactobacillus* strains with different enzymes. Lanes: 1-D1, 2-D3, 3-D2, 4-D41, 5- *Lb. delbrueckii* 11842, 6- *Lb. delbrueckii* 12315, 7-D44, 8-D7, 9-D6, 10-D8, 11-D9, 12-D10, 13- *Lb. acidophilus* 4356, 15-D42, 16- *Lb. helveticus* 15009, 17-D45

After comparison between the ARDRA profiles of reference strains and new isolated strains by cluster analysis three groups were created (*Table 1*). The first group included the reference strains of three species: *Lactobacillus delbrueckii*,
Lactobacillus helveticus and Lactobacillus acidophilus and thirteen isolates from dairy foods. The second group included the species Lactobacillus fermentum, Lactobacillus plantarum and 7 isolates. To the third group belonged only one isolate (D12), showing similarity with the species Lactobacillus casei and Lactobacillus rhamnosus.

### TABLE 1

<table>
<thead>
<tr>
<th>Lb. acidophilus</th>
<th>Lb. fermentum</th>
<th>Lb. casei</th>
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<tbody>
<tr>
<td>Lb. delbrueckii</td>
<td>Lb. plantarum</td>
<td>Lb. rhamnosus</td>
</tr>
<tr>
<td>D41, D42, D43, D45, D9, D1, D2, D7, D3, D6, D8, D10, D44</td>
<td>D13, D14, D15, D32, D34, D35, D37</td>
<td>D12</td>
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In the second stage of the study the isolates from each group were tested with specific primers or probes for the respective species. By the following PCR reactions and hybridization, as Lactobacillus delbrueckii were identified the isolates: D 41, D 42, D 43, D 9, D 1, D 2, D 6 and D 8. The isolates D 7, D 44 belonged to Lactobacillus helveticus and isolates D 13, D 14, D 32, D 34, D 35, D 37 were identified as Lactobacillus plantarum. From isolates D 45, D 3, D 10, D 15, D 12 we didn’t receive any amplification product using species-specific primers pair, nor positive signal in hybridization with the probe pY85. As a result, we could not successfully identify these strains to species level by the methods used.

These results confirmed other authors’ work on the species diversity and identification of Lactobacillus strains of dairy foods (7, 4). The significance of these studies is related to the requirement for all microorganisms to be clearly identified before their application in food or products consumed by humans.

Another part of our work was aimed at investigation of contaminant microflora in dairy foods. The quality of the food is directly related to the health of consumers. The home made dairy foods are usually not prepared under strict hygienic conditions and the presence of polluting microflora is somewhat expected and acceptable. We decided to verify whether industrially produced yoghurts available on the market contain contaminant microflora. Yogurt is made by fermenting milk with friendly bacteria, mainly Lactobacillus bulgaricus and Streptococcus thermophilus. Food is considered contaminated when unwanted microorganisms are presented. From five commercial products "yogurt", produced by different company were isolated pure cultures microorganisms. Following the morphological characteristics, these strains were identified as representatives of the family of the yeasts. After amplification of the D1-D2 region of 26S rRNA gene these yeast strains were identified as representatives of the species: Kluyveromyces marxianus, Kluyveromyces lactis, Rhodotorula mucilaginosa and Candida parapsilosis.

As a result of our studies, 16 strains of the genus Lactobacillus isolated from traditional home-made dairy products were identified to species level. The potentially useful properties of these lactobacilli, related to their possible use in probiotic foods and products will be tested.

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### REFERENCES