DNA BARCODING OF YEASTS FROM SELECTED BULGARIAN FOOD PRODUCTS

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ABSTRACT
Studies on biodiversity of yeasts in Bulgarian food ecosystems are scarce. In present article 14 yeast strains were isolated from various commercial Bulgarian food products and characterized by DNA barcoding analysis. The analysis has shown occurrence in food products various yeasts from the following genera- Saccharomyces, Kazachstania, Kluyveromyces, Pichia, Isatchenkia, Candida and Rhodotorula.

Introduction
The production and maintenance of good food products contribute to the quality of life. Yeasts are important microorganisms for the food industry, and have positive role in the processing of wine, beer, bread and diary products (2). Yeasts survive better than bacteria and molds under food environmental conditions in terms of pH, water activity and low temperature and therefore frequently involved in spoilage of food products. Moreover, some yeast species can utilize organic acids such as lactic, citric and acetic acids and weak acid preservatives such as benzoic acid, propionic acid, sorbic acid used for the food production (13, 17, 18). In recent years, the interest in food yeast ecosystems surveys has increased considerably because control of microbial spoilage is becoming an increasing challenge for the food industry. (10). Little knowledge is available on yeast ecosystem of various Bulgarian food products. Among the most popular traditional Bulgarian products are yogurt, boza, bialo salamureno sirene. The aim of the present study was to explore yeast ecosystem of these food products using DNA barcoding analysis (12).

Materials and Methods
Isolation of yeasts from food products
Commercial food products such as boza, cheeses, yogurt produced in Bulgaria were sampled. Yeast strains were isolated by spreading of diluted aliquots of various food samples on Petri dishes with YM agar (3% yeast extract, 3 % malt extract, 5% peptone, 1% glucose, 2% agar, pH 3.4). Yeast isolates were purified several times to obtain pure cultures. The yeast strains obtained in this study are listed in Table 1. All strains were grown on YM agar at 24°C, maintained on YM agar slopes at 4°C, and frozen in YPD medium containing 20% of glycerol at -80°C. The strains initially were characterized phenotypically (6). The list of yeasts and food products examined are presented in Table 1.

DNA barcoding analysis
Partial region of 26S rDNA were PCR-amplified directly from an individual yeast colonies using oligonucleotide primers NL1 and NL4 (14). The amplified 26S rDNA were purified using a QIAquick PCR purification kit (Qiagen, USA) according to the manufacturer’s instructions. Direct sequencing of the purified 26S rDNA PCR products was performed using NL1 and NL4 as sequencing primers with a Taq DyeDeoxy terminator cycle sequencing kit (Applied Biosystems, USA) according to the manufacturer’s recommendations. Purified sequencing reaction mixtures were separated with a 3730XL automated DNA analyser (Applied Biosystems, USA) by Macrogen Inc. (South Korea).

For DNA barcoding identification 26S rDNA sequences of yeasts strains were compared with sequences maintained in the GenBank database at www.ncbi.nlm.nih.gov/ using BLAST search analysis. The analysis has shown that the yeasts sequences were affiliated with sequences from the database with high percent of similarity (99%, Table 1).

Results and Discussion
Yeasts been used during several thousands of years for the production of a wide range of foods such as bread, wine, beer and kefyr, and more recently for the production of ethanol for fuel, biochemicals for the pharmaceutical industry and many other substances. Yeasts present on fruits, vegetables, equipments, in homemade starters, and in all kinds of foods (2, 12).

Identifying, naming and placing yeasts in their proper evolutionary framework is of importance to many areas of science, including agriculture, medicine, the biological sciences, biotechnology, for determining industrial-property rights and food industry. At present, approximately 1000 yeast species are recognized, but only a few are frequently isolated (12). Traditional yeast identification methods based on phenotype include
fermentation reactions on a select set of sugars and growth responses on various carbon and nitrogen sources or on other diagnostic compounds. Isolates were further characterized phenotypically from the microscopic appearance of vegetative cells as well as sexual states. Molecular methods for yeast identification include faster procedure of sequence analysis of regions of genomic DNA i.e. DNA barcoding.

### TABLE 1

List yeasts strains, their origin (foods products) and percentage of 26S rDNA sequence similarity with closest sequences from GenBank database.

<table>
<thead>
<tr>
<th>Yeasts strains</th>
<th>Food products</th>
<th>% Sequence similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kazachstania exigua IMB1</td>
<td>Boza</td>
<td>99%</td>
</tr>
<tr>
<td>Kazachstania exigua IMB2</td>
<td>Boza</td>
<td>99%</td>
</tr>
<tr>
<td>Isatchenkia orientalis IM80</td>
<td>Boza</td>
<td>99%</td>
</tr>
<tr>
<td>Candida inconspicua IM81</td>
<td>Boza</td>
<td>99%</td>
</tr>
<tr>
<td>Pichia fermentans IMB2-1</td>
<td>Boza</td>
<td>99%</td>
</tr>
<tr>
<td>Pichia fermentans IMB2-1</td>
<td>Boza</td>
<td>99%</td>
</tr>
<tr>
<td>Kluyveromyces marxianus IMB1</td>
<td>Cheese</td>
<td>99%</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae IMC1</td>
<td>Cheese</td>
<td>99%</td>
</tr>
<tr>
<td>Kluyveromyces marxianus IM95</td>
<td>Cheese</td>
<td>99%</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae IM7</td>
<td>Cheese</td>
<td>99%</td>
</tr>
<tr>
<td>Rhodotorula mucilaginosa IM4</td>
<td>Cheese</td>
<td>99%</td>
</tr>
<tr>
<td>Kluyveromyces marxianus IMA6</td>
<td>Yogurt</td>
<td>99%</td>
</tr>
<tr>
<td>Rhodotorula mucilaginosa IMH7</td>
<td>Yogurt</td>
<td>99%</td>
</tr>
<tr>
<td>Kluyveromyces marxianus IMH8</td>
<td>Yogurt</td>
<td>99%</td>
</tr>
</tbody>
</table>

Food industry has a considerable economical value. The same time spoilage of food has a huge impact on consumer’s health. More than 150 species yeasts from over 40 different genera were recorded as food contaminants (11). But very few yeast are considered as significant spoilage species. In a review of yeasts associated with food spoilage (15) authors listed only 12 yeast species. The major food spoilage species is Zygosaccharomyces bailii. It is characterized by resistance to preservatives, osmotolerance and high fermentative ability. (4, 5) Other yeasts frequently occurring in food products are Candida parapsilosis, Pichia membranifaciens, Debaryomyces hansenii and Rhodotorula mucilaginosa (13).

We have analyzed yeast ecosystem of boza, bialo salamureno sirene and kiselo mliako. Boza is a traditional cereal-based fermented cold drink with a slightly acidic sweet flavour. Boza is produced in most Balkan countries- in Bulgaria, Albania, Romania and Turkey. Different cereals (wheat, millet and rye) can be used for boza production. Interactions between microorganisms are uncontrolled during the process, which leads to variable product quality. DNA barcoding analysis of yeasts isolated from three boza products contained yeasts- Kazachstania exigua, Isatchenkia orientalis, Candida inconspicua and Pichia fermentans.

Bialo salamureno sirene is a type of brine cheese made from goat’s, cow’s or sheep’s milk. DNA analysis demonstrated that five bialo salamureno cheeses contained yeasts Kluyveromyces marxianus, Saccharomyces cerevisiae and Rhodotorula mucilaginosa.

Kiselo mlyako -yogurt is the best known of all Bulgarian products that are available to consumers nowadays. DNA barcoding identification shown that three kiselo mliako products contained yeasts Kluyveromyces marxianus and Rhodotorula mucilaginosa.

All yeasts strains obtained from Bulgarian products in this study were frequently isolated from various foods by other authors (2, 9).

Unlike bacteria, viruses and some filamentous fungi yeasts rarely associated with foodborne infections and inoxications. As a part of normal daily food consumption, humans unknowingly ingesting large amount of viable diversity of yeasts species without adverse impact on their health. Nevertheless, caution is required –Escherichia coli not considered to be serious foodborne pathogen 25 years ago, now is classified in high risk category (9). Also, there are data showing correlation between presence of yeasts in foods and allergic and hypersensitive reactions in humans (3, 7). Moreover, individuals with weakened health and immune system (AIDS and cancer patients) are at greatest risk of yeast infections caused by Kluyveromyces marxianus, Saccharomyces cerevisiae and Rhodotorula mucilaginosa (1, 6, 8, 16).

### Conclusions

DNA barcoding identification of the strains isolated from selected Bulgarian food products has shown that they do not belong to the yeasts that can cause a risk for healthy individuals. But some of them could be source of serious infections in individuals with compromised immune system.
Acknowledgments
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REFERENCES