MICROBIOTA OF THE TRADITIONAL SLOVAK SHEEP CHEESE “BRYNDZA”

Miroslava Kačániová1,2, Simona Kunová3, Jana Šefániková4, Soňa Felšöciová5, Lucia Godočíková3, Elena Horská6, Ľudmila Nagyová6, Peter Haščík2, Margarita Terentjeva8

Address(es):
1 Slovak University of Agriculture in Nitra, Faculty of Horticulture and Landscape Engineering, Department of Fruit Sciences, Viticulture and Enology, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia, phone number: +421 37 641 4715.
2 University of Rzeszow, Faculty of Biology and Agriculture, Department of Bioenergy and Food Technology, Zelwerowicza St. 4, PL-35601 Rzeszow, Poland.
3 Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Food Safety and Hygiene, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia, phone number: +421 37 641 5807.
4 Slovak University of Agriculture in Nitra, AgroBioTech - Research Center, Tr. A. Hlinku 2, 949 76 Nitra, Slovakia.
5 Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Microbiology, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia, phone number: +421 37 641 5812.
6 Slovak University of Agriculture, Faculty of Economics and Management, Department of Marketing and Trade, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia, phone number: +421 37 641 4102.
7 Slovak University of Agriculture in Nitra, Faculty of Biotechnology and Food Sciences, Department of Technology and Quality of Animal Products, Trieda Andreja Hlinku 2, 949 76 Nitra, Slovakia, phone number: +421 37 641 4708.
8 Latvia University of Life Sciences and Technologies, Institute of Food and Environmental Hygiene, Faculty of Veterinary Medicine, Latvia K. Helmanja iela 8, LV-3004, Jelgava, Latvia.

*Corresponding author: miroslava.kacaniova@gmail.com

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ABSTRACT
The aim of the present study was to describe microbiota of the traditional Slovak cheese „Bryndza“. A total of 30 cheese samples were collected from seven different farms during in 2019. The microbiota studies included the total bacterial count, coliforms, enterococci, lactic acid bacteria, and yeasts. The total bacterial counts were cultivated on plate count agar at 30°C in aerobic conditions, lactic acid bacteria on MRS, API and MASE at 37°C in anaerobic conditions, coliform on VRBL at 37 °C in aerobic condition and yeasts on MEA at 25°C in aerobic condition. Gram-positive, Gram-negative and yeasts isolates were identified by MALDI-TOF MS profiling. Totally, a number of 870 isolates were identified with score higher than 2. Hafnia alvei and Klebsiella oxytoca were the most frequently identified species of Gram-negative and Lactococcus lactis and Lactobacillus paracasei from Gram-positive bacteria. Dipodascus candidum and Yarrowia lipolytica were the most distributed yeasts. Lactic acid bacteria group was represented by Lactobacillus, Lactococcus and Pediococcus. The most abundant genera of lactic acid bacteria were Lactobacillus with 7 species. This study describes the indigenous microbiota of the traditional raw milk cheeses from Slovakia. Our results provide useful information on occurrence of valuable microbial strains for the industrialization of producing of the traditional cheese products.

Keywords: Gram-positive and Gram-negative bacteria, yeasts, „Bryndza“, MALDI-TOF MS Biotype

INTRODUCTION
Raw milk was found to be contaminated with non-pathogenic and pathogenic microorganisms. Pathogens possibly present in raw milk may be originated from sick or apparently healthy animals or as a contamination from the environment or personnel during the collection or storage of milk. Contamination from animals can appear directly, e.g. an endogenous infection then the milk is contaminated directly from the blood stream (systemic infection) or from udder in case of mastitis. Milk cross-contamination could be a result from contamination of faeces, the skin or the environment (Claeys et al., 2013). D’Amico and Donnelly (2010) did not find significant difference between the total microbial counts in raw milk from goats, sheep and cows. The total microbial counts goat and sheep milk were variable depending on milking, the number of milking sessions making up the milk mix, the type of milking system and herd size (Alexopoulos et al., 2011).

Consumer health has become a priority concern for food production. Sheep milk is expected to be an excellent source of nutrients (Balthazar et al., 2017). Sheep milk is rarely consumed as itself; mostly it is used for production of cheese and yogurt (Haenlein and Wendorff, 2006). Consequently, the milk quality has direct impact to the production of high-quality products and high cheese yield per liter of milk used in the cheese manufacture. Functional volume of milk used in the traditional manufacturing process depends on the type of cheese (Santillo and Albenzio, 2015).

The risks and benefits of traditional cheeses, which are frequently produced from raw milk, could be detected objectively by studying the microbiota of cheese inhabiting the product (Bhowmik and Marth, 1990). The microbial diversity and the benefits related to consumption of raw milk cheese depends on both the milk microbiota and traditional manufacturing practices, including a quality of inoculation practices. Traditional processing from farming to cheese making helps to maintain the diversity of microbiota of individual cheeses and the between lots of cheeses throughout processing (Litopoulou-Tzanetaki et al., 1989). More than 400 lactic acid bacteria species, Gram and catalase-positive bacteria, Gram-negative bacteria, yeasts and moulds have been detected in raw milk. The cheese surface is inhabited by numerous species of bacteria, yeasts and moulds, but the cheese cores reveals the smaller degree of biodiversity with a number of lactic acid bacteria species are numerically dominant (Montel et al., 2014). Raw milk can contain pathogenic bacteria that have been raising public health concern and many of raw milk related outbreaks were describe since the beginning of dairy industry. The most common pathogenic bacteria found in raw milk and milk products were Salmonella, Listeria monocytogenes, Staphylococcus aureus and Escherichia coli (Markov et al., 2011).

Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) is a tool in microbiological diagnostics which allow the identification of identification of microorganisms in various matrices. Conventional identification methods rely on biochemical reactions and require additional pre-testing and incubation. In comparison, MALDI-TOF MS can
directly identify bacteria and yeast from their colonies in very short period of time. This new and methodically simple approach reduces the cost of materials and duration of diagnostics (Wieser et al., 2012). Identification of microorganisms by MALDI-TOF MS is done by comparing the peptide mass fingerprint (PMF) of tested organism with previously described PMFs from the database, or by matching the masses of biomarkers of tested microorganisms with the proteome database. In PMF matching, the MS spectrum of unknown microbial isolates is compared with the MS spectra of known microbial isolates in the database (Murray, 2012).

The aim of our study was to determine microbiota of traditional sheep cheese with mass spectrometry MALDI-TOF MS Biotyper.

MATERIAL AND METHODS

Samples

There were 30 samples of the Slovak national cheese „Bryndza“ examined in our study. Additionally, a total of 30 sheep milk cheese samples from the Slovak producers located in Slovakia were collected (Bukovina, Turčianské Teplice, Vazec, Zvolenská Slatina). All samples were placed in sterile sample containers and transported to laboratory on ice for microbiological investigations. Samples were kept in a refrigerator (4±1°C) until the testing began. The primary dilution of the milk products was made for preparing the samples for testing: a 5 ml of sample material was added to 45 ml of 0.87 % sterile saline. Then the serial dilutions (10−2 to 10−7) were done and a 100 µl of each dilution was plated out.

Isolation of coliform bacteria

Violet red bile lactose agar (VRBGA, Sigma-Aldrich®, St. Louis, USA) for enumeration of coliforms bacteria was used. Inoculated plates were incubated at 37°C for 24-48 h and then examined for the characteristics of typical colonies.

Isolation of enterococci

Enterococcus selective agar (ESA, Sigma-Aldrich®, St. Louis, USA) for enumeration of enterococci was used. Inoculated plates were incubated at 37°C for 24-48 h and then examined for the characteristics of typical colonies.

Isolation of Lactic Acid Bacteria (LAB)

MRS (Main Rogose agar, Oxoid, UK), MSE (Mayeux, Sandine and Elliker in 1962, Oxoid, UK), and APT (All Purpose TWEEN® agar, Oxoid, UK) agars were used for enumeration of LAB including lactobacilli, leuconostocs and lactic acid streptococci as well as other microorganisms with high requirements for thiamine (Sigma-Aldrich®, St. Louis, USA). Inoculated agars were incubated at 30°C for 72 h anaerobically and then the bacterial growth was evaluated.

Isolation of yeasts

Malt extract agar (Sigma-Aldrich®, St. Louis, USA) and acid base indicator bromocresol green (Sigma-Aldrich®, St. Louis, USA) (0.020 g.L−1) were used for yeasts identification. Inoculated plates were incubated at 25°C for 5 days aerobically and then the growth was evaluated.

Sample preparation and MALDI-TOF MS measurement

Prior to identification, the bacterial colonies were subcultured on TSA agar (Tryptone Soya Agar, Oxoid, UK) at 37°C for 18-24 h. One colony of eight bacterial isolate was selected. Subsequently, the identification was performed using the Maldi TOF MS Biotyper as was described by Kačániová et al. (2019). Totally, a number of 870 isolates were identified with a score higher than 2.

RESULTS AND DISCUSSION

Traditional bryndza is sharp, salty, greyish, grated and pin-rolled, crumbly, semi-spreadable 100% sheep cheese. There is no close equivalent in taste and texture among sheep, cow or goat cheeses. Unique food and drinks make up a significant part of Slovak culture, as the country produces several products which cannot be found or replicated in any other part of the world; bryndza cheese is one of those products (EC, 2008). The numbers of microorganisms in sheep cheese in our study is shown in table 1. Total count of bacteria in bryndza ranged from 3.83 to 3.78 log cfu.g−1. Enterococci were from 2.97 to 3.24 log cfu.g−1 in the studied samples. Coliform bacteria counts ranged from 3.07 to 3.85 log cfu.g−1, lactic acid bacteria counts ranged from 3.05 to 3.13 log cfu.g−1. The counts of yeasts ranged from 2.19 to 2.54 log cfu.g−1.

### Table 1 The number of isolated group of microorganisms from sheep cheese „Bryndza“ in cfu·g⁻¹

<table>
<thead>
<tr>
<th>Sample</th>
<th>Coliforms</th>
<th>Enterococcus</th>
<th>Total Bacterial Counts</th>
<th>Lactic Acid Bacteria</th>
<th>Yeasts</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>3.63±0.05</td>
<td>3.05±0.03</td>
<td>3.68±0.18</td>
<td>3.05±0.04</td>
<td>2.37±0.13</td>
</tr>
<tr>
<td>2.</td>
<td>3.42±0.03</td>
<td>2.97±0.02</td>
<td>3.78±0.12</td>
<td>3.07±0.07</td>
<td>2.37±0.14</td>
</tr>
<tr>
<td>3.</td>
<td>3.85±0.06</td>
<td>3.01±0.02</td>
<td>3.83±0.09</td>
<td>3.09±0.07</td>
<td>2.30±0.20</td>
</tr>
<tr>
<td>4.</td>
<td>3.07±0.02</td>
<td>3.08±0.06</td>
<td>3.73±0.13</td>
<td>3.13±0.02</td>
<td>2.54±0.62</td>
</tr>
<tr>
<td>5.</td>
<td>3.72±0.02</td>
<td>3.04±0.07</td>
<td>3.70±0.09</td>
<td>3.11±0.05</td>
<td>2.19±0.07</td>
</tr>
<tr>
<td>6.</td>
<td>3.54±0.02</td>
<td>3.07±0.04</td>
<td>3.72±0.12</td>
<td>3.10±0.04</td>
<td>2.63±0.06</td>
</tr>
<tr>
<td>7.</td>
<td>3.81±0.03</td>
<td>3.13±0.02</td>
<td>3.78±0.05</td>
<td>3.11±0.02</td>
<td>2.19±0.07</td>
</tr>
<tr>
<td>8.</td>
<td>3.71±0.11</td>
<td>3.24±0.22</td>
<td>3.67±0.13</td>
<td>3.09±0.03</td>
<td>2.19±0.10</td>
</tr>
<tr>
<td>9.</td>
<td>3.60±0.04</td>
<td>3.13±0.02</td>
<td>3.62±0.11</td>
<td>3.11±0.01</td>
<td>2.20±0.09</td>
</tr>
<tr>
<td>10.</td>
<td>3.52±0.12</td>
<td>3.11±0.05</td>
<td>3.72±0.12</td>
<td>3.13±0.02</td>
<td>2.30±0.09</td>
</tr>
</tbody>
</table>

A total of 40 species of 10 microbial families and 20 genera (14 Gram-negative (G−), 17 Gram-positive (G+) and 9 yeasts species) were identified in sheep cheese by MALDI-TOF Mass Spectrometry. The G−, G+ and yeasts comprised 25.86% (225 isolates), 49.43% (430 isolates) and 24.71% (215 isolates), respectively. Isolated species of bacteria from cheese „Bryndza“ are shown in table 2.
The most distributed bacterial species were Lactobacillaceae (with 7 species). Altogether, 14 species of Gram negative bacteria were isolated. Klebsiella spp. were the most abundant bacterial genus, while Dipodascus candidum was the most frequently isolated yeast species (table 5).

<table>
<thead>
<tr>
<th>Family</th>
<th>Genera</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moraxellaceae</td>
<td>Acinetobacter</td>
<td>Acinetobacter baumannii</td>
</tr>
<tr>
<td>Moraxellaceae</td>
<td>Acinetobacter</td>
<td>Acinetobacter tandoii</td>
</tr>
<tr>
<td>Bacillaceae</td>
<td>Bacillus</td>
<td>Bacillus pumilus</td>
</tr>
<tr>
<td>Saccharomycetaceae</td>
<td>Candida</td>
<td>Candida catenulata</td>
</tr>
<tr>
<td>Saccharomycetaceae</td>
<td>Candida</td>
<td>Candida kruisi</td>
</tr>
<tr>
<td>Saccharomycetaceae</td>
<td>Candida</td>
<td>Candida lastiniae</td>
</tr>
<tr>
<td>Saccharomycetaceae</td>
<td>Candida</td>
<td>Candida rugosa</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Citrobacter</td>
<td>Citrobacter braakii</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Citrobacter</td>
<td>Citrobacter koseri</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Enterobacter</td>
<td>Enterobacter cloacae</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Enterobacter</td>
<td>Enterobacter ludwigii</td>
</tr>
<tr>
<td>Enterococcaceae</td>
<td>Enterococcus</td>
<td>Enterococcus faecalis</td>
</tr>
<tr>
<td>Enterococcaceae</td>
<td>Enterococcus</td>
<td>Enterococcus faecium</td>
</tr>
<tr>
<td>Enterococcaceae</td>
<td>Enterococcus</td>
<td>Enterococcus hirae</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Escherichia</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Hafnia</td>
<td>Hafnia alvei</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Klebsiella</td>
<td>Klebsiella oxytoca</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Klebsiella</td>
<td>Klebsiella pneumoniae spp.</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Klebsiella</td>
<td>Klebsiella pneumoniae spp.</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Klebsiella</td>
<td>Klebsiella pneumoniae spp.</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>Lactobacillus</td>
<td>Lactobacillus brevis</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>Lactobacillus</td>
<td>Lactobacillus harbinensis</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>Lactobacillus</td>
<td>Lactobacillus johnsonii</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>Lactobacillus</td>
<td>Lactobacillus plantarum</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>Lactobacillus</td>
<td>Lactobacillus paracasei spp.</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>Lactobacillus</td>
<td>Lactobacillus paraplanatum</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>Lactobacillus</td>
<td>Lactobacillus suebicus</td>
</tr>
<tr>
<td>Streptococcaceae</td>
<td>Lactococcus</td>
<td>Lactococcus lactis ssp lactis</td>
</tr>
<tr>
<td>Streptococcaceae</td>
<td>Lactococcus</td>
<td>Lactococcus lactis</td>
</tr>
<tr>
<td>Microbacteriaceae</td>
<td>Microbacterium</td>
<td>Microbacterium liquefaciens</td>
</tr>
<tr>
<td>Lactobacillaceae</td>
<td>Pediococcus</td>
<td>Pediococcus acidilactici</td>
</tr>
<tr>
<td>Saccharomycetaceae</td>
<td>Pichia</td>
<td>Pichia catrophila</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Raoultella</td>
<td>Raoultella ornithinolytica</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Serratia</td>
<td>Serratia liquefaciens</td>
</tr>
<tr>
<td>Staphylococcaceae</td>
<td>Staphylococcus</td>
<td>Staphylococcus aureus ssp.</td>
</tr>
<tr>
<td>Staphylococcaceae</td>
<td>Staphylococcus</td>
<td>Staphylococcus aureus ssp.</td>
</tr>
<tr>
<td>Staphylococcaceae</td>
<td>Staphylococcus</td>
<td>Staphylococcus pasteurii</td>
</tr>
<tr>
<td>Xanthomonadaceae</td>
<td>Stenotrophomonas</td>
<td>Stenotrophomonas maltophilia</td>
</tr>
<tr>
<td>Dipodascaceae</td>
<td>Yarrowia</td>
<td>Yarrowia lipolytica</td>
</tr>
</tbody>
</table>

A total of 14 species of Gram-negative bacteria were isolated. Klebsiella spp. were represented by three species and were the most widespread bacterial genus. The most distributed bacterial species were Hafnia alvei, Klebsiella oxytoca and Enterobacter cloacae (table 3).

Previous culture-independent studies showed the diversity of bacteria and fungi and the changes is their population during the production of bryndza (Chebeňová-Turecovská et al., 2011; Pangalo et al., 2014). Interactions between the lactic acid bacteria and Galactomyces/Geistrichum group and coagulase-positive staphylococci were studied as well (Hudecová et al., 2011, Medveďová and Valík, 2012). The culture-dependent methods showed that the bryndza samples contained lactococci, lactobacilli and Galactomyces/Geistrichum in high numbers. Majority of lactobacilli were identified as Lactobacillus paracasei and Lactobacillus plantarum and lactococci as Lactococcus lactis with PCR-based identification methods. Culture-independent analysis revealed that Lactococcus spp. followed by Streptococcus spp. and Leuconostoc spp were the most abundant bacterial genera (Šaková et al., 2015).

Altogether, nine yeast species were isolated from sheep cheese. Candida spp. were the most abundant yeast genus, while Dipodascus candidum was the most frequently isolated yeast species (table 5).

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>No. of isolates</th>
<th>No. of isolates in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter baumannii</td>
<td>15</td>
<td>6.7</td>
</tr>
<tr>
<td>Acinetobacter tandoii</td>
<td>6</td>
<td>2.7</td>
</tr>
<tr>
<td>Citrobacter braakii</td>
<td>8</td>
<td>3.6</td>
</tr>
<tr>
<td>Citrobacter koseri</td>
<td>7</td>
<td>3.1</td>
</tr>
<tr>
<td>Enterobacter cloacae</td>
<td>21</td>
<td>9.3</td>
</tr>
<tr>
<td>Enterobacter ludwigii</td>
<td>15</td>
<td>6.7</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>15</td>
<td>6.7</td>
</tr>
<tr>
<td>Hafnia alvei</td>
<td>45</td>
<td>20.0</td>
</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>25</td>
<td>11.1</td>
</tr>
<tr>
<td>Klebsiella pneumoniae spp. ozenae</td>
<td>10</td>
<td>4.4</td>
</tr>
<tr>
<td>Raoultella ornithinolytica</td>
<td>20</td>
<td>8.9</td>
</tr>
<tr>
<td>Serratia liquefaciens</td>
<td>15</td>
<td>6.7</td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
<td>8</td>
<td>3.6</td>
</tr>
</tbody>
</table>

Galactomyces/Geistrichum, and yeasts Yarrowia lipolytica, Kluyveromyces lactis and Debaryomyces Hansenii were the main representative of eukaryotic microbiota in study of Šaková et al., 2015. Yarrowia lipolytica was identified as one of the most abundant species in the present study. Composition and activity of microflora is believed to have a great impact on the flavour of bryndza cheese. The compounds contained in ewes’ milk and from the products of fermentation of the substrate by microflora were responsible for typical sensory characteristics (Šidečka et al., 2014). In previous studies, Lactobacillus spp. (Berta et al., 1990), Lactococcus spp., Streptococcus spp., Enterococcus spp., Kluyveromyces marxianus and Galactomyces geotrichum were identified as the main microorganism of bryndza cheese (Görner, 1980;
Microbiologically, the 40 species of 20 bacterial genera of three main groups of microorganisms were identified with MALDI-TOF Mass Spectrometry. The Gram-negative, Gram-positive bacteria and yeasts comprised 25.86% (225 isolates) 49.43% (430 isolates) and 24.71% (215 isolates), accordingly. Fast microbial identification is in high demand in industry for improving of HACCP-based procedures, reduce biocide consumption and to avoid the distribution of contaminated products. The speed and precision of microbial identification with MALDI-TOF-MS were well described for clinical isolates, but the present study show that the methods could be applicable for dairy producing and industrial applications.

CONCLUSION

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