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THE CHARACTERISTIC OF SHEEP CHEESE "BRYNDZA" FROM DIFFERENT REGIONS OF SLOVAKIA BASED ON MICROBIOLOGICAL QUALITY

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ABSTRACT
The aim of our study was to describe microorganisms which occur in the traditional Slovak cheese „Bryndza". There were a total of 60 cheese samples collected from ten different farms during May 2019. The microbiota studies included the total bacterial count, coliforms, enterococci, lactic acid bacteria, yeasts and microscopic fungi. The total bacterial counts were cultivated on plate count agar at 30 °C in aerobic conditions, lactic acid bacteria on MRS at 37 °C in anaerobic conditions, coliform on VRBL and VRBG at 37 °C in aerobic condition, yeasts and microscopic fungi on MEA at 25 °C under aerobic condition. Gram-positive, Gram-negative and yeasts isolates were identified with MALDI-TOF MS Biotyper. Totally, a number of 1175 isolates of G^-, G^+ and yeast were identified with score higher than 2 and moulds. *Escherichia coli* and *Stenotrophomonas maltophilia* were the most frequently identified species of Gram-negative and *Leuconostoc mesenteroides ssp. mesenteroides* and *Lactococcus lactis ssp. lactis* from Gram-positive bacteria. *Yarrowia lipolitica* and *Kluyveromyces lactis* were the most distributed yeasts. Lactic acid bacteria group was represented by *Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus.* The most abundant genera of lactic acid bacteria were *Lactobacillus* with 11 species. This study describes the indigenous microbiota of the traditional ewe's milk cheeses from Slovakia.

Keywords: isolation and identification of microorganisms; MALDI TOF MS Biotyper; Slovak ewe's cheese

INTRODUCTION
Slovak „Bryndza" is a natural white, gently spreadable, slightly moist fresh ripened cheese with curds and own texture, made in a traditional way from well-fermented ripened ewe's lump cheese.

A characteristic feature of the production of Slovak „Bryndza" is the crushing and grinding of mature ewe's or a mixture of ewe's and cow's lump cheese and their mixing with salt or specially prepared saline solution to achieve the required composition, which distinguishes this production from the production of other ewe's cheese produced outside Slovakia. Its characteristic sensory attributes are due to the natural microflora contained in raw ewe's milk and ewe's lump cheese and to the characteristic production method. The basic raw material for the production of Slovak „Bryndza" is ewe's lump cheese or a mixture of ewe's and cow's lump cheese, or a mixture of cured ewe's lump cheese and cow's lump cheese aged under specific conditions **(Commission Regulation No. 676/2008)**.

Microorganisms represent without doubt the largest group of living organisms in the world, with only a small fraction of microbial species which have been identified until now. They can be highly diverse in their biochemistry, physiology and nutritional modes. Most of them are reproducing swiftly and the significant plasticity of their genome allows them to easily adapt to changing environmental conditions, as well as perform a variety of essential ecosystem functions, on which food production depends on. According to **FAO (2009)**, the main functional groups for food processing are beneficial microorganisms (fermentation and probiotics). Microbial food cultures include bacterial food cultures, fungi and yeasts. These microorganisms determine the characteristics of the fermented food, e.g., acidity, flavour and texture, as well as health benefits that go beyond elementar nutrition **(Vogel et al., 2011)**.

The aim of our study was to isolate and identificate the microorganisms from Slovak ewe's cheese „Bryndza" obtained from different Slovak regions.

Scientific hypothesis
Slovak „Bryndza" is specific traditional food product with various microorganisms, which has positive and negative role of quality.

Hypothesis no. 1: There are a lot of different bacteria and yeast species presented in the traditional Slovak sheep cheese called „Bryndza".

Hypothesis no. 2: There are microscopic filamentous fungi presented in the traditional Slovak sheep cheese „Bryndza".

MATERIAL AND METHODOLOGY

There were 60 samples of Slovak ewe's cheese „Bryndza“ from east, middle and west part of Slovakia evaluated for microbiological quality in our study. All samples were obtained in May 2019. These samples were placed in sterile sample containers and transported on ice to the laboratory for microbiological investigations. Samples were kept in a refrigerator (4 ±1 °C) until the testing began. The primary dilution of the ewe's cheese 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^{-4}) were done and a 100 μL of each dilution was plated out.

Determination of total bacterial count

Plate count agar (PCA, Sigma-Aldrich®, St. Louis, USA) for total microbial count enumeration was used. Inoculated plates were incubated at 30 °C for 24 – 48 h and then examined for the characteristics of bacterial colonies.

Isolation of coliform bacteria

The 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 the identification, the bacterial and yeasts 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)**. We identified totally 870 isolates with a score higher than 2 **(Kačániová et al., 2019)**.

Identification of microscopic fungi

Microscopic fungi were identified to species level according to the manuals of **Samson et al. (2002)**, **Samson and Frisvad (2004)**, **Pitt and Hocking (2009)**.

Statistical analysis

All experiments were carried out in triplicate and the results reported are the results of those replicate determinations with standard deviations.

RESULTS AND DISCUSSION

Different groups of microorganisms were isolated from the 60 ewe's cheese „Bryndza“ samples (Table 1). Total bacterial count in ewe's cheese ranged from 3.87 ±0.58 $CFU.g^{-1}$ from west Slovak producers to 4.32 ±0.17 $CFU.g^{-1}$ from middle Slovak producers. Generally, the coliform bacteria ranged from 3.46 ±0.26 $CFU.g^{-1}$ from east Slovak producers to 3.64 ±0.19 $CFU.g^{-1}$ for bryndza from middle Slovak producers. The number of lactic acid bacteria ranged from 3.14 ±0.09 $CFU.g^{-1}$ from east Slovak producers to 3.24 ±0.21 $CFU.g^{-1}$ in the bryndza cheeses from west Slovak producers. Table 2a and Table 2b showes isolated species of bacteria.

The eukaryotic microorganisms were represented largely by members of the genera of *Dipodascus* and *Kluyveromyces*, which were present at a level of 99 isolates and 60 isolates, and by other yeasts, which were present in *Candida* genera (Figure 3). The moulds were present generally at middle levels, with the most colonies of *Rhizopus* spp. With 21 isolates. All samples contained high numbers of lactic acid bacteria belonging to genera *Lactobacillus*, *Lactococcus* and *Leuconostoc*. In order to obtain a better view of the lactic acid bacteria isolates, the *Enterococcus, Lactobacillus, Lactococcus, Leuconostoc and Pediococcus* strains were identified by mass spectrometry assays. Representatives of the species *Leuconostoc mesenteroides* ssp. *mesenteroides* were isolated and identified in all bryndza cheese samples (Figure 2).

Different *Lactobacillus* species, such as *Lb. brevis*, *Lb. delbrueckii*, *Lb. fermentum*, *Lb. helveticus*, *Lb. harbinensis*, *Lb. jonsonii*, *Lb. paracasei* ssp. *paracasei*, *Lb. plantarum*, *Lb. paraplantarum*, *Lb. rhamnosus* and *Lb. suebicus*, were identified in the bryndza cheese samples from all producers (Figure 2). The most isolated species were *Lb. brevis*, *Lb. fermentum* and *Lb. plantarum.*

There were totally 1175 isolates identified by mass spectrometry include G^-, G^+ and microscopic filamentous fungi in our study. Together 199 isolates were (Figure 1) isolated and identified from G^- and most frequently species was *Escherichia coli*, 599 isolates from G^+ with most isolated species *Leuconostoc mesenteroides* ssp. *mesenteroides* (Figure 2) and 377 isolates of yeast and molds where the most frequently isolated species was *Yarrowia lipolitica* (Figure 3).

Table 1 Groups of microorganisms in ewe's cheese „Bryndza".

Microorganisms	Content CFU.g^{-1}		
	east	middle	west
Total bacterial count	4.05 ±0.45	4.32 ±0.17	3.87 ±0.58
Coliforms bacteria	3.64 ±0.19	3.60 ±0.21	3.46 ±0.26
Enterococci	2.77 ±0.23	2.71 ±0.17	2.67 ±0.29
Lactic acid bacteria	3.24 ±0.21	3.17 ±0.10	3.14 ±0.09
Yeats and molds	2.41 ±0.19	2.28 ±0.14	2.18 ±0.10

Table 2a Isolated family, genera and species from ewe's cheese.

Family	Genera	Species
Moraxellaceae	*Acinetobacter*	*Acinetobacter baumannii*
Moraxellaceae	*Acinetobacter*	*Acinetobacter tandoii*
Bacillaceae	*Bacillus*	*Bacillus pumilus*
Saccharomycetaceae	*Candida*	*Candida catenulate*
Saccharomycetaceae	*Candida*	*Candida krusei*
Saccharomycetaceae	*Candida*	*Candida lusitaniae*
Saccharomycetaceae	*Candida*	*Candida rugose*
Saccharomycetaceae	*Candida*	*Candida utilis*
Enterobacteriaceae	*Citrobacter*	*Citrobacter braakii*
Enterobacteriaceae	*Citrobacter*	*Citrobacter koseri*
Davidiellaceae	*Cladosporium*	*Cladosporium* spp.
Dipodascaceae	*Dipodascus*	*Dipodascus candidum*
Dipodascaceae	*Dipodascus*	*Dipodascus silvicola*
Enterobacteriaceae	*Enterobacter*	*Enterobacter cloacae*
Enterobacteriaceae	*Enterobacter*	*Enterobacter ludwigii*
Enterococcaceae	*Enterococcus*	*Enterococcus faecalis*
Enterococcaceae	*Enterococcus*	*Enterococcus faecium*
Enterococcaceae	*Enterococcus*	*Enterococcus hirae*
Enterobacteriaceae	*Escherichia*	*Escherichia coli*
Enterobacteriaceae	*Hafnia*	*Hafnia alvei*
Enterobacteriaceae	*Klebsiella*	*Klebsiella oxytoca*
Enterobacteriaceae	*Klebsiella*	*Klebsiella pneumoniae* ssp. *ozaenae*
Enterobacteriaceae	*Klebsiella*	*Klebsiella pneumoniae* ssp. *pneumonia*
Saccharomycetaceae	*Kluyveromyces*	*Kluyveromyces lactis*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus brevis*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus delbrueckii*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus fermentum*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus helveticus*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus harbinensis*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus johnsonii*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus paracasei* ssp. *paracasei*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus plantarum*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus paraplantarum*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus rhamnosus*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus suebicus*
Streptococcaceae	*Lactococcus*	*Lactococcus lactis* ssp. *lactis*
Streptococcaceae	*Lactococcus*	*Lactococcus lactis* ssp. *cremoris*
Lactobacillaceae	*Leuconostoc*	*Leuconostoc mesenteroides* ssp. *mesenteroides*

Table 2b Isolated family, genera and species from ewe's cheese.

Family	Genera	Species
Microbacteriaceae	*Microbacterium*	*Microbacterium liquefaciens*
Lactobacillaceae	*Pediococcus*	*Pediococcus acidilactici*
Saccharomycetaceae	*Pichia*	*Pichia cactophila*
Saccharomycetaceae	*Pichia*	*Pichia fermentas*
Enterobacteriaceae	*Raoultella*	*Raoultella ornithinolytica*
Mucoraceae	*Rhizopus*	*Rhizopus* spp.
Enterobacteriaceae	*Serratia*	*Serratia liquefaciens*
Staphylococcaceae	*Staphylococcus*	*Staphylococcus aureus* ssp. *aureus*
Staphylococcaceae	*Staphylococcus*	*Staphylococcus pasteuri*
Xanthomonadaceae	*Stenotrophomonas*	*Stenotrophomonas maltophilia*
Dipodascaceae	*Yarrowia*	*Yarrowia lipolytica*

Figure 1 Gram negative bacteria isolated from ewe's cheese bryndza.

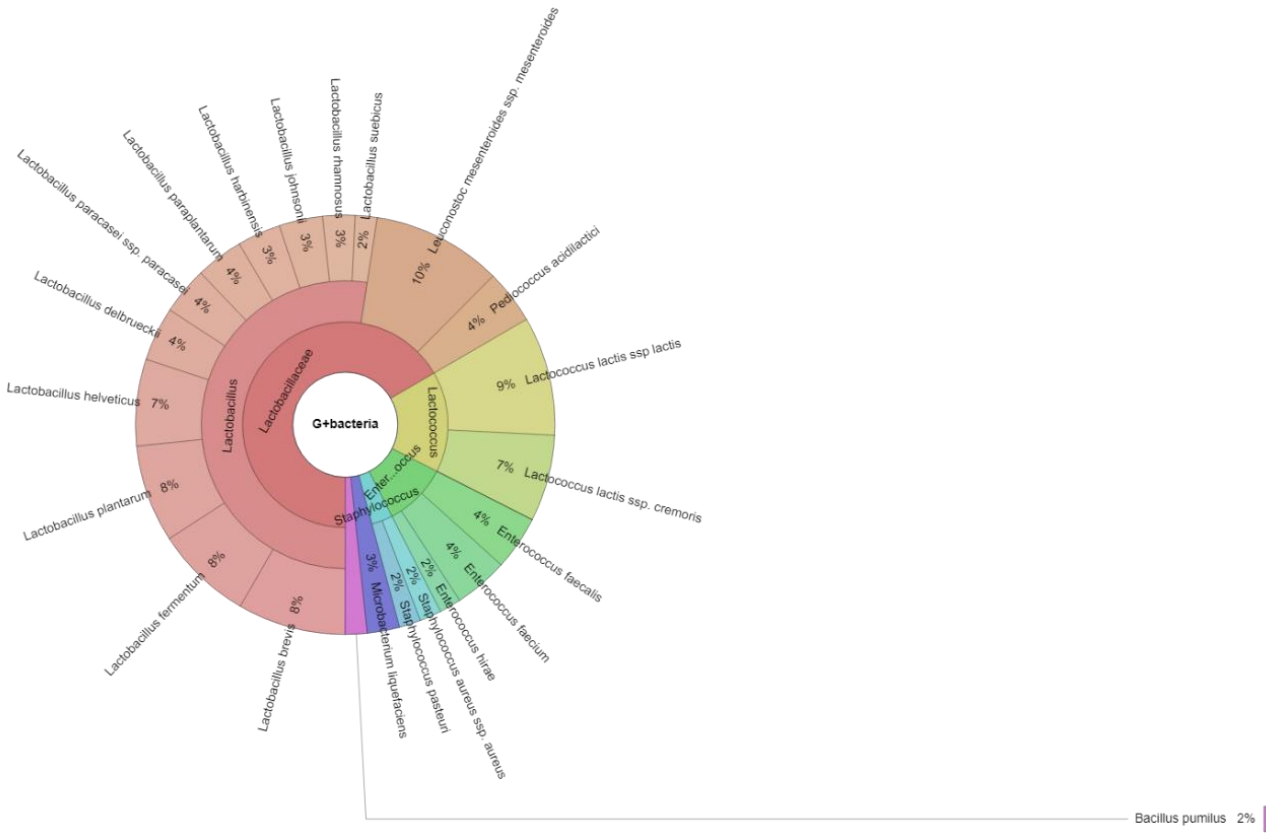

Figure 2 Gram positive bacteria isolated from ewe's cheese bryndza.

Figure 3 Microscopic filamentous fungi isolated from ewe's cheese bryndza.

The distinctive flavour of bryndza cheese produced in month May is apparently composed from compounds contained in ewes' milk and from the products of fermentation of the substrate by microflora. Principal volatile aroma-active compounds of May bryndza cheese have been characterized by **Sádecká et al. (2014)**.

Due to composition and activity of microflora is estimated to have a great impact on the flavour of bryndza cheese, several culture-based as well as culture-independent microbiological studies were carried out in this regard. Data from older culture based studies, which identified *Lactobacillus* spp., *Lactococcus* spp., *Streptococcus* spp., *Enterococcus* spp., *Kluyveromyces marxianus* and *Galactomyces geotrichum* as main components of the microflora of bryndza cheese **(Palo and Kalab, 1984; Görner and Valík, 2004; Görner, 1980)** were updated by a study of **Berta et al. (2009)**, in which a range of *Lactobacillus* spp. isolates were identified by 16S rDNA sequencing. Enterococci **(Jurkovič et al., 2006)**, staphylococci **(Mikulášová et al., 2014)** and fungal species **(Laurenčík et al., 2008)** were cultured and identified in bryndza cheese. Culture-independent studies **(Chebeňová-Turcovská et al., 2011; Pangallo et al., 2014)** provided information on the diversity of bacteria and fungi and its dynamics during the production of bryndza cheese. In the production of bryndza cheese, also interactions between lactic acid bacteria and *Galactomyces*/*Geotrichum* group **(Hudecová et al., 2011)** and competition between lactic acid bacteria and coagulase-positive staphylococci **(Medveďová and Valík, 2012)** were studied.

Although basic information on May bryndza cheese is available regarding microbiological composition as well as aroma-active compounds, most of the previous experiments were done on a limited geographical basis, sometimes with products of just one factory. In order to obtain a more reliable and representative view, this study aimed to gain data for the products from the entire territory of Slovakia that is relevant to bryndza production, i.e. specified mountainous regions of Slovakia **(Commission Regulation (EC) No. 676/2008)**. In Slovakia, the presence of Carpathian Mountains creates different climatic conditions that can have influence various characteristics of the produced bryndza cheese. These can relate, in particular, to the ewe's diet in terms of different plant species composition in the pasture and, therefore, to the quality of milk used for the production of bryndza **(Ostrovský et al., 2009)** and to different temperatures at which the lump cheese is produced, which can affect the microbial consortia in the beginning of the ripening process **(Görner and Valík, 2004)**.

CONCLUSION

The aim of our study was to evaluate the microbiological quality of Slovak ewe's cheese bryndza from producers of east, middle and west Slovakia. The number of isolated group of microorganisms was accurate for the traditional cheese produced in Slovakia. Totally 1175 isolates of bacteria with score more than 2 were identified with MALDI TOF MS Biotyper.

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