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DIVERSITY OF MICROORGANISMS IN THE TRADITIONAL SLOVAK CHEESE

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ABSTRACT
The aim of the present study was to describe the microbial groups of the traditional Slovak cheese Parenica during rippening. The microbial group included the total bacterial count, coliform bacteria, enterococci, lactic acid bacteria, and microscopic filamentous fungi, which may affect the organoleptic characteristics of this product. A total of 42 cheese samples were collected from four different farms during three months. The total bacterial counts were cultivated on Plate count agar at 30 °C, lactic acid bacteria (LAB) on MRS, APT and MSE at 37 °C, coliform bacteria on VRBL at 37 °C. Gram-positive and Gram-negative isolates were identified by MALDI-TOF MS profiling. *Bacillus* sp. and *Enterococcus faecium* were the most frequently identified species of bacteria. *Candida kefyr* was the most distributed yeast according to microbiological methods. Lactic acid bacteria group was represented by *Lactobacillus helveticus, L. jensenii, L. alimentarius, L. crispatus, L. curvatus, L. fermentum, L. suebicus, L. delbrueckii* ssp. *lactis, L. paracasei* ssp. *paracasei, Lactococcus lactis* ssp. *lactis, Leuconostoc lactis* and *Le. mesenteroides* ssp. *mesenteroides* . This report describing the indigenous microbiota of the traditional raw milk cheeses from Slovakia. Our results provide useful information on occurrence of valuable microbial strain for the industrialization of producing of the traditional dairy products in Slovakia.

Keywords: diversity; microbiota; smoked and non-smoked cheese; mass spectrometry

INTRODUCTION
Cheese is one of the oldest fermented foods **(Oyetunji and Adebisi, 2018; Franke and Cwiková, 2019)**. The history of cheese lasts for thousands of years with changed related to the technical, social and economic conditions in different parts of the globe. Therefore the cheese fermentation process is attributed to culture and tradition **(Štefániková et al., 2019)**. Especially cheese making traditions are pronounced in rural households and village communities. There are around 1000 types of (most artisanal) cheese known worldwide. Cheeses are very different in textures, aromas, visual presentations and flavours that is attributed to microbial activity. The microorganisms are present and growing in cheese-making process in large numbers and which degrade the components of the curd **(Montel et al., 2014)**.

Diversity of sensory characteristics as flavor, smell, and texture of cheese are linked to microbiological activity in the product. Microorganisms show large metabolic capacities, and contribute the sensory parameters through the production of digestive enzymes and small molecules. Different cheese production technologies can lead to the growth of different microbial groups or microorganisms. The source and raw milk treatment (raw or pasteurized) used for cheesemaking can result in different microbiota of cheese. Changes in product characteristics during aging can significantly influence the cheese-associated microbiota with pH, salt, moisture, and temperature of cheese reveal the biggest impact on microorganisms **(Button and Dutton, 2012)**.

The microbiota of cheese rinds vary from simple to complex with *Firmicutes*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, yeasts and moulds are represented. The abundance and diversity of the microorganisms depend on cheese ripening process, type of rind (bloomy, washed or natural) and the processing technology (soft, hard or semi-hard). The lactic acid bacteria (LAB) from starter cultures are predominant in the initial stages of cheese ripening. Later, during ripening, the yeasts and/or moulds colonize the cheese surface and the yeast cell count may reach about 6 – 8 $\log_{10}$ cfu.cm^{-2}. Those counts remains unchanged until the end of ripening. The growth of yeasts and/or moulds lead to lower pH of the cheese surface that favours the development of acid-sensitive, salt-tolerant bacteria. The final bacterial counts were 1 – 2 $\log_{10}$ higher than that for yeast counts **(Cogan et al., 2014)**.

From a technological point of view, the contamination of processed cheese by Gram-positive spore-forming rod-shaped bacteria of the genera *Bacillus*, *Geobacillus*, and *Clostridium* is the most significant problem in cheese-making. The shelf-life and microbiologicl quality of processed mostly are affected by the microbiological quality of the raw material, hygienic conditions during the manufacturing process, type of packaging materials and

storage conditions. Other parameters such as the water activity, pH, salts and emulsifying salts and fat in the product influence the overall quality of processed cheese as well **(Buňková and Buňka, 2017)**.

The aim of our study was to detect the microbiota of the traditional Slovak cheese „Parenica" and to identify the microorganisms by mass spectrometry.

Scientific hypothesis

Are there different bacteria and yeast species present in smoked and non-smoked traditional Slovak cheese?

Are microscopic filamentous fungi presented in traditional Slovak cheese?

MATERIAL AND METHODOLOGY

Samples of cheese

In our study, 42 samples of Slovak traditional cows cheese "Parenica" were examined during three moths. The cheese samples included non-smoked cheese (n = 21) and smoked cheese (n = 21). Additionally, 42 cow milk cheese samples from the western and middle Slovak producers were collected (Bánovce nad Bebravou, Liptovský Mikuláš, Červený Kameň, Važec). Samples were collected in sterilized sample containers and brought to laboratory in icebox for microbiological investigations. Samples were kept in a refrigerator (4 ±1 °C) until the testing began.

The primary dilution of cheese were made by adding of 5 g of sample to 45 mL of 0.87% sterile saline. Then, the serial dilutions (10^{-2} to 10^{-4}) were done and 100 µL of each dilution was plated out onto agars.

Detection of total bacterial counts (TCB)

Plate count agar (PCA, (Sigma-Aldrich®, St. Louis, USA) for total count bacteria enumeration was used. Inoculated plates were incubated for 48 – 72 h at 30 °C and then examined for the presence of bacterial colonies.

Isolation of coliform bacteria (CB)

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 examined for the presence of typical colonies.

Isolation of enterococci (E)

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 examined for the presence of typical colonies.

Isolation of lactic acid bacteria (LAB)

MRS (Main Rogose agar), MSE (Mayeux, Sandine and Elliker) and APT (All Purpose TWEEN® agar, Sigma-Aldrich®, St. Louis, USA) agars were used for cultivation of lactic acid bacteria. Inoculated plates were incubated at 37 °C for 72 h anaerobically and then the bacterial growth was evaluated.

Isolation of microscopic fungi and yeasts (MFY)

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 microscopic fungi and yeasts identification. Inoculated plates were incubated at 25 °C for 5 days aerobically and then the growth was evaluated.

The colonies from total bacterial counts, *Enterobacteriales*, enterococci and lactic acid bacteria were selected for further confirmation with MALDI-TOF. Selected colonies were cultured overnight on TSA agar (Tryptone Soya Agar) aerobically or anaerobically and used for identification.

Sample preparation and MALDI-TOF MS measurement

One colony of each bacterial isolate was transferred into an Eppendorf tubes and mixed with 300 µL of sterile water. After addition of ethanol (900 µL), the suspension was mixed and centrifuged (13,000 g, 2 min). After removal of supernatant, the pellets were dried at room temperature at least for 5 min. The bacterial pellets were resuspended in 20 – 50 µL of formic acid (70%) and the same amount of acetonitrile. After centrifugation (2 min at 13,000 g), a 1 µL of supernatant was spotted onto a sample position of a polished steel MALDI target plate and dried at room temperature. A 1 µL of MALDI matrix (solution of α-cyano-4-hydroxycinnamic acid (HCCA) in 50% acetonitrile/2.5% trifluoro-acetic acid) was added to the spot and dried.

The MALDI target plate was introduced into the MALDI-TOF mass spectrometer for automated measurement and data interpretation. MALDI-TOF profile mass spectra were imported into the MALDI Biotyper 3.0 software and processed automatically after measurement. The logarithm of the score (log[score]) was displayed as the matching result. The MALDI Biotyper output was a log(score) between 0 and 3.0, which was calculated from a comparison of the peak list from an unknown isolate with the reference MSP in the database. A log(score) ≥1.7 indicated identification at the genus level, log(score) ≥2.0 was set as the threshold for a match at the species level. Isolates with ≥2.0 were accepted as a correct identification.

Statistic analysis

All experiments were carried out in triplicate. Standard deviations were calculated for replications. The experimental data were subjected to analysis of variance (Duncan's test) at the 95% confidence level of 0.05 (software XL STAT, 2019).

RESULTS AND DISCUSSION

The results on non-smoked and smoked cheese bacterial counts, including coliform bacteria, enterococci, total bacterial count, lactic acid bacteria and microscopic filamentous fungi are shown in Table 1. Coliforms bacteria were not found in samples with exception of sample no.6, where coliform bacteria counts ranged from 3.17 to 3.43 log $CFU.g^{-1}$. Number of enterococci ranged from 2.09 till 4.32 log $CFU.g^{-1}$. Total count of bacteria in cheese samples ranged from 3.17 till 4.36 log $CFU.g^{-1}$. Microscopic filamentous fungi ranged from 2.26 till 3.38 log $CFU.g^{-1}$. How we can see in our results the worst results were found in sample number 6. The composition of the cheese surface microbiota has been studied for

several decades via the application of conventional, culture-based analyses **(Valdés-Stauber, Scherer and Seiler, 1997; Maoz, Mayr and Scherer, 2003; Viljoen, Khoury and Hattingh, 2003; Feurer et al., 2004; Mounier et al., 2005, Mounier et al., 2009; Callon et al., 2006; Florez and Mayo, 2006; Larpin et al., 2006; Lopandic et al., 2006; Rea et al., 2007; Goerges et al., 2008; Bleicher et al., 2010; Roth et al., 2010; Larpin-Laborde et al., 2011; Amato et al., 2012; Lavoie et al., 2012; Panelli et al., 2012; Gori et al., 2013; Cogan et al., 2014; Gkatzionis et al., 2014)**.

From the genus Acinetobacter 4 different strains were isolated: *A. baumannii, A. dijkshoorniae, A. johnsonii* and *A. junii. Acinetobacter* were found in 33.33% of samples (Table 2). Candida was also widelt distributed and *C. kefyr, C. parapsilosis, C. rugosa, C. guilliermondii, candida zeylanoides* and *C. lusitaniae* were isolated. From Enterobacteriaceae *Enterobacter cloacea, Hafnia alvei, Citrobacter braakii, C. freundii, Klebsiella oxytoca* and *Serratia ureilytica* were found. The most abundant genus was Enterococcus with three species were detected: *E. durans, E. faecium* and *E. faecalis*. Among yeasts *Kluyveromyces, Saccharomyces* and *Yarrowia* were identified.

Secondary microflora such as yeasts could be frequently isolated from different types of cheeses with counts from $10^4 – 10^6$ cfu.g^{-1} to $10^7 – 10^8$ cfu.g^{-1}. The presence of yeasts in cheese could be attributed to the favourable conditions for their growth and the wide distribution in dairy environment **(Wyder, 2003; Alessandria et al., 2010; Mirzaei, 2011)**. The role of the presence of yeast depends on the particular type of cheese **(Wyder, 2003; Colombo, Borgo and Fortina, 2009)**. Yeasts are important for bacterial development in cheese but their influence on bacteria may vary **(Mounier et al., 2009)**. Yeasts can cause sensory defects as excessive gas production and cheese blowing, bitter taste, fruit flavours, changes in acidity and texture profile **(Wyder, 2003)**. Nevertheless, the yeasts can alter the unique characteristics of several cheeses due to their lipolytic and proteolytic activities, formation of aromatic compounds, and degradation of the lactic acid **(Wyder, 2003; De Freitas et al., 2009)**.

Among lactic acid bacteria the following species were isolated: *Lactobacillus helveticus, L. jensenii, L. alimentarius, L. crispatus, L. curvatus, L. fermentum, L. suebicus, L. delbrueckii* ssp. *lactis, L. paracasei* ssp. *paracasei Lactococcus lactis* ssp. *lactis, Leuconostoc lactis* and *Leu. mesenteroides* subsp. *mesenteroides* (Table 2).

Lactic acid bacteria are the most studied microorganisms in milk fermentation **(Olson, 1990; Urbach, 1995; Maragkoudakis et al., 2006)**. The LAB in milk fermentation can be either as contaminants or content starter cultures. Milk itself serve as one of the natural source of LAB **(Delavenne et al., 2012; Wouters et al., 2002)**. Under spontaneous fermentations, the growth of LAB can not be controlled, but this procedure has been done during traditional cheese production. Backslopping also is often used in traditional cheese production and under this procedure fermented milk products as artisanal cheese klila **(Mennane et al., 2007),** kumis **(Chaves-López et al., 2011),** iben **(Ouadghiri et al., 2008)** and kurut **(Sun et al., 2010)** are produced. In general, the technology of milk fermentation is relatively simple and cost-effective. The large-scale production of standardized fermented milk products in controlled conditions require the industrial application of LAB as starter cultures. There are significant differences in the composition of industrally produced cheese with additional of starter culture from naturally fermented product with high microbial diversity in the traditional, naturally fermented products **(Widyastuti, Rohmatussolihat and Febrisiantosa, 2014)**.

Among staphylococci *Staphylococcus warneri, S. epidermidis, S. saprophyticus subsp. saprophyticus, S. sciuri subsp. carnaticus, S. cohnii, S. xylosus* and *S. hominis* were isolated.

Three *Staphylococcus* species were isolated previously with distribution of certain species in particular cheese: *S. equorum* was unique to ardrahan and milleens cheeses, *S. saprophyticus* to gubbeen cheese, and *S. epidermidis* to durrus cheese. *S. equorum* and *S. saprophyticus* have been

Table 1 Microbial counts in traditional Slovak cheese.

	CB	E	TCB	LAB	MFY
Non- smoked cheese 1	0.00 ±0.00	2.46 ±0.05^{def}	3.67 ±0.06^{cde}	3.77 ±0.16^{a}	2.72 ±0.14^{bc}
Smoked cheese 1	0.00 ±0.00	2.29 ±0.07^{fgh}	3.54 ±0.09^{ef}	3.47 ±0.20^{bcde}	2.30 ±0.13^{ef}
Non- smoked cheese 2	0.00 ±0.00	2.60 ±0.07^{d}	3.62 ±0.09^{de}	3.87 ±0.09^{a}	2.79 ±0.05^{b}
Smoked cheese 2	0.00 ±0.00	2.23 ±0.02^{hi}	3.17 ±0.05^{h}	3.52 ±0.06^{bcd}	2.46 ±0.14^{cde}
Non- smoked cheese 3	0.00 ±0.00	2.41 ±0.15^{efg}	3.74 ±0.15^{cd}	3.64 ±0.10^{abc}	2.52 ±0.07^{cd}
Smoked cheese 3	0.00 ±0.00	2.17 ±0.02^{hi}	3.24 ±0.04^{gh}	3.46 ±0.19^{bcde}	2.26 ±0.05^{f}
Non- smoked cheese 4	0.00 ±0.00	2.55 ±0.02^{de}	3.70 ±0.14^{cde}	3.70 ±0.17^{ab}	2.75 ±0.10^{b}
Smoked cheese 4	0.00 ±0.00	2.32 ±0.11^{fgh}	3.34 ±0.08^{gh}	3.40 ±0.17^{cdef}	2.43 ±0.09^{def}
Non- smoked cheese 5	0.00 ±0.00	2.44 ±0.11^{defg}	3.88 ±0.09^{c}	3.51 ±0.19^{bcd}	2.72 ±0.17^{b}
Smoked cheese 5	0.00 ±0.00	2.09 ±0.04^{i}	3.41 ±0.13^{fg}	3.23 ±0.09^{ef}	2.38 ±0.15^{def}
Non- smoked cheese 6	3.17 ±0.03^{b}	4.32 ±0.23^{a}	4.36 ±0.14^{a}	3.37 ±0.09^{def}	3.38 ±0.12^{a}
Smoked cheese 6	3.43 ±0.10^{a}	3.53 ±0.06^{b}	4.08 ±0.03^{b}	3.19 ±0.07^{f}	3.28 ±0.06^{a}
Non- smoked cheese 7	0.00 ±0.00	2.84 ±0.09^{c}	3.60 ±0.07^{de}	3.68 ±0.10^{ab}	2.75 ±0.09^{b}
Smoked cheese 7	0.00 ±0.00	2.27 ±0.06^{gh}	3.27 ±0.03^{gh}	3.50 ±0.14^{bcd}	2.37 ±0.05^{def}

Note: mean ± standard deviation; different letters in column mean that the differences were significant.

Table 2 Microorganisms isolated from non-smoked and smoked cheese "Parenica".

Sample	Isolated microorganisms
1N	*Enterococcus faecalis, Enterococcus faecium, Candida kefyr, Lactobacillus helveticus, Lactobacillus jensenii, Lysinibacillus boronitolerans Sphingomonas parapaucimobilis*
1U	*Candida kefyr, Kocuria kristinae, Lactococcus lactis* subsp. *lactis, Lactobacillus alimentarius, Lactobacillus jensenii, Lactobacillus crispatus, Lactobacillus curvatus, Lysinibacillus boronitolerans, Staphylococcus epidermidis, Staphylococcus saprophyticus* subsp. *saprophyticus, Streptococcus lutetiensis, Sphingomonas parapaucimobilis,*
2N	*Bacillus megatherium, Bacillus safensis, Bacillus* sp., *Candida kefyr, Candida parapsilosis, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Lysinibacillus boronitolerans, Staphylococcus saprophyticus* ssp. *saprophyticus, Staphylococcus warneri,*
2U	*Acinetobacter dijkshoorniae, Bacillus badius, Candida kefyr, Enterobacter cloacae, Enterococcus faecalis, Enterococcus faecium, Lactobacillus fermentum, Lactobacillus helveticus, Lactobacillus suebicus, Lysinibacillus boronitolerans, Micrococcus luteus, Paenibacillus lactis, Raoultella ornithinolytica, Staphylococcus warneri*
3N	*Bacillus* sp., *Candida kefyr, Lactobacillus delbrueckii* subsp. *lactis, Leuconostoc lactis, Enterococcus faecium*
3U	*Bacillus* sp., *Candida kefyr, Lactobacillus delbrueckii* subsp. *lactis, Leuconostoc lactis, Staphylococcus hominis*
4N	*Enterococcus durans, Enterococcus faecalis, Klebsiella oxytoca, Lactobacillus oligofermentans, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus rhamnosus, Lysinibacillus fusiformis, Serratia ureilytica, Stenotrophomonas acidaminiphila, Staphylococcus sciuri* ssp. *carnaticus*
4U	*Bacillus safensis, Bacillus* sp., *Candida kefyr, Enterococcus faecalis, Enterococcus faecium, Enterococcus durans, Lysinibacillus boronitolerans, Proteus hauseri, Staphylococcus saprophyticus* subsp. *saprophyticus,*
5N	*Bacillus* sp., *Candida parapsilosis, Cupriavidus metallidurans, Enterococcus durans, Enterococcus faecalis, Enterococcus faecium, Kluyveromyces lactis, Lactobacillus paracasei* subsp. *paracasei, Lactobacillus rhamnosus, Leuconostoc mesenteroides* subsp. *mesenteroides, Micrococcus luteus, Staphylococcus epidermidis*
5U	*Bacillus* sp., *Bacillus safensis, Enterococcus durans, Lactobacillus paracasei* subsp. *paracasei, Sphingomonas parapaucimobilis*
6N	*Acinetobacter baumannii, Acinetobacter junii, Bacillus infantis, Bacillus sp., Candida parapsilosis, Candida rugosa, Citrobacter braakii, Chryseobacterium ureilyticum, Enterococcus faecalis, Enterococcus durans, Hafnia alvei, Leuconostoc lactis, Paenibacillus lactis, Raoultella ornithinolytica, Staphylococcus cohnii, Staphylococcus saprophyticus* subsp. *saprophyticus, Staphylococcus xylosus, Yarrowia lipolytica*
6U	*Acinetobacter johnsonii, Bacillus infantis, Bacillus safensis, Bacillus subtilis, Candida parapsilosis, Candida rugosa, Candida zeylanoides, Citrobacter freundii, Enterococcus durans, Enterococcus faecalis, Enterobacter cloacae, Hafnia alvei, Kluyvera cryocrescens, Lactobacillus suebicus, Raoultella ornithinolytica, Saccharomyces cerevisiae*
7N	*Acinetobacter baumannii, Alcaligenes faecalis* subsp. *faecalis, Bacillus* sp., *Candida guilliermondii, Candida zeylanoides, Enterococcus faecium, Lactobacillus paracasei* subsp. *paracasei, Sphingomonas parapaucimobilis, Streptococcus lutetiensis*
7U	*Bacillus infantis, Bacillus* sp., *Enterococcus faecalis, Candida guilliermondii, Candida lusitaniae, Enterococcus faecium, Gluconacetobacter liquefaciens, Hafnia alvei, Streptococcus lutetiensis, Sphingomonas parapaucimobilis*

isolated previously from the surfaces of traditional French cheeses **(Irlinger et al., 1997)**.

Bacillus megatherium, Bacillus sp., B. infantis, B. safensis, B. subtilis, B. badius were representatives of the genus Bacillus.

CONCLUSION

This study was designed to evaluate coliform bacteria, enterococci, yeasts and LAB population's in Slovak traditional non-smoked and smoked cheese. Our results show that the traditional Slovak cheese contains very diverse microbiota. In our study, the mass spectrometry method allowed the accurate identification of microorganisms and this method was reliable and easy done for identification in comparison with molecular methods.

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