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# Identification of the Slovak traditional cheese "Parenica" microflora

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**Summary**

Numerous studies have demonstrated the higher accuracy, faster time-to-results and lower costs provided by MALDI Biotyper systems compared to classical methods. In this study, the culturable population of total count of bacteria, enterococci, coliforms bacteria, lactic acid bacteria (LAB) and microscopic fungi and yeasts from cow's dairy products was identified using the MALDI-TOF MS Biotyper. Altogether, 50 samples of the Slovak cheese "Parenica" were examined. Total numbers of bacteria were cultured on Plate count agar at 37 °C for 24–48 h, aerobically; enterococci were cultured on Enterococcus selective agar at 37 °C for 24–48 h, aerobically; coliforms bacteria were cultured on Violet Red Bile lactose agar at 37 °C for 24–48 h, aerobically. The LAB were cultured on MRS (Main Rogosa agar), MSE and APT agar at 30 °C in microaerophilic conditions. The microscopic fungi and yeasts were cultured on Malt extract agar at 25 °C for 5 days, aerobically. Isolated strains (total 669) were subjected to identification by the MALDI-TOF MS. Among total count the identified bacteria mostly were *Acinetobacter baumannii, Bacillus cereus, Micrococcus luteus* and *Staphylococcus warneri*. *Escherichia coli* and *Enterobacter cloacae* were the most abundant coliform bacteria representatives identified. Coliform bacteria included *Citrobacter, Hafnia* and *Klebsiella.* Altogether three genera belonged to the LAB – *Lactobacillus, Lactococcus* and *Leuconostoc* were identified with *Lactococcus lactis*, *Lactobacillus plantarum, Lactobacillus coryniformis*, *L. fructivorans* and *Leuconostoc mesenteroides* were considered as the dominated LAB species in dairy products. Among yeasts, *Kluyveromyces lactis, Candida zeylanoides* and *Yarrowia lipolytica* were among the most isolated.

**Keywords:** microorganisms, identification, MALDI TOF MS Biotyper, cow traditional cheese

## Introduction

The main task of cheese microbiologists is to develop a clear view of the cheese microflora and its evolution during ripening. It is important that the complete flora is monitored and that the individual components are accurately identified and characterised (Beresford et al., 2001).

The indigenous flora of milk is the main factor affecting the specific consistency, aroma and flavour of raw milk cheese (Poznanski et al., 2004; Edalatian et al., 2012). Furthermore, the LAB, including wild type of cultures, represents a natural reservoir of microorganisms that contains diverse genetic information. Isolation and screening of LAB from natural processes have always been the most powerful means for obtaining useful cultures for commercial purposes. The use of commercial LAB cultures and pasteurized milk for industrial cheese production has led to the loss of flavour and a reduction in the diversity of dairy microflora. Sensorial differences between raw and pasteurized milk cheeses could be minimized by using LAB strains isolated from raw milk cheeses (Menendéz et al., 2004; Leboš Pavunc, 2012).

The reason for studying of the prevalence of enterococci in dairy products is the hygienic evaluation of the processing environment. The presence of enterococci has long been considered as a result of unhygienic conditions during the production and processing of milk. However, their presence in foods has often been shown to be unrelated with direct faecal contamination. Enterococci may enter the milk either directly from human or animal faeces or indirectly from contaminated water sources, exterior of the animal and/or from the milking equipment and bulk storage tank. Therefore, enterococci are a part of both the raw and pasteurised milk microflora. Different species of enterococci are found in dairy products, but *E. faecalis* and *E. faecium* remain the species of greatest importance (Franz et al., 1999; Gelsomino et al., 2001).

Coliforms were widely found in many cheeses (Brooks et al., 2012). However, in contrast to the presence of these microbes in raw and pasteurized fluid milk, and even in some other cultured products (e.g., yogurt), the presence of coliforms in cheese may be observed. The vast variety of types of cheese manufactured contributes to the complexity of fully understanding the role of coliforms in cheese quality and safety. Cheese product characteristics, including moisture content, pH, salt content, ripening conditions, age of product, and culture all influence

potential levels of and roles for coliforms and other microorganisms in the final product (Wolfe et al., 2014; Trmčič et al., 2016).

Molds and yeasts were recognized as an important cause of spoilage of various dairy products (Khalifa et al., 2013; Pal and Jadhav, 2013; Pal et al., 2014).The contamination of milk products with different types of fungi particularly of species of *Aspergillus, Fusarium* and *Penicillium* constitute a public health hazard as these fungi are known to produce mycotoxins that are injurious to human health (Pal, 2002; Sengum et al., 2008; Khalifa et al., 2013). Common contaminating yeasts of cheeses include *Candida* spp., *Kluyveromyces marxianus, Geotrichum candidum, Debaryomyces hansenii* and *Pichia* spp. (Johnson, 2001).

The conventional methods for identifying microorganisms in clinical microbiology laboratories are based on biochemical methods and gene sequencing identification techniques. However, these procedures take considerable time, and the results may be difficult to interpret occasionally because of indistinct reactions or outdated databases (Cherkaoui et al., 2010; Cloud et al., 2010). Recently, the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been effectively used as a rapid method for identifying a wide array of microbial species (Croxatto et al., 2012; Patel, 2015).

The aim of this study was to identify the culturable population of total count of bacteria, enterococci, coliforms bacteria, lactic acid bacteria (LAB) and microscopic fungi and yeasts from cow's dairy products with MALDI-TOF MS Biotyper.

## Material and methods

*Material*

In our study, 50 samples of the Slovak national cheese "Parenica" were examined. The cheese samples included non-smoked cheese (n=25) and smoked cheese (n=50). Additionally, 50 milk products samples from the western and middle parts of Slovakia directly from the producers (Bánovce nad Bebravou, Liptovský Mikuláš, Červený Kameň, Važec) were collected (*Figure 1*). Samples were kept sterilized sample containers and brought to laboratory with icebox for microbiological investigation.

Samples were stored at 4±1 °C until the testing began. The primary dilution of the milk products were made by adding of 5 g of sample material to 45 ml of 0.9% sterile saline. Then, the serial dilutions ($10^{-1}$ to $10^{-4}$) were done and 100 µl of each dilution was plated out on to agars.

Figure 1. *Map of Slovak republic*


Source: www.google.sk

## Methods

*Isolation of total counts of bacteria*

Plate count agar (PCA, (Sigma-Aldrich®, St. Louis, USA) for total count bacteria enumeration were used. Inoculated plates were incubated for 24–48 h at 37 °C and then examined for the presence of bacterial colonies.

*Isolation of coliforms bacteria*

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

*Isolation of Lactic Acid Bacteria (LAB)*

MRS (Main Rogose agar), MSE (Mayeux, Sandine and Elliker) for the detection and enumeration of *Leuconostoc* in milk and dairy products and APT (All Purpose TWEEN® Agar) for enumeration and cultivation of heterofermentative lactic acid bacteria including lactobacilli, leuconostocs

and lactic acid streptococci as well as other microorganisms with high requirements for thiamine (Sigma-Aldrich®, St. Louis, USA) agars were used for LAB identification. Inoculated plates were incubated for 72 h at 30 °C anaerobically and then the bacterial growth was evaluated.

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

*Identification of bacteria with MALDI-TOF MS Biotyper*

The suspicious colonies from total count of bacteria, coliforms bacteria, enterococci, lactic acid bacteria and fungi and yeasts 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.

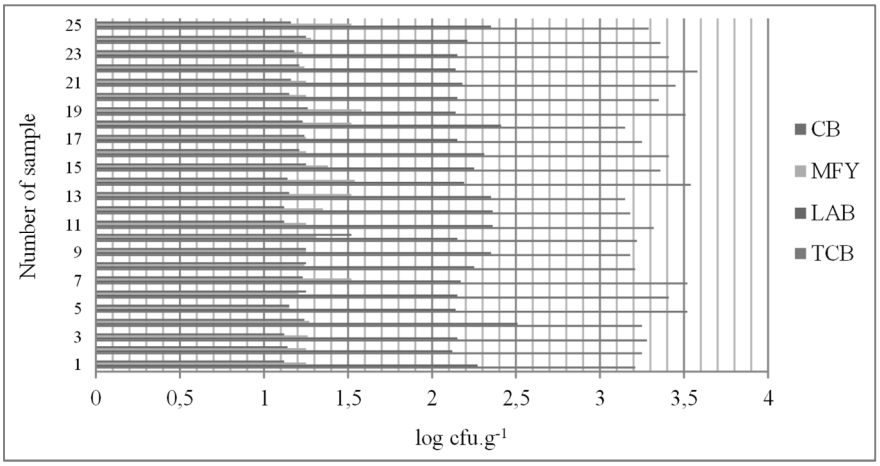
A sample for analysis with MALDI-TOF MS analysis was prepared in accordance with extraction procedure provided by the manufacturer (Bruker Daltonik, Bremen, Germany). Bacterial colony was suspended in 300 µL of water (Sigma-Aldrich, St. Louis, USA) and 900 µL of absolute ethanol (Bruker Daltonik, Bremen, Germany), mixed and centrifuged at 13000 rpm for 2 min. After removal of supernatant, the pellet was mixed with 10 µL of 70% formic acid (v/v) (Sigma-Aldrich, USA) and an equal volume of acetonitrile (Sigma-Aldrich, USA). The mixture was repeatedly centrifuged and1 µL of the supernatant was spotted onto a polished steel target plate and air dried at room temperature. Each sample was overlaid with 1 µL of MALDI matrix (a saturated solution of α-cyano-4-hydroxycinnamic acid, HCCA, Bruker Daltonik, Germany) in 50% acetonitrile and 2.5% trifluoroacetic acid (Sigma-Aldrich, USA). Mass spectra were automatically generated using the microflex LT MALDI-TOF mass spectrometer (Bruker Daltonik, Germany) operated in the linear positive mode within a mass range of 2000–20000 Da. The instrument was calibrated using the Bruker bacterial test standard.

Results of mass spectra were processed with the MALDI Biotyper 3.0 software (Bruker Daltonik, Germany). The identification criteria used were: a score of 2.300 to 3.000 indicated highly probable identification on species level; a score of 2.000 to 2.299 secure genus identification with probable species identification; a score of 1.700 to 1.999 probable identification to the genus level; <1.700 was considered as unreliable identification.

## Results and discussion

The number of microorganisms in non-smoked cheese samples are shown in *Figure 2*. Total count of bacteria in non-smoked cheese ranged from 3.15 to 3.58 log cfu.g$^{-1}$. Enterococci were not identified in the samples studied. Coliform bacteria counts ranged from 1.12 to 1.52 log cfu.g$^{-1}$, but lactic acid bacteria counts ranged from 2.12 to 2.51 log cfu.g$^{-1}$. Microscopic filamentous fungi and yeasts counts ranged from 1.12 to 1.54 log cfu.g$^{-1}$.

Figure 2. *Number of isolated microorganisms in non-smoked cheese*




Note: TCB – total count of bacteria, LAB – lactic acid bacteria, MFY – microscopic filamentous fungi and yeasts.

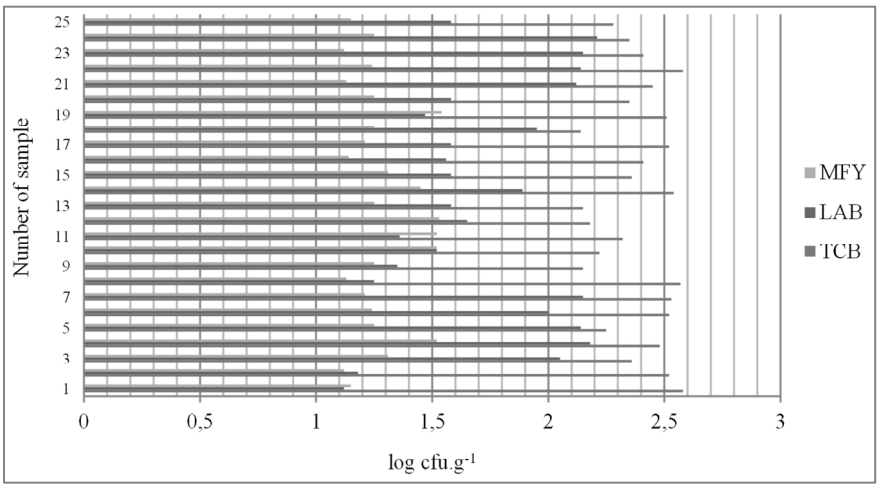
Many types of fermented foods arex produced throughout the world. Fermentation improves the shelf life of these products and contributes to their typical sensory and nutritional properties. Cheeses are fermented dairy products whose manufacturing involves different types of bacteria, yeasts, and molds (Montel et al., 2014; Irlinger et al., 2015). Cheese manufacturing can be considered as a process in which a nutrient-rich environment, milk, is colonized by adventitious and deliberately inoculated microorganisms. Two different habitats may be considered, the interior of the cheese and the cheese rind. The rind microbiota can be considered as an interesting model system for the field of ecosystems biology (Wolfe et al., 2014).

Mounier et al. (2006) showed that the microorganisms that developed on the cheese surface were an adventitious microflora from the cheese environment (brine, ripening shelves, and personnel) which rapidly outnumbered the commercial cultures. Several hypotheses have been

advanced to explain these findings. These ripening cultures may be unfit for the cheese habitat, or negative interactions may occur between them and the adventitious microflora. Bacterial and yeast strains have also been selected for their antilisterial activity (Eppert et al., 1997; Maoz et al., 2003).

The number of microorganisms in smoked cheese samples are shown in *Figure 3*. Total count of bacteria in smoked cheese ranged from 2.14 to 2.58 log cfu.g$^{-1}$. Enterococci and coliforms bacteria were not identified in the samples studied. Lactic acid bacteria counts ranged from 1.12 to 2.18 log cfu.g$^{-1}$. Microscopic filamentous fungi and yeasts counts ranged from 1.12 to 1.54 log cfu.g$^{-1}$.

Figure 3. *Number of isolated microorganisms in smoked cheese*




Note: TCB – total count of bacteria, LAB – lactic acid bacteria, MFY – microscopic filamentous fungi and yeasts.

From the non-smoked and smoked cheese a total of 47 species of 28 bacterial genera represented by 17 Gram-negative and 12 Gram-positive, and 10 species of yeasts belonging to 5 genera were identified with MALDI-TOF Mass Spectrometry. From a total of 669 isolates, the percentage representation of each microbial group reached the following values: 166 isolates of Gram-negative (24.81%), 297 isolates of Gram-positive (44.39%) and 206 isolates of yeasts (30.79%). The most common microorganisms isolated from cheese are shown in *Table 1*. Yeast and bacteria were isolated from each sample. Bacterial species were identified in highest counts in comprasion with yeasts and fungi.

At the beginning of the cheese-making process, lactic acid bacteria (LAB) starter cultures (i.e. *Lactococcus lactis, Streptococcus thermophilus*) grow rapidly and produce acid in milk (Delbes et al., 2007. During the first days of ripening, yeasts and/or moulds (i.e. *Debaryomyces hansenii, Geotrichum candidum, Penicillium camemberti*) colonize the surfaces of cheese and utilize lactate (Callon et al., 2006).

Table 1. *Number of isolates identified with Maldi TOF MS Biotyper in cheese*

| Microorganisms | Non- smoked cheese | Smoked cheese | Total |
|---|---|---|---|
| Yeasts | | | |
| *Candida catenulata* | 8 | 5 | 13 |
| *Candida famata* | 8 | 5 | 13 |
| *Candida guilliermondii* | 8 | 7 | 15 |
| *Candida kefyr* | 8 | 5 | 13 |
| *Candida lusitaniae* | 10 | 5 | 15 |
| *Candida zeylanoides* | 19 | 15 | 34 |
| *Kluyveromyces lactis* | 20 | 15 | 35 |
| *Saccharomyces cerevisiae* | 10 | 8 | 18 |
| *Torulaspora delbrueckii* | 10 | 7 | 17 |
| *Yarrowia lipolytica* | 18 | 15 | 33 |
| | 119 | 87 | 206 |
| Gram-negative bacteria | | | |
| *Acinetobacter pittii* | 3 | 2 | 5 |
| *Acinetobacter baumannii* | 15 | 10 | 25 |
| *Acinetobacter junii* | 3 | 2 | 5 |
| *Chryseobacterium oranimense* | 4 | 5 | 9 |
| *Citrobacter braakii* | 5 | 0 | 5 |
| *Citrobacter youngae* | 3 | 0 | 3 |
| *Enterobacter cloacae* | 4 | 3 | 7 |
| *Escherichia coli* | 25 | 5 | 30 |
| *Ewingella americana* | 2 | 2 | 4 |
| *Gluconobacter cerinus* | 5 | 5 | 10 |
| *Hafnia alvei* | 8 | 1 | 9 |
| *Pantoea agglomerans* | 2 | 5 | 7 |
| *Pluralibacter gergoviae* | 3 | 2 | 5 |
| *Pseudomonas rhodesiae* | 1 | 4 | 5 |
| *Rahnella aquatilis* | 3 | 1 | 4 |
| *Raoultella ornithinolytica* | 3 | 0 | 3 |
| *Raoultella planticola* | 2 | 1 | 3 |
| *Rhizobium radiobacter* | 2 | 3 | 5 |
| *Serratia liquefaciens* | 5 | 0 | 5 |
| *Sphingomonas melonis* | 5 | 5 | 10 |
| *Sphingomonas parapaucimobilis* | 5 | 2 | 7 |
| | 108 | 58 | 166 |

*The rest of Table 1 is on the next page ...*

*... second part of Table 1*

| Microorganisms | Non- smoked cheese | Smoked cheese | Total |
|---|---|---|---|
| Gram-positive bacteria | | | |
| *Bacillus cereus* | 20 | 12 | 32 |
| *Bacillus pumilus* | 4 | 2 | 6 |
| *Bacillus thuringiensis* | 1 | 2 | 3 |
| *Brevibacterium casei* | 3 | 0 | 3 |
| *Enterococcus durans* | 5 | 4 | 9 |
| *Enterococcus faecalis* | 4 | 3 | 7 |
| *Enterococcus faecium* | 5 | 5 | 10 |
| *Enterococcus italicus* | 1 | 2 | 3 |
| *Kocuria kristinae* | 2 | 2 | 4 |
| *Lactobacillus acidophilus* | 2 | 0 | 2 |
| *Lactobacillus coryniformis* | 12 | 12 | 24 |
| *Lactobacillus curvatus* | 5 | 5 | 10 |
| *Lactobacillus fermentum* | 8 | 9 | 17 |
| *Lactobacillus plantarum* | 10 | 10 | 20 |
| *Lactobacillus fructivorans* | 10 | 10 | 20 |
| *Lactococcus lactis* | 20 | 19 | 39 |
| *Leuconostoc mesenteroides* | 15 | 10 | 25 |
| *Macrococcus caseolyticus* | 2 | 3 | 5 |
| *Micrococcus luteus* | 2 | 0 | 2 |
| *Pediococcus pentosaceus* | 5 | 2 | 7 |
| *Staphylococcus epidermidis* | 3 | 2 | 5 |
| *Staphylococcus haemoliticus* | 2 | 4 | 6 |
| *Staphylococcus saprophyticus* | 2 | 2 | 4 |
| *Staphylococcus succinus* | 5 | 2 | 7 |
| *Staphylococcus warneri* | 10 | 12 | 22 |
| *Streptococcus equinus* | 1 | 1 | 2 |
| *Streptococcus salivarius _ssp._ thermophilus _* | 2 | 1 | 3 |
| | 161 | 136 | 297 |
| Total | 388 | 281 | 669 |

This leads to the deacidification of the cheese surface, enabling the establishment of a bacterial community which is less acid-tolerant (i.e. *Arthobacter arilaitensis, Brevibacterium aurantiacum, Brevibacterium linens, Corynebacterium casei*) (Belén Flórez and Mayo, 2006; Rademaker et al., 2006; Licitra et al., 2007; Parayre et al., 2007; Rea et al., 2007; Saubusse et al., 2007; Abriouel et al., 2008). Studies of traditional smear cheeses have also revealed the presence of moderately halophilic *Proteobacteria* i.e. *Halomonas* spp., bacteria from the *Enterobacteriaceae* family (i.e. *Hafnia alvei*), and halophilic and alkaliphic LAB i.e. *Marinilactibacillus psychrotolerans* or *Alkalibacterium olivapovliticus*. The roles of these organisms in cheese ripening have not yet been determined (Ishikawa et al., 2006; Mounier et al., 2009).

Cheese is a ready-to-eat food easily contaminated on the surface by undesirable microorganisms. Some are spoilage microorganisms which

may produce uncharacteristic visual appearance and diminish the commercial value of the cheeses, such as *Yarrowia lipolytica, Pseudomonas aeruginosa* and *Penicillium* spp. but others are pathogenic such as *Listeria monocytogenes*, which have been associated with foodborne listeriosis by consumption of cheese (McLauchlin et al., 2004). The Gram-negative bacteria *Pseudomonas* spp. are the most important of the psychrotrophs that dominate in microflora of raw milk (Sorhaug and Stepaniak, 1997). Strains of *Ps. aeruginosa* have been associated with undesirable browning reactions on cheese rind, and some are pathogenic. The *Y. lipolytica* yeast, frequently found in cheeses, was also reported to be associated with browning phenomenon (Carreira et al., 1998). Mold *Penicillium* was the most frequently isolated from naturally contaminated cheese rind samples and include mycotoxigenic strains. All these microorganisms comprise strains with psychrotrophic characteristics that could increase in number during cold storage (Sorhaug and Stepaniak, 1997).

## Conclusion

Microbiological analysis of 50 cheese samples revealed the three main groups of microorganisms: 47 species of 29 bacterial genera (17 Gram-negative and 12 Gram-positive) and 10 species of yeasts belonging to 5 genera were identified with MALDI-TOF Mass Spectrometry. From a total of 669 isolates, the percentage representation of each microbial group reached the following values: 166 isolates of Gram-negative (24.81%), 297 isolates of Gram- (44.39%), and 206 isolates of yeasts (30.79%).

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