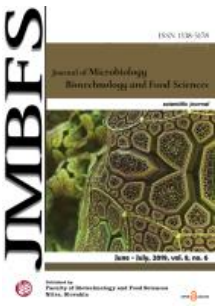

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APPLICATION OF MALDI-TOF MASS SPECTROMETRY FOR IDENTIFICATION OF BACTERIA ISOLATED FROM TRADITIONAL SLOVAK CHEESE "PARENICA"

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ABSTRACT

In this study, the cultivable population of bacteria from a traditional Slovak cheese "Parenica" made from cow milk were identified using MALDI-TOF mass spectrometry (MS). A total of 100 "Parenica" cheese packages from four producers were examined, including n=50 smoked and n=50 non-smoked cheese samples. The lactic acid bacteria (LAB) were cultured on MRS, APT and MSE agars at 30 °C, coliform bacteria on VRBL agar at 37 °C, total count of bacteria (TCB) on Plate count agar at 30 °C and enterococci on Enterococcus selective agar at 37 °C. Gram-positive and gram-negative strains were subjected to identification by MALDI-TOF MS profiling. MALDI-TOF MS identification revealed four genera belonging to LAB including *Lactococcus*, *Lactobacillus*, *Enterococcus* and *Leuconostoc*. *Lactobacillus* was the most represented genus with seven species: *Lactobacillus curvatus* (*L. curvatus*), *L. delbrueckii*, *L. fermentum*, *L. casei*, *L. paraplantarum*, *L. plantarum* and *L. sakei*.. *Escherichia coli*, *Enterobacter asburiae*, *Klebsiella oxytoca* and *K. pneumoniae* were the most identified bacteria species from *Enterobacteriales* order.

Keywords: bacteria, mass spectrometry, identification, Slovak "Parenica" cheese

INTRODUCTION

Manufacture of most of cheese varieties involves a combining of four ingredients: milk, rennet, microorganisms and salt, which are processed. The common steps of cheese-making include gel formation, curd whey expulsion, acid production and salt addition, followed by a period of ripening. A variation in ingredient blends and processing has led to the evolution of cheese varieties. While variations in processing parameters such as processing temperature and curd handling techniques play a major role in production of each cheese type, but the cheese microflora play a critical and pivotal role in the development of the unique characteristics of each cheese variety (**Beresford *et al.*, 2001**). Traditional raw-milk cheeses are highly valued for their flavors, while large-scale products are often perceived by the consumer as ''boring'' (**Law, 2001**). This difference is a consequence of the elimination of raw milk microflora by pasteurization that has a key role in flavor development. To compensate the sensory characteristics of product the food industry looks for alternative LAB (Lactic Acid Bacteria) cultures capable of improving products flavor (**Leroy and De Vuyest, 2004**). However, the LAB are only a part of the complete microflora of raw milk (**Kongo *et al.*, 2007**). Complex approach then the addition of LAB is associated to other technological methods such as pressing allows the production of diverse of traditional cheeses (**Parguel, 2011**). The raw-milk microbiota also represents the contamination from the environment (air, utensils, the animal skin), and the load and its diversity will vary with location, season and livestock species and milking procedures.
Food spoilage is an enormous economic worldwide problem. Approximately one-fourth of the world's food supply is lost through microbial activity alone (**Huis in't Veld, 1998**). Milk is highly nutritious food that serves as an excellent growth medium for a wide range of microorganisms (**Ruegg, 2003; Rajagopal *et al.*, 2005**). The microbiological quality of milk and dairy products is influenced by the initial microbiota of raw milk, the processing conditions, and post-heat treatment contamination (**Richter *et al.*, 1992**). Undesirable microbiota that can cause a spoilage of dairy products includes Gram-negative psychrotrophs, coliforms, lactic acid bacteria, yeasts, and molds. In addition, the various pathogens of public health concern such as *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter jejuni*, *Yersinia enterocolitica*, pathogenic strains of *Escherichia coli* and enterotoxigenic strains of *Staphylococcus aureus* may also be found in milk and dairy products (**Tatini and Kauppi, 2003; Al-Sahlany, 2016; Verma and Niamah, 2017**). This is one of the reasons why the increased emphasis should be focused on the microbiological examination of milk and dairy foods. Microbiological analyses of milk and milk products are critical for assessment of quality and safety, conformation with standards and specifications, and regulatory compliance (**Vasavada, 1993**).
The aim of this study was to evaluate microbiological quality of the traditional Slovak non-smoked and smoked cheese "Parenica" made from cow milk and to identify bacterial strains with MALDI-TOF MS Biotyper.

MATERIAL AND METHODS

Samples

There were 50 samples of the Slovak national cheese "Parenica" examined in this study. The cheese samples included non-smoked cheese (n=25) and smoked cheese (n=25). Additionally, a total of 50 cow milk cheese samples from the Slovak producers located in the western and the middle part of Slovakia were collected (Bánovce nad Bebravou, Liptovský Mikuláš, Červený Kameň, Važec). 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. For that a 5 ml of sample material was added to 45 ml of 0.87 % sterile

saline, then the serial dilutions (10^{-1} to 10^{-4}) were done and a 100 µl of each dilution was plated out.

Isolation of total count of bacteria

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

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 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 at 37 °C for 24-48 h and then examined for the presence 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.

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 each bacterial isolate was selected. Subsequently, the identification was performed using the Maldi TOF MS Biotyper described by **Kluga *et al.* (2017)**. Totally, a number of 512 isolates were identified with score higher than 2.

RESULTS AND DISCUSSION

Number of isolated bacterial group

Cheeses are fermented dairy products whose manufacturing involves different types of bacteria (**Montel *et al*., 2014; Irlinger *et al*., 2015**). Cheese producing is a process when a nutrient-rich substrate as milk is colonized by adventitious and deliberately inoculated microorganisms. Two different habitats of bacteria in cheese 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**).

Total count of bacteria in non-smoked cheese ranged from 5.25 to 5.58 log cfu.g^{-1}. Enterococci were not identified in the studied samples. Coliform bacteria counts ranged from 1.25 to 1.80 log cfu.g^{-1}, but lactic acid bacteria counts ranged from 4.12 to 4.51 log cfu.g^{-1}. Total count of bacteria in smoked cheese ranged from 5.45 to 5.85 log cfu.g^{-1}. Enterococci and coliform bacteria number of bacteria were not identified in the samples studied. Lactic acid bacteria counts ranged from 4.12 to 4.48 log cfu.g^{-1}.

Kačániová *et al*., (2018) found similar results in cheese samples, and the 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 studied samples. 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}. 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 studied samples. Lactic acid bacteria counts ranged from 1.12 to 2.18 log cfu.g^{-1}.

Total counts of bacteria are the most useful indicator for the overall microbiological quality of the cheese. High viable count often indicates a contamination of the raw material, unsatisfactory sanitation, or unsuitable time and temperature during storage and/or production. The attention has been focused on coliform bacteria because of their public health importance. Coliforms are widely distributed in nature. They gain entry to milk and milk products through the water supply, equipment, unhygienic conditions of production and handling (**El-Leboudy *et al*., 2014**).

Isolated bacteria with MALDI-TOF MS Biotyper

Table 1 Isolated species of bacteria from smoked and non-smoked cheese "Parenica"

Family	Genera	Species
Moraxellaceae	*Acinetobacter*	*Acinetobacter pittii*
Moraxellaceae	*Acinetobacter*	*Acinetobacter baumannii*
Moraxellaceae	*Acinetobacter*	*Acinetobacter junii*
Bacillaceae	*Bacillus*	*Bacillus cereus*
Bacillaceae	*Bacillus*	*Bacillus pumilus*
Bacillaceae	*Bacillus*	*Bacillus thuringiensis*
Brevibacteriaceae	*Brevibacterium*	*Brevibacterium casei*
Flavobacteriaceae	*Chryseobacterium*	*Chryseobacterium oranimense*
Enterobacteriaceae	*Citrobacter*	*Citrobacter braakii*
Enterobacteriaceae	*Citrobacter*	*Citrobacter youngae*
Burkholderiaceae	*Cupriavidus*	*Cupriavidus metallidurans*
Enterobacteriaceae	*Enterobacter*	*Enterobacter asburiae*
Enterobacteriaceae	*Enterobacter*	*Enterobacter cloacae*
Enterobacteriaceae	*Enterococcus*	*Enterococcus durans*
Enterococcaceae	*Enterococcus*	*Enterococcus faecalis*
Enterococcaceae	*Enterococcus*	*Enterococcus faecium*
Enterococcaceae	*Enterococcus*	*Enterococcus italicus*
Enterobacteriaceae	*Escherichia*	*Escherichia coli*
Enterobacteriaceae	*Ewingella*	*Ewingella americana*
Acetobacteraceae	*Gluconobacter*	*Gluconobacter cerinus*
Enterobacteriaceae	*Hafnia*	*Hafnia alvei*
Micrococcaceae	*Kocuria*	*Kocuria kristinae*
Enterobacteriaceae	*Klebsiella*	*Klebsiella oxytoca*
Enterobacteriaceae	*Klebsiella*	*Klebsiella pneumoniae*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus curvatus*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus delbruckii*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus fermentum*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus paracasei*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus paraplantarum*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus plantarum*
Lactobacillaceae	*Lactobacillus*	*Lactobacillus sakei*
Streptococcaceae	*Lactococcus*	*Lactococcus lactis*
Leuconostocaceae	*Leuconostoc*	*Leuconostoc mesenteroides*
Staphylococcaceae	*Macrococcus*	*Macrococcus caseolyticus*
Micrococcaceae	*Micrococcus*	*Micrococcus luteus*
Enterobacteriaceae	*Pantoea*	*Pantoea agglomerans*
Lactobacillaceae	*Pediococcus*	*Pediococcus pentosaceus*
Enterobacteriaceae	*Pluralibacter*	*Pluralibacter gergoviae*
Pseudomonadaceae	*Pseudomonas*	*Pseudomonas rhodesiae*
Enterobacteriaceae	*Rahnella*	*Rahnella aquatilis*
Enterobacteriaceae	*Raoultella*	*Raoultella ornithinolytica*
Enterobacteriaceae	*Raoultella*	*Raoultella planticola*
Rhizobiaceae	*Rhizobium*	*Rhizobium radiobacter*
Enterobacteriaceae	*Serratia*	*Serratia liquefaciens*
Sphingomonadaceae	*Sphingomonas*	*Sphingomonas melonis*
Sphingomonadaceae	*Sphingomonas*	*Sphingomonas parapaucimobilis*
Staphylococcaceae	*Staphylococcus*	*Staphylococcus epidermidis*
Staphylococcaceae	*Staphylococcus*	*Staphylococcus haemoliticus*
Staphylococcaceae	*Staphylococcus*	*Staphylococcus saprophyticus*
Staphylococcaceae	*Staphylococcus*	*Staphylococcus succinus*
Staphylococcaceae	*Staphylococcus*	*Staphylococcus warneri*
Streptococcaceae	*Streptococcus*	*Streptococcus equinus*
Streptococcaceae	*Streptococcus*	*Streptococcus salivarius_ssp._thermophilus*

Table 2 Number of isolates identified with MALDI-TOF MS Biotyper in cheese

Microorganisms	Non-smoked cheese	Smoked cheese	Total
Acinetobacter pittii	5	2	7
Acinetobacter baumannii	10	10	20
Acinetobacter junii	2	2	4
Chryseobacterium oranimense	2	2	4
Citrobacter braakii	4	4	8
Citrobacter youngae	4	4	8
Cupriavidus metallidurans	2	1	3
Enterobacter asburiae	8	9	17
Enterobacter cloacae	4	3	7
Escherichia coli	15	12	27
Ewingella americana	2	2	4
Gluconobacter cerinus	1	5	6
Hafnia alvei	8	2	10
Klebsiella oxytoca	10	12	22
Klebsiella pneumoniae	12	12	24
Pantoea agglomerans	2	2	4
Pluralibacter gergoviae	2	2	4
Pseudomonas rhodesiae	2	2	4
Rahnella aquatilis	3	2	5
Raoultella ornithinolytica	3	3	6
Raoultella planticola	1	1	2
Rhizobium radiobacter	2	2	4
Serratia liquefaciens	4	4	8
Sphingomonas melonis	3	3	6
Sphingomonas parapaucimobilis	2	2	4
Gram-negative bacteria	**113**	**105**	**218**
Microorganisms	**Non- smoked cheese**	**Smoked cheese**	**Total**
Bacillus cereus	5	2	7
Bacillus pumilus	4	2	6
Bacillus thuringiensis	1	2	3
Brevibacterium casei	3	3	6
Enterococcus durans	3	5	8
Enterococcus faecalis	4	4	8
Enterococcus faecium	5	5	10
Enterococcus italicus	1	1	2
Kocuria kristinae	2	2	4
Lactobacillus curvatus	15	10	25
Lactobacillus delbruckii	15	15	30
Lactobacillus fermentum	10	10	20
Lactobacillus paracasei	15	15	30
Lactobacillus paraplantarum	10	10	20
Lactobacillus plantarum	10	10	20
Lactobacillus sakei	15	12	27
Lactococcus lactis	10	9	19
Leuconostoc mesenteroides	5	5	10
Macrococcus caseolyticus	3	3	6
Micrococcus luteus	4	4	8
Pediococcus pentosaceus	2	2	4
Staphylococcus epidermidis	2	2	4
Staphylococcus haemoliticus	2	2	4
Staphylococcus saprophyticus	2	3	5
Staphylococcus succinus	1	2	3
Staphylococcus warneri	1	2	3
Streptococcus equinus	2	2	4
Streptococcus salivarius ssp. *thermophilus*	8	2	10
Gram-positive bacteria	**149**	**145**	**294**
Total	**262**	**250**	**512**

A total of 53 species of 30 bacterial genera (18 gram-negative G^- and 12 Gram positive G^+) were identified in smoked and non-smoked cheese by MALDI-TOF Mass Spectrometry. The percentage representation of each bacterial group (G^- and G^+) were 42.58% for G^- (218 isolates) (and 57.42% for G^+ (294 isolates). (). Isolated species of bacteria from smoked and non-smoked cheese "Parenica" are shown in Table 1.

Percentages of the number of isolates of each species for G^- and G^+ are shown in Table 2. The most abundant G^- bacterium was *Escherichia coli*, *Klebsiella oxytoca* and *Klebsiella pneumoniae*. *Lactobacillus* was the most abundant within 12 different species of G^+ bacteria with *Lactobacillus casei*, *L. delbrueckii* and *L. sakei* were the most distributed.

Nevertheless, many LAB species were found in both kinds of French cheeses, e.g. *L. plantarum*, *L. paracasei*, *L. curvatus*, *L. rhamnosus*, *L. fructivorans*, *L. parabuchneri*, *L. brevis* (**Nacef *et al.*, 2017**). As previously were reported, some *Lactobacilli* are present in the natural microflora of dairy products and arise from animals, farms and dairies: *L. casei* ssp. *casei*/*L. paracasei* ssp. *paracasei*, *L. rhamnosus*, *L. plantarum*, *L. fermentum*, *L. brevis*, *L. buchneri*, *L. curvatus*, *L. acidophilus* and *L. pentosus* (**Corbo *et al.*, 2001, Gobbetti *et al.*, 2002, Medina *et al.*, 2001**). *Lactobacilli*, especially *L. curvatus*, represents a type of milk microbiota that is resistant to pasteurization. Moreover the presence of LAB could be also attributed to contamination occurring after pasteurization (**Martley and Crow, 1993**).

Kačániová *et al.* (2018) found in microbiological analysis of 50 cheese samples three main groups of microorganisms: gram-negative and gram-positive bacteria and fungi. Althogether, 47 species of 18 bacterial genera (17 Gram negative G^- and 12 Gram positive G^+) and 10 species of yeasts of 5 genera were identified with MALDI-TOF Mass Spectrometry. The percentage representation of each microbial group (G^-, G^+ and yeasts) from a total of 669 isolates, reached the following values: 166 isolates of G^- (24.81%), 297 isolates of G^+ (44.39%), and 206 isolates of yeasts (30.79%).

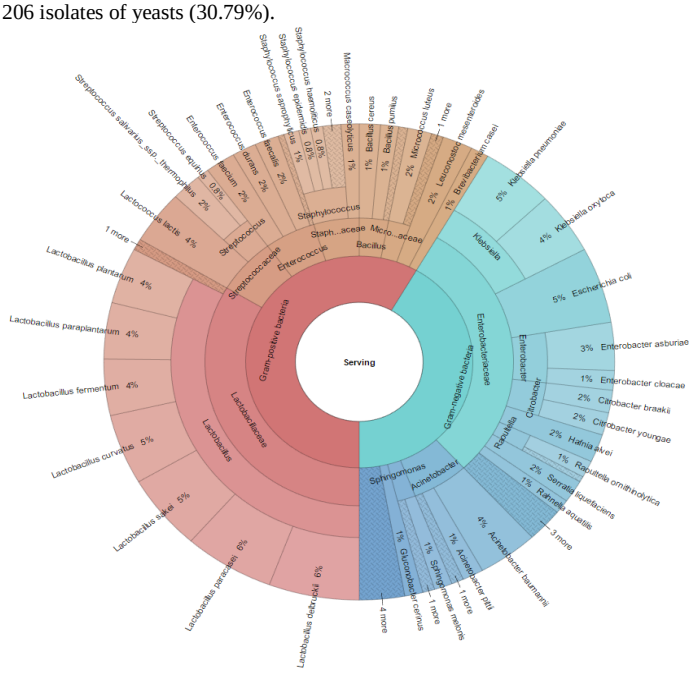

Figure 1 Percentage of isolated bacterial species in cheese samples

Mounier *et al.* (2006) found out 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 **(Maoz *et al.*, 2003).**

CONCLUSION

Microbiological analysis of 100 cheese samples revealed the two main groups of microorganisms comprising 53 species of 30 bacterial genera (18 Gram-negative G^- and 12 Gram-positive G^+) identified with MALDI-TOF Mass Spectrometry. The percentage representation of each bacterial group (G^- and G^+) were 42.58% (218 isolates of G^-) (and 57.42% (294 isolates of G^+). Fast microbial identification is becoming increasingly necessary in industry to improve microbial control, reduce biocide consumption, to avoid cost-intensive recall of contaminated products and damage to brand reputation. While MALDI-TOF-MS has revolutionized speed and precision of microbial identification for clinical isolates, in contrast few performance studies have been published so far focusing on suitability for particularly industrial applications.

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