Microorganisms Isolated from the Slovak Traditional Cheese "Parenica"and their Identification with Mass Spectrometry

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Abstract

Cheese is one of the most important products of dairy industry from the economic and nutritional points of view that demands the high processing technology and production hygiene. The aim of the study was to evaluate microbiological quality of traditional Slovak cheese "Parenica" produced from sheep milk. The coliforms, enterococci, lactic acid bacteria and microscopic filamentous fungi were detected in cheese samples. MALDI-TOF MS was applied for identification of isolates. In group of lactic acid bacteria three genera were identified: *Lactococcus* and *Leuconostoc*. *Lactobacillus* spp. was the most abundant genus, which was found in 25, *Leuconostoc* in 10 and *Lactococcus* in 7 samples. *Lactobacillus* was mostly represented with four species: *Lactobacillus plantarum*, *L. acidophilus*, *L. ultunensis*, *L. casei*. Another bacterial isolates included Gram-positive and Gram-negative *Enterococcus faecium*, *Streptococcus*, *Raoutella ornithinolytica* and *Klebsiela oxytoca*. Our study shows that MALDI-TOF MS could serve as an important method for identifying microorganisms for dairy industry needs.

Keywords: cheese, microorganisms, identification, mass spectrometry.

1. Introduction

Cheese is a source of various groups of microorganisms with different function. The microorganism including those contributing food spoilage and the microorganisms, which help to develop the typical sensory characteristics of cheese are the most important. The microorganisms of a great importance in cheese-making particularly include the lactic acid bacteria group. A lactic acid bacteria group produces high proportion of lactic acid from lactose fermentation as well as flavor materials [1,2]. These bacteria includes various species with different physiological characteristics [3]. They are Gram-positive microaerophilic or anaerobic and non-animated except *Lactobacillus agilis* and *Lactobacillus ghanensis* [4] and *Lactobacillus capillatus* [5]. Lactic acid bacteria are non-sporeforming, non-producing catalase enzyme and has a low content of guanine and cytosine.

The transformation of milk to cheese involves a complex and dynamic microbial ecosystem in which numerous biochemical reactions occur.

In cheese manufacture and ripening the starter bacteria (mainly *Lactococcus*) that are added to milk during cheese manufacture for providing typical cheese characteristics [6].

Alongside with the valuable lactic acid bacteria, the contaminants as coliforms may be present.

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Typical representatives of ubiquitous coliform genera are *Enterobacter* and *Citrobacter*. Both genera include several species that can be found in many known environments, including milk and cheese [7-10]. Fecal coliforms represent only a small proportion of the coliform group, and several different reports suggest that *Escherichia coli* is the only coliform to exclusively represent the fecal environment [11,-13] which could be find also in cheese.

Rapid detection and identification of microorganisms is a challenging and important aspect in a wide range of fields, from medical to industrial, affecting human lives. Unfortunately,

The classical methods of microorganism identification are based on time-consuming and labor-intensive approaches with limited sensitivity. MALDI-TOF MS is the latest next generation tool being used for the rapid identification and classification of microorganisms. The principle of this method is based on the gentle ionization of intact microorganism cells with short laser pulses and then accelerating the particles in a vacuum by way of an electric field. As a result of microorganism ionization, a specific molecular fingerprint (spectra profile) of the microorganism can be registered. Identification of the microorganism occurs as a result of comparing the spectral profile of the analyzed microorganism against a database using an automated program. The most commonly used MS technique for the identification of microorganisms is protein analysis. Currently, MALDI-TOF MS is being successfully used in clinical microbiology laboratories, in the veterinary field, in monitoring water quality and food, and in detecting and identifying fungi, yeasts and bacteria [14].

The aim of the study was to evaluate microbiological quality of traditional Slovak cheese "Parenica" produced from sheep milk.

2. Materials and methods

Sampling

Altogether, 26 samples of Slovak national cheese "Parenica" were examined. The cheese samples included non-smoked parenica cheese (n=13) and smoked parenica cheese (n=13). Additionally, 50 milk products samples from the middle Slovak producers were collected. Samples were collected in sterilized sample containers and brought to laboratory with icebox for microbiological investigation. Samples were kept in a refrigerator (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 1 ml of each dilution was plated out on to agars.

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 and APT (Sigma-Aldrich, St. Louis, USA) agars were used for Lactic acid bacteria 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) 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.

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 aerobically or anaerobically and used for identification.

Identification of bacteria with MALDI-TOF MS Biotyper

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.

3. Results and discussion

The number of microorganisms in non-smoked cheese samples are shown in Table 1.

Total count of bacteria in non-smoked cheese ranged from 2.25 to 3.58 log cfu.g^{-1}. Enterococci were not identified in the samples studied. Coliform bacteria counts ranged from 0.00 to 1.45 log cfu.g^{-1}, but lactic acid bacteria counts ranged from 1.12 to 2.85 log cfu.g^{-1}. Microscopic filamentous fungi and yeasts counts ranged from 1.12 to 1.51 log cfu.g^{-1}.

Table 1 Number of isolated microorganisms from non-smoked cheese in log cfu.g^{-1}

Number of samples	TCB	E	CB	Lactic acid bacteria	MFF
1.	3.22	0.00	1.12	1.12	1.15
2.	2.82	0.00	1.25	1.32	1.21
3.	2.25	0.00	1.24	1.15	1.25
4.	3.24	0.00	1.45	2.27	1.24
5.	3.52	0.00	1.25	2.75	1.51
6.	3.45	0.00	0.00	2.85	1.23
7.	3.58	0.00	0.00	2.65	1.18
8.	2.87	0.00	1.15	1.25	1.21
9.	2.58	0.00	0.00	1.98	1.19
10.	2.46	0.00	1.25	1.23	1.15
11.	3.48	0.00	1.12	1.12	1.14
12.	3.52	0.00	1.10	1.15	1.21
13.	3.48	0.00	0.00	1.21	1.12

TBC – total counts of bacteria, E – enterococci, CB – coliform bacteria, MFF – yeasts and fungi

The microbial counts in smoked cheese are shown in Table 2. Total count bacteria counts in smoked cheese ranged from 2.25 to 2.92 log cfu.g^{-1}. Enterococci were not identified but coliform counts were from 0.00 to 1.32 log cfu.g^{-1}. Lactic acid bacteria counts ranged from 1.12 to 2.15 log cfu.g^{-1} and counts of microscopic filamentous fungi and yeasts ranged from 1.12 to 1.52 log cfu.g^{-1}. Lower yeast and mould counts were found in the naturally smoked and the liquid smoke-applied cheeses compared with the control cheese. The reason for this is considered to be related to the lack of inhibitory compounds in the non-smoked cheese against the yeast, moulds. The number of yeast, moulds detected during ripening was lower than that reported by Atasever et al. [15] in smoked Kasar cheese. Ozcan et al. [18] note that the number of microorganisms was lower and that the shelf life of cheese could be extended due to the bactericidal and bacteriostatic effect of the wood smoke.

E. coli and total coliform group bacteria are assumed to be an indicator of direct or indirect faecal contamination of foods [16]. Uysal et al. [17] did not identify coliform bacteria in Circassian cheese.

Analyzing the most abundant bacterial genera, the *Enterobacter* sp. (70%) was the prevalent with four bacterial species. *Escherichia* sp. (50%) was represented by one species – *E. coli*. Other genera were characterized with lower distribution and *Klebsiella* sp., *Citrobacter* sp., *Hafnia* sp. were isolated from 20% of investigated samples.

Table 2 Number of isolated microorganisms from smoked cheese in log cfu.g^{-1}

Number of samples	TCB	E	CB	Lactic acid bacteria	MFF
1.	2.58	0.00	0.00	1.12	1.12
2.	2.45	0.00	1.12	1.25	1.21
3.	2.25	0.00	0.00	1.52	1.31
4.	2.45	0.00	1.21	1.41	1.14
5.	2.45	0.00	0.00	1.25	1.15
6.	2.52	0.00	1.15	1.32	1.12
7.	2.45	0.00	1.16	1.12	1.21
8.	2.58	0.00	1.22	1.25	1.25
9.	2.58	0.00	1.32	1.52	1.21
10.	2.78	0.00	1.25	1.47	1.25
11.	2.89	0.00	0.00	2.12	1.52
12.	2.80	0.00	0.00	2.15	1.25
13.	2.92	0.00	0.00	2.14	1.23

TBC – total counts of bacteria, E – enterococci, CB – coliform bacteria, MFF – yeasts and fungi

Table 3 Isolated species of microorganisms from non-smoked cheese

Sample numbero.	Product	Microorganisms
1.	Parenica	*Enterobacter cloacae, Lactobacillus plantarum, Lactococcus lactis, Candida catenulata, Candida zeylanoides* *E. ludwigii, E. asburiae, Klebsiella oxytoca, Sphingomonas parapaucimobilis, Candida lusitaniae*
2.	Parenica	*Enterobacter cloacae, E. ludwigii, Kluyveromyces lactis* *Citrobacter braakii, Lactobacillus plantarum, Candida lusitaniae*
3.	Parenica	*Enterobacter asburiae, E. cloacae, E. kobei, Lactobacillus acidophilus, L. ultunensis, Candida catenulata*
4.	Parenica	*E. coli, Enterobacter cloacae, Lactobacillus acidophilus, L. ultunensis, Leuconostoc lactis, Leuconostoc mesenteroides, Kluyveromyces lactis, Sphingomonas parapaucimobilis, Candida lusitaniae*
5.	Parenica	*Enterobacter cloacae, E. ludwigii, E. asburiae, E. kobei, Citrobacter koseri, Lactobacillus casei, Lactobacillus coryniformis, Lactobacills fructivorans, Kluyveromyces lactis, Candida zeylanoides, Sphingomonas parapaucimobilis*
6.	Parenica	*Escherichia coli, Enterobacter cloacae, Lactobacillus acidophilus, Lactobacillus coryniformis, Leuconostoc mesenteroides, Candida catenulata,*
7.	Parenica	*Escherichia coli, Klebsiela pneumoniae, Kluyveromyces lactis, Candida zeylanoides*
8.	Parenica	*Escherichia coli, Lactobacillus plantarum, Lactobacills fructivorans, Leuconostoc mesenteroides, Kluyveromyces lactis, Candida zeylanoides,*
9.	Parenica	*Escherichia coli, Enterobacter cloacae, E. ludwigii, E. asburiae, E. kobei, Lactobacillus acidophilus, L. ultunensis, Kluyveromyces lactis, Sphingomonas parapaucimobilis*
10.	Parenica	*Hafnia alvei, Enterobacter cloacae, Lactobacillus acidophilus, L. ultunensis, Candida catenulata, Candida zeylanoides*
11.	Parenica	*Enterobacter asburiae, E. cloacae, E. kobei, Lactobacillus plantarum, Candida zeylanoides, Candida lusitaniae*
12.	Parenica	*Escherichia coli, Lactobacillus plantarum, Lactobacills fructivorans, Leuconostoc mesenteroides, Candida zeylanoides*
13.	Parenica	*Enterobacter asburiae, E. cloacae, E. kobei, Lactobacillus plantarum, Candida catenulata*

Coliforms are among the many groups of microorganisms that are normally present in raw milk, i.e., 96% of all bulk tank milk samples collected during a 2002 study in the U.S. were coliform-positive [19]. Reported coliform levels in raw milk vary greatly, with mean coliform counts for milk sampled in the U.S. ranging from 31 cfu.mL^{-1} [20] to 2,570 cfu.mL^{-1} [21]. Similar results have been reported by others [22-24]. Common coliform genera in raw milk included *Citrobacter*, *Enterobacter*, *Escherichia*, and *Klebsiella* that is similar to our findings [21]. Coliforms could be originated from a variety of sources in the dairy farm environment including water, plant materials, equipment, dirt, and fecal sources [24]. High levels of coliforms (e.g., >1,000 cfu/mL) in raw milk may indicate unsanitary practices on the farm, inadequate refrigeration, or the presence of coliform mastitis [21,23,25]. Additionally, certain management practices at the farm level, including milking machine wash failures, rate of cluster washes and rate of milking unit fall-off during milking also correlate to variations in levels of coliforms in raw milk [23].

Table 4 Isolated species of microorganisms from smoked cheese

Sample number	Product	Microorganisms
1.	Parenica	*Enterobacter cloacae,* *E. ludwigii, E. asburiae, Klebsiella oxytoca, Lactobacillus casei, Lactobacillus coryniformis, Lactobacills fructivorans, Torulaspora delbrueckii, Bacillus cereus*
2.	Parenica	*Enterobacter cloacae, E. ludwigii, Lactobacillus plantarum, Lactococcus lactis* *Citrobacter braakii, Saccharomyces cerevisiae*
3.	Parenica	*Enterobacter asburiae, E. cloacae, E. kobei, Lactobacillus plantarum, Lactobacills fructivorans, Leuconostoc mesenteroides, Torulaspora delbrueckii, Bacillus cereus*
4.	Parenica	*E. coli, Enterobacter cloacae, Lactobacillus acidophilus, L. ultunensis, Leuconostoc lactis, Leuconostoc mesenteroides*
5.	Parenica	*Enterobacter cloacae, E. ludwigii, E. asburiae, E. kobei, Citrobacter koseri, Lactobacillus acidophilus, Lactobacillus coryniformis, Leuconostoc mesenteroides, Saccharomyces cerevisiae, Candida famata, Bacillus cereus*
6.	Parenica	*Escherichia coli, Enterobacter cloacae, Lactobacillus plantarum, Lactobacills fructivorans, Leuconostoc mesenteroides, Torulaspora delbrueckii, Bacillus cereus*
7.	Parenica	*Escherichia coli, Klebsiela pneumoniae, Lactobacillus plantarum, Lactobacills fructivorans, Leuconostoc mesenteroides, Torulaspora delbrueckii, Candida famata*
8.	Parenica	*Escherichia coli, Lactobacillus casei, Lactobacillus coryniformis, Lactobacills fructivorans, Yarrowia lipolytica, Bacillus cereus*
9.	Parenica	*Escherichia coli, Enterobacter cloacae, E. ludwigii, E. asburiae, E. kobei, Lactobacillus plantarum, Lactobacills fructivorans, Leuconostoc mesenteroides, Saccharomyces cerevisiae, Candida famata, Bacillus cereus*
10.	Parenica	*Hafnia alvei, Enterobacter cloacae, Lactobacillus acidophilus, Lactobacillus coryniformis, Leuconostoc mesenteroides, Yarrowia lipolytica*
11.	Parenica	*Enterobacter cloacae, Lactobacillus casei, Lactobacillus coryniformis, Lactobacills fructivorans, Yarrowia lipolytica* *E. ludwigii, E. asburiae, Klebsiella oxytoca, Torulaspora delbrueckii, Candida famata, Bacillus cereus*
12.	Parenica	*Escherichia coli, Enterobacter cloacae, E. ludwigii, E. asburiae, E. kobei, Lactobacillus casei, Lactobacillus coryniformis, Yarrowia lipolytica Lactobacills fructivorans, Yarrowia lipolytica, Candida famata, Bacillus cereus*
13.	Parenica	*Enterobacter asburiae, E. cloacae, E. kobei, Lactobacillus plantarum, Lactobacills fructivorans, Leuconostoc mesenteroides, Yarrowia lipolytica, Bacillus cereus*

4. Conclusions

Our study revealed the high contamination of dairy products with microorganisms related to the producing hygiene. Standard hygienic procedures should be revised to ensure the qualitative and safe products to consumers. MALDI-TOF was effective in identification of microorganisms in dairy products and could be used for screening of microbiological quality and safety of dairy products.

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