Identification of Lactic Acid Bacteria in Milk and Milk Products with MALDI-TOF Mass Spectrometry

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Abstract
Recently, the Matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) has been developed as vigorous method for the identification of microorganisms. In this study, the culturable population of lactic acid bacteria (LAB) from nonpasteurized milk and milk products was identified using the MALDI-TOF MS. Altogether, 20 samples of Slovak milk and milk products were tested, including 10 samples of milk and 10 samples of dairy products. The LAB were cultured on MRS agar at 30 °C. Gram-positive and catalase-negative strains were subjected to identification by the MALDI-TOF MS profiling. MALDI-TOF MS identification allowed the identification of three genera belonging to the LAB - *Lactobacillus, Lactococcus* and *Leuconostoc*. Presence of *Lactobacillus* was found in 17, *Leuconostoc* in three and *Lactococcus* only in one sample. *Lactobacillus* spp. was the most abundant genus and comprised 84% of all strains were isolated. *Lactobacillus* was mostly represented with four species: *Lactobacillus plantarum*, *L. acidophilus*, *L. ultunensis*, *L. casei.* Our study shows that the MALDI-TOF MS could serve an important method in identidication of microorganisms for dairy industry needs.

Keywords: MALDI TOF MS Biotyper, *Lactobacillus*, milk and milk products

1. Introduction

Lactic acid bacteria (LAB) are Gram-positive, non-spore former, usually nonmotile, nonacid-fast, non-respiring rods or coccobacilli or cocci. LAB frequently arranged in chains, devoid of cytochromes, catalase-negative and facultatively anaerobic with possible growth in in microaerophilic as well as aerobic conditions. The optimum growth observed in slightly lower acidic condition (pH 5.5 – 6.0) but is often restricted at neutral alkaline condition (pH > 7.0 to 7.5). LAB are strictly fermentative and the with lactic acid as the major end product during sugar fermentation [3,4]. LAB have two different metabolic pathways for hexose fermentation, which are used for their classification. In homofermentative pathway, lactic acid (more than 85%) is the major end product whereas in heterofermentative pathway thelactic acid, ethanol/acetone and CO_2 are the terminal products [3,4]. *Lactobacillus* (*Lb. delbruckii* subsp. *bulgaricus*, *Lb. acidophilus*) *Lactococcus*, *Streptococcus*, *Enterococcus*, *Pediococcus* and *Aerococcus* belong to homofermentative while the *Leuconostoc*, *Lb. brevis*, *Lb. fermentum* and *Lb. reuteri* to heterofermentative LAB.
LAB are common in nature, were isolated from plant material and identified as a part of resident microflora of animals and humans. LAB are important microorganisms in biotechnology,

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including dairy production. LAB are used in milk fermentation processes to assign typical characteristics for dairy products as cheese or acidophilic milk. However, the wild strains of LAB bacteria could be involved in spoilage processes by producing of undesirable flavours and gas, altering the quality of dairy products. Identification of LAB can help to evaluate the composition of LAB and possible impact on dairy product quality and production [1,2].

Traditional microbiological methods usually are time consuming. The Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can be good alternative to provide the quick results for dairy industry needs. MALDI-TOF ensure the quick identification of bacterial isolates to the species or even sub-species level. This is an effective tools in comparison with time-consuming and expensive methods, including 16S rRNA gene sequencing and 16S-ARDRA, which require DNA extraction, amplification and electrophoretic separation [5,6]. MALDI-TOF MS allow to identify the mass-to-charge ratio (m/z) of chemical compounds of intact cells and cell extracts, which are ionized into charged molecules. This technology generates the mass of highly abundant proteins, including ribosomal proteins specific for each bacterial species. The identification relies on comparison of the mass spectrum of the tested isolate with those of strains in reference databases [6]. The reliability of the identification by MALDI-TOF MS was studied before and this was found to be comparable to that of genetic typing methods, including 16S rDNA sequencing [5,7,8,9].

For quality testing of milk and milk products the fast and reliable methods are needed, the aim of the present study was to identify the microflora of milk and milk with MALDI-TOF.

2. Materials and methods

Sampling

The study was conducted from November 2015 to September 2016. A total of ten raw cow and sheep milk samples were collected from lactating animals in middle Slovakia. Samples were collected using the sterilized sample bottles and brought to laboratory with icebox for microbiological investigation. Samples were kept in a refrigerator (4 ±1°C). For milk product testing, altogether 10 samples of cottage cheese and butter were collected. The samples included cottage cheese (n=5) and butter (n=5) from private farm.

Isolation of Lactic Acid Bacteria (LAB)

Primary dilution of sample (1:10) in 0.9% sterile saline solution was made for each sample. Then, the serial dilutions (10^{-2} to 10^{-4}) were performed and 1 mL of each dilution was plated onto MRS agar (de Man, Rogosa and Sharpe, Sigma-Aldrich, St. Louis, USA). . Inoculated plates were incubated for 72 h at 30 °C anaerobically and then the bacterial growth was evaluated. The colonies with morphology typical for LAB were selected for further confirmation with MALDI-TOF. Selected colonies were cultured overnight at 30 °C on MRS agar anaerobically and used for identification. Bacterial colonies only from countable plates (30 to 300 CFU) were selected.

Identification of bacteria with MALDI-TOF MS Biotyper

A sample for 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) ten 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

LAB counts in milk ranged from 3.11 to 4.67 log cfu.mL^{-1} (Table 1). Lactococci were the dominant lactic acid bacteria in raw milk. Their initial numbers ranged from 5 to 7 log CFU.mL^{-1}.

Table 1. Isolated LAB from milk (log cfu.mL^{-1})

Sample number	log cfu.mL^{-1}
1.	3.96
2.	3.62
3.	3.91
4.	4.40
5.	3.62
6.	4.60
7.	4.67
8.	3.11
9.	4.30
10.	4.45

Viable LAB count was from 2.5 x 10^{6} to 7.3 x 10^{6} CFU/ ml that is less than identified in our study [11].

LAB counts in milk product (Table 2) ranged from 0.00 to 4.60 log cfu.g^{-1}.

Table 2. Isolated LAB from milk product (log cfu.g^{-1})

Sample number	log cfu.g^{-1}
1.	0.00
2.	4.09
3.	4.60
4.	4.35
5.	3.95
6.	3.08
7.	3.52
8.	3.59
9.	3.53
10.	3.49

The differences between LAB counts in different mil products were reported previously [12-15]. LAB counts in milk products ranged between 4.78 and 9.68 log cfu.g^{-1} in different geographical areas and products. The differences between the products and our results may be linked to regional differences in production conditions and processing technology as well as to differences in the diet and farming conditions of dairy animals. In the Mediterranean Basin, the number of lactic acid bacteria ranged between 4 and 9 log cfu/g in many cheese varieties that produced traditionally from raw milk [12,13,14,15].

However, the total count of viable lactic bacteria in the present study was less than reported for rippened milk products. The counts of LAB should be at least 10^{6} cfu.mL^{-1} in the final product during the entire validity period that is significantly higher that reported in the present study [11].

Lactobacillus genus was the most often isolated from milk and milk products (Table. 3). *Lactobacillus plantarum* was isolated from 10 samples and the presence of the bacterim was indetified in samples No. 1, 4, 6, 10, 12, 14-18. *L. acidophilus* and *L. casei* were isolated from six and three samples, respcetively. *Lactobacillus ultunensis* was isolated only from goat products. Less frequently occurring bacteria were *Leuconostoc lactis* and *Lactococcus lactis*.

The genera *Lactobacillus*, *Pediococcus* and *Lactococcus* belong to the lactic acid bacteria (LAB) and the strains of these genera are frequently used on a large scale in the production of milk products, preservation of many foods as well as probiotics for human and animals [16,17].

Among the LAB, genus *Lactobacillus* sp. (84%) was isolated most frequently. *L. plantarum*, *L. acidophilus* 24% comprised 40%, *L. casei* and *L. ultunensis* 8% of the all isolates. *Leuconostoc* composed 12% but genus *Lactococcus* 4% of all LAB isolates (Table 4).

Table 3. Isolated LAB of milk and milk products with MALDI-TOF MS Biotyper

Sample number	Product	Microorganisms
1.	Cow milk	*Lactobacillus plantarum*
2.	Cow milk	*L. acidophilus*
3.	Cow milk	*Lactococcus lactis*
4.	Cow cottage cheese	*Lactobacillus plantarum*
5.	Cow milk	*Leuconostoc lactis*
6.	Sheep milk	*Lactobacillus plantarum*
7.	Goat milk	*Lactobacillus acidophilus, L. ultunensis, Leuconostoc lactis*
8.	Cow cottage cheese	*Lactobacillus casei*
9.	Sheep cheese	*Lactobacillus acidophilus*
10.	Cow cottage cheese	*Lactobacillus plantarum*
11.	Cow cottage cheese	*Lactobacillus acidophilus*
12.	Cow cottage cheese	*Lactobacillus plantarum*
13.	Cow cheese	*Leuconostoc lactis*
14.	Cow butter	*Lactobacilus plantarum*
15.	Cow butter	*Lactobacilus plantarum*
16.	Cow cheese	*Lactobacilus plantarum*
17.	Cow milk	*Lactobacilus plantarum, L. acidophilus*
18.	Cow milk	*Lactobacilus plantarum, L. acidophilus*
19.	Goat milk	*Lactobacillus casei, Lactobacillus ultunensis*
20.	Sheep milk	*Lactobacillus casei*

Table 4. Percentage of isolated LAB species within the samples

Species of bacteria	Number of samples	Percentage %
Lactobacillus plantarum	10/20	50
Lactobacillus acidophilus	6/20	30
Lactobacillus casei	3/20	15
Leuconostoc lactis	3/20	15
Lactobacillus ultunensis	2/20	10
Lactococcus lactis	1/20	5

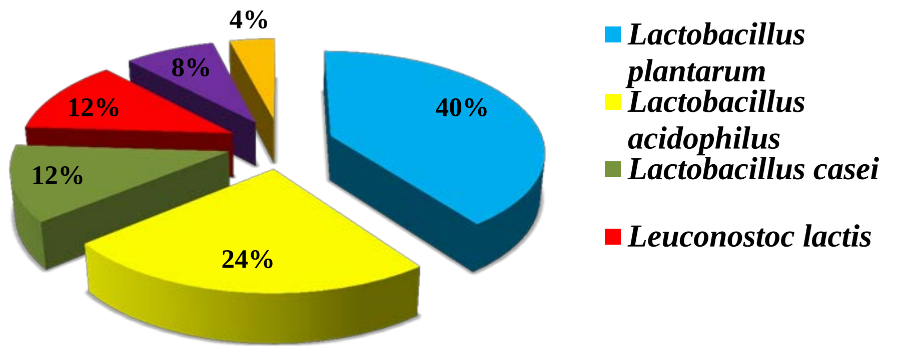

Figure 1. Percentage of isolated LAB species

Our study showed that MALDI-TOF could be an effective method in identification of LAB. The several advantages for the method was reported ranging from high-throughput, robustness, recognizing-based method, including ultrafast test results, ease of use and low cost per test. MALDI-TOF MS profiling can be easily implemented and applied in routine analysis [18] and the use of the method could be effective tool to enhance capacity of sample examinations by the phenotypic methods for fast identification and characterization of the culturable microorganisms in dairy products. Fast identification of microorganisms is important in food and dairy industry for improvement of hygiene and quality control in processing, controlling of microbial growth, reducing biocide consumption, to avoid the recall of contaminated products. While Despite the MALDI-TOF MS application are mostly limited to clinical microbiology because of speed and precision of examination, the industrial application of the method is very promising area. The MALDI-TOF MS based microbial identification is suggested to be fulfilling the gap between the reliability of classical microbiological method and genotypic identification systems providing the exact results. It is important to point out that the MALDI-TOF MS bacteria identification takes approximately 24 h shorter time than other classical methods and suitable for data management. As the new method, the MALDI-TOF MS has application limitations -. the dependence to database, the needs of global quality control program, the inability to identify non-viable microorganisms, the need of fresh cultures, challenging discrimination between phylogenetically closely-related strains. All those application limitations should be addressed in the future to improve the bacterial identification [19].

The present study showed composition of LAB im milk and milk products. Information about microbial populations, composition and their development is important for understanding the special properties and effect of these populations on milk and milk products. This information can be used for assessment of microbiological quality of milk and milk products [10].

4. Conclusions

Raw cow, goat and sheep milk and milk products are a source of LAB. Raw cow, goat and sheep milk and milk products were showed to containe both homofermentative and hetrofermentative LAB with Lactobacillus to be the predominating LAB genus both in milk and milk products. This Slovak raw milk and milk products could serve as source for beneficial lactic acid bacteria for consumers. Further studies should be done on the characterization and identification of the LAB species and their probiotic potential.

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