Rapid Identification of *Enterobacteriaceae* in Milk and Dairy Products with the Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS)

Miroslava Kačániová[1,2*], Margarita Terentjeva[3], Lucia Godočíková[1], Czeslaw Puchalski[2], Maciej Kluz[2], Rafal Kordiaka[2], Simona Kunová[1], Peter Haščík[1]

[1]*Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Nitra 949 76, Tr. A. Hlinku 2, Slovakia*
[2]*University of Rzeszow, Faculty of Biology and Agriculture,, 35-601 Rzeszow, Zelwerowicza St. 4, Poland*
[3]*Latvia University of Agriculture, Faculty of Veterinary Medicine, Institute of Food and Environmental Hygiene, LV-3004, Jelgava, K. Helmaņa iela 8, Latvia*

Abstract
Identification of microorganisms by MALDI-TOF MS Biotyper has been demonstrated to be accurate, rapid and lower cost than conventional food investigation methods. Rapid identification of pathogenic and spoilage microorganisms is crucial for dairy industry to ensure the quality and safety of milk and milk products. In this study, the bacterial species representing the *Enterobacteriaceae* family were identified in raw milk and milk products using the MALDI-TOF MS mass spectrometry. Altogether, 20 samples of Slovak milk and milk products were examined. Samples were cultured on VRBG agar at 37 °C for 24-48 h. Typical bacterial colonies were selected for identification with MALDI-TOF MS Biotyper. *Escherichia coli* and *Enterobacter* sp. were the most abundant *Enterobacteriaceae* family representatives identified. *E. coli* was found in nine and *Enterobacter* sp. in eight samples. *Enterobacter* sp. comprised 49 % and *Escherichia coli* 23 % of all bacterial isolates. The study shows that MALDI-TOF MS Biotyper was reliable identification method of *Enterobacteriaceae* in milk and dairy products. This method was helpful in evaluation of bacterial contamination of milk.

Keywords: mass spectrometry, *E. coli*, *Enterobacter*, milk, dairy products

1. Introduction

Enterobacteriaceae in food is considered to be an indicator of quality and safety for raw material and processed foods, especially with regard to good manufacturing and hygienic practices [1]. *Enterobacteriaceae* has been adopted by the European Union as an index of food safety and evaluation of processing hygiene replacing the detection of coliforms [2].

Enterobacteriaceae is a group of Gram-negative, facultative anaerobic, catalasa-positive and glucose-fermentating microorganisms, representatives of which primary imhabit the gastro-intestinal tract of animal of human. Some of the *Enterobacteriaceae* are well-known agent of human food-borne infections with gastro-intestinal manifestations. Bacteria that affect the gastro-intestinal tract include the *Escherichia coli*, *Salmonella*, *Shigella* and *Yersinia*. Alongside, other *Enterobacteriaceae* could serve as opportunistic pathogens causing infection in

* Corresponding author: Miroslava Kačániová, +421376414494, Email: miroslava.kacaniova@gmail.com

immunocompromised patients. These conditions include septicemia, pneumonia, meningitis and urinary tract infections. *Citrobacter, Enterobacter, Hafnia, Morganella, Providencia* and *Serratia* are an examples of opportunistic pathogens [3].

Nowadays, MALD-TOF is a powerful tool in microbiological diagnostic. Initially, MALDI-TOF MS technique was developed for industrial use to analyze and identify proteins. But two decades later was demonstrated that it could be useful for rapid identification of bacteria based on whole–cell spectral pattern [4,5]. But its capacity for routine microbial identification in clinical microbiology laboratory was revealed only after years [6-9]. This method is based on the analysis of microbial proteins, when the ionization process devoid the fragmentation by coordinated action of laser and small organic acids of the matrix. Different mass-to-charge ratios allow to separate them resulting in characteristic mass spectral profile. The identification of microorganisms is based on the comparison of the protein spectrum from whole microbial cells to the database specific reference protein profiles, indicating the bacterial species. Protein analysis by MALDI-TOF MS does not require the biochemical confirmation, thereby making the more rapid identification than traditional methods [8,9].

The purpose of the study was to identify the composition of *Enterobacteriaceae* in milk and milk products with the MALDI-TOF Biotyper method.

2. Materials and methods

Sample collection

A total of ten raw cows, goat sheep milk samples were collected from lactating cows, goats and sheep in middle Slovakia from November 2015 to September 2016. Additionally, ten milk products samples from private farms were collected. The milk product samples included cottage cheese (n=5) and butter (n=5). Samples were collected in sterilized sample bottles and brought to laboratory with icebox for microbiological investigation. Samples were kept in a refrigerator (4±1°C) untill the testing began. For analyses were collected 10 different samples of cow, goat and sheep milk and 10 different samples of cottage cheese and butter.

Isolation of *Enterobacteriaceae* from milk and milk products

The primary dilution of the milk and milk products were made by adding of 10 g of sample material to 90 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 onto violet red bile glucose agar (VRBGA, Sigma-Aldrich, St. Louis, USA). Inoculated plates were incubated for 24-48 h at 37 °C and then examined for the presence of typical colonies of *Enterobacteriaceae*. Suspected colonies were subcultured in tryptone soya agar (TSA, Oxoid) incubating at 37°C for 24 ± 4 h. Finally, the isolated *Enterobacteriaceae* from milk and milk products were identified with MALDI-TOF MS Biotyper.

Identification of *Enterobacteriaceae* with MALDI-TOF MS Biotyper

A sample for MALDI-TOF MS analysis was prepared according to the ethanol/formic acid extraction protocol recommended by the manufacturer (Bruker Daltonik, Bremen, Germany). A bacterial colony was suspended in 300 µL of water (Sigma-Aldrich, St. Louis, USA), and 900 µL of absolute ethanol (Bruker Daltonik, Bremen, Germany). Suspension was cetrifuged at 13000 rpm for 2 min and the supernatant was discarded. The pellet was mixed with 10 µL of 70% formic acid (v/v) (Sigma-Aldrich, USA) and identical volume of acetonitrile (Sigma-Aldrich, USA). The mixture was repeatedly centrifuged and 1 µL of the supernatant was spotted onto a polished steel target plate. 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) and air dried at room temperature. Mass spectra were automatically generated with microflex LT MALDI-TOF mass spectrometer (Bruker Daltonik, Germany) operated in the linear positive mode within a mass range of 2000-20000 Da. Calibration with Bruker bacterial test standard was done. Recorded mass spectra were processed with the MALDI Biotyper 3.0 software (Bruker Daltonik, Germany). The MALDI Biotyper output was a log score value in the range of 0-3.0 representing the probability of correct identification of the isolate. The identification criteria used were: a score of 2.300 to 3.000 indicated highly probable identification of species;

2.000 to 2.299 the secure genus with probable species identification; 1.700 to 1.999 identification to the genus level; <1.700 was unreliable identification.

3. Results and discussion

Enterobacteriaceae counts isolated from milk ranged from 0.00 to 4.34 log cfu.mL^{-1}, while from milk products were from 0.00 to 4.51 log cfu.g^{-1}. *Enterobacteriaceae* counts from milk and milk products are shown in Table 1 and 2.

Table 1. *Enterobacteriaceae* counts in milk (log cfu.mL^{-1})

Sample number	log cfu.mL^{-1}
1.	4.34
2.	3.94
3.	3.30
4.	2.30
5.	4.00
6.	3.92
7.	3.30
8.	2.00
9.	3.88
10.	0.00

Milk could become contaminated with *Enterobacteriaceae* from animal, farm environment, milking equipment and personnel. Overall contamination with *Enterobacteriaceae* indicates the hygienic performance at the farm. Consumption of milk contaminated with *Enterobacteriaceae* in the high level is unacceptable; therefore the quality of raw milk and milk products mainly did not meet the acceptable microbiological quality.

During ripening of the milk and milk products, the *Enterobacteriaceae* and total coliforms has a trend to decrease with becoming undetectable from the 60th ripening day [11-13]. Raw sheep milk and cheese was negative for E. coli, Enterobacteriaceae and total coliforms or show values lower than 1 log cfu.g^{-1} [11-13] that was significantly lower than in our study. Our study indicates the necessity for introduction of measures for improvement of milking and product processing hygiene in small private farms.

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

Sample number	log cfu.g^{-1}
1.	0.00
2.	0.00
3.	4.16
4.	3.28
5.	4.60
6.	2.00
7.	4.37
8.	4.51
9.	4.51
10.	4.37

Other studies reported the isolated of *Enterobacteriaceae* from milk with pathogenic microorganism were involved. *E. coli* was isolated from 14.2%, *Enterobacter* spp. 12.8%, *Shigella* spp. 20% and *Citrobacter* 21.4% of raw milk samples. The higher percentage of *E. coli* may be due to the fact that *E. coli* may grow in raw milk in high ambient temperatures in the absence of appropriate cooling system. The *Enterobacteriaceae* species isolated were in agreement with Kagkli [15] and Jayarao and Wang [16] who found the presence of *E. coli*, *Enterobacter*, *Klebsiella* spp. and *Citrobacter* as the major *Enterobacteriaceae* contaminants of milk. In should be stressed that the presence of *Enterobacteriaceae* resulting in unsatisfactory microbiological safety and was associated with lowering the quality of raw milk.

Moreoften *E. coli* was found in milk and milk products samples with nine samples (No. 6, 7, 9, 11, 13, 17, 18, 19 and 20) were positive. *Enterobacter cloacae*, were isolated from eight samples (No. 1, 2, 3, 5, 9, 15, 16 and 20). Other representatives of *Enterobacteriaceae* included *Klebsiella*, *Raoultella*, *Cronobacter*, *Citrobacter*, *Serratia* (Table 3, 4).

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

Sample number	Product	Microorganisms
1.	Cow milk	*Enterobacter cloacae, E. ludwigii, E. asburiae, Klebsiella oxytoca, Raoultella ornithinolytica*
2.	Cow milk	*Enterobacter cloacae, E. ludwigii, Cronobacter sakazakii,Citrobacter braakii, Raoultella ornithinolytica, R. planticola*
3.	Cow milk	*Enterobacter asburiae, E. cloacae, E. kobei*
4.	Cow cottage cheese	negative
5.	Cow milk	*Enterobacter cloacae, E. ludwigii, E. asburiae, E. kobei, Citrobacter koseri, Serratia liquefaciens*
6.	Sheep milk	*Escherichia coli*
7.	Goat milk	*Escherichia coli*
8.	Cow cottage cheese	negative
9.	Sheep cheese	*Escherichia coli, Enterobacter cloacae, E.ludwigii, E. asburiae, E. kobei*
10.	Cow cottage cheese	negative
11.	Cow cottage cheese	*Escherichia coli*
12.	Cow cottage cheese	negative
13.	Cow cheese	*Escherichia coli, Klebsiella pneumoniae*
14.	Cow butter	negative
15.	Cow butter	*Raoultella ornithinolytica, Klebsiella oxytoca, Enterobacter cloacae*
16.	Cow cheese	*Enterobacter cloacae*
17.	Cow milk	*Escherichia coli*
18.	Cow milk	*Escherichia coli*
19.	Goat milk	*Escherichia coli*
20.	Sheep milk	*Escherichia coli, Enterobacter cloacae*

Table 4. Percentage of isolated *Enterobacteriaceae* species within the samples

Species of bacteria	Number of samples	Percentage %
Escherichia coli	9/20	45
Enterobacter cloacae	8/20	40
Enterobacter ludwigii, E. asburiae	4/20	20
Enterobacter kobei	3/20	15
Raoultella ornithinolytica	3/20	15
Klebsiella oxytoca	2/20	10
Klebsiella pneumoniae	1/20	5
Raoultella planticola	1/20	5
Cronobacter sakazakii	1/20	5
Citrobacter braakii, C. koseri	1/20	5
Serratia liquefaciens	1/20	5

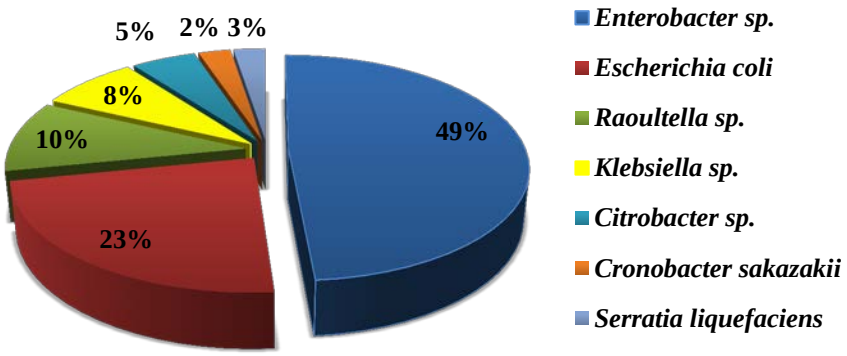

Figure 1. Distribution of isolated *Enterobacteriaceae* species

Analyzing the most abundant bacterial genera, the *Enterobacter* sp. (49%) was the prevalent with four bacterial species. *Escherichia* sp. (23%) was respresented by one species – *E. coli*. Other genera were characterized with lower distribution and *Raoultella* sp. from 10%, *Klebsiella* sp. from 8%, *Citrobacter* sp., *Cronobacter sakazakii* and *Serratia liquefaciens* from 5% of investigated samples (Table 4; Figure 1) .

Our results were in accordance with Ntuli et al., 2016 who found *E. coli, Raoultella ornithinolytica, K. oxytoca, E. cloacae, E. asburiae, Serratia liquefaciens,* and *Hafnia alvei* among the most abundant *Enterobacteriaceae* representatives [17]. *E. coli* was isolated from 36% (n = 258) of samples with mostly the raw milk samples (59%) were found *E. coli* positive. Only milk samples from 2 provinces were negative for *E. coli* with prevalence in pasteurized milk of 40%. [17]. Van Kessel et al. [20] reported that presence of *E. coli* in raw milk can be via intramammary secretion or via fecal contamination of udder or milking equipment, despite the contamination of pasteurized milk is mostly related to insufficient thermal treatment and problems related to good hygienic practice.

Since the *Enterobacteriaceae* cause the spoilage of milk occurring in high numbers in food products and are potential opportunistic pathogens, they presence should be eliminated [18, 19].

Rapid and precise detection of *Enterobacteriaceae* in milk are required for microbiological quality assurance of milk and milk products and for accessing the milk processing hygiene. Rapid and reliable methods identification of *Enterobacteriaceae* may help to replace the time-consuming confirmation step with more exact detection and enumeration of contaminations [10]. Better control and characterization of milk microflora with MALDI-TOF could be a solution for providing the better microbiological quality of raw milk and milk products.

4. Conclusions

Our study revealed the high contamination of milk and milk products with *Enterobacteriaceae* from farm producers. Standard hygienic procedures should be revised to ensure the qualitative and safe products to consumers. MALDI-TOF was effective in identification of *Enterobacteriaceae* in milk and milk products and could be used for screening of microbiological quality and safety of milk and milk products.

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