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Composition of raw cow's milk microbiota and identification of enterococci by MALDI-TOF MS - short communication

Vesna Dobranić[1], Snježana Kazazić[2], Ivana Filipović[1], Nataša Mikulec[3], and Nevijo Zdolec[1]*

[1]*Department of Hygiene, Technology and Food Safety, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia*

[2]*Laboratory for Chemical Kinetics and Atmospheric Chemistry, Division of Physical Chemistry, Ruđer Bošković Institute, Zagreb, Croatia*

[3]*Department of Dairy Science, Faculty of Agriculture, University of Zagreb, Zagreb, Croatia*

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ABSTRACT

Raw milk consumption could present a potential risk for public health due to the presence of foodborne pathogens and spoilage bacteria. In this research, microbiota or raw cow's milk was studied with particular reference to the presence of enterococcal opportunistic pathogens. Total viable count, psychrophilic bacteria, lactic acid bacteria, staphylococci, *Escherichia coli,* enterococci, enterobacteria, *Listeria* spp. and sulfite-reducing clostridia were evaluated using standard culture methods. Milk samples originated from healthy (n = 17) and antibiotic-treated cow's udders (n = 19). Presumptive enterococci (n = 43) were randomly selected from chromogenic media and subjected to phenotypic identification by API 20 Strep, followed by Matrix-Assisted Laser Desorption Ionization - Time of Flight Mass Spectometry (MALDI-TOF MS). Statistically significant differences ($P<0.05$) regarding the udder status were found in the total viable count and lactic acid bacteria. MALDI-TOF MS determination showed 100% concordance with API 20 Strep in the identification of *Enterococcus faecalis.* Strains determined to the genus level only, or non-determined strains by API 20 Strep were successfully identified using MALDI-TOF MS to the strain level as *E. faecalis, E. faecium* and *E. durans.* Results showed that MALDI-TOF MS could be applied as a reliable method in the routine identification of enterococcal species from raw milk.

Key words: milk, enterococci, MALDI-TOF MS

*Corresponding author:

Assist. Prof. Nevijo Zdolec, DVM, PhD, Department of Hygiene, Technology and Food Safety. Faculty of Veterinary Medicine, University of Zagreb, Heinzelova 55, Zagreb, Croatia, Phone: +385 1 2390 199, Fax: +385 1 2390 123; E-mail: nzdolec@vef.hr

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Introduction

The composition of raw milk microbiota depends on several factors, such as: udder health and farm management, milking hygiene and sanitation procedures, as well as storage conditions after milking. The main microbiological hazards for raw milk consumers are the pathogens *Salmonella* spp, *Campylobacter* spp., *Listeria monocytogenes* and *Escherichia coli* O157:H7 (TREMONTE et al., 2014). Alongside these pathogens, additional public-health hazards may involve the presence of antibiotic resistance in milk commensal or contaminating bacteria (ZDOLEC, 2016). Enterococci are examples of such microorganisms and are often considered as controversial bacteria within food safety (FRANZ et al., 1999). Namely, they may possess probiotic characteristics, such as strong antimicrobial activity by producing enterocins (BANWO et al., 2013; LAUKOVÁ, 2012). On the other hand, they are known as opportunistic pathogens in humans and animals, producers of biogenic amines in fermented food, or carriers of antimicrobial resistant genes (ZDOLEC, 2016; ZDOLEC et al., 2016). Raw milk and dairy products from unpasteurized milk are rich sources of enterococci, with potential beneficial or hazardous determinants. Many dairy-related enterococcal strains are characterized and intended for experimental application as starter or protective cultures in fermented food production (LEBOŠ PAVUNC et al., 2013). Their role in cow mastitis etiology is limited compared to other microbial pathogens, such as *Streptococcus agalactiae* or *Streptococcus uberis* (WYDER et al., 2011). However, primary milk production is one of the contamination routes involved in the spread of pathogenic enterococci through the food chain (QUIGLEY et al., 2013). Determination of milk microbiota is often very complex, because of the many influencing factors, including intrinsic (udder related) or extrinsic (environmental) factors (QUIGLEY et al., 2013). Traditional microbiological methods of isolation and identification are costly and time-consuming, with uncertain results of bacterial identification at the species level due to high biochemical (enzymatic) variability, especially in lactic acid bacteria. This is particularly evident in biochemical identification of enterococci, which include phenotypically and genotypically very similar species (WYDER et al., 2011). A very high discordance was observed using API tests in identification of enterococci from milk, compared to genetic methods (WYDER et al., 2011, GOMES et al., 2007). Genetic methods are reliable tools in epidemiological studies and scientific work, but hard to apply in routine laboratory work with food or clinical isolates. Matrix-Assisted Laser Desorption Ionization - Time of Flight Mass Spectometry (MALDI-TOF MS) represents a reliable, cost-effective and rapid identification technique for clinically relevant bacteria, including enterococci (BENAGLI et al., 2011). This technology identifies microorganisms through the generation of fingerprints of highly abundant proteins, followed by correlation to reference spectra in a database (PAVLOVIC et al., 2013). There are limited data on the implementation of this method in food-related enterococci identification (QUINTELA BALUJA et al., 2013; PAVLOVIC et al., 2013). In this respect, the aim of the present study

was to evaluate the applicability of MALDI-TOF MS for identification of presumptive enterococci from raw cow's milk, and compare it with traditional API identification.

Materials and methods

Milk sampling and microbiological analyses. Milk samples were aseptically taken into sterile microbiological tubes from dairy cows with healthy udders (n = 17) and cows with cured mastitis (n = 19), and transported in a portable cooler to the laboratory. One mL of the milk samples was decimally diluted in sterile salt peptone water to 10^{-8}. Appropriate dilutions were chosen and 0.1 mL or 1 mL was used for enumeration of the following bacteria: aerobic mesophilic bacteria, psychrophilic bacteria, lactic acid bacteria, staphylococci, *Escherichia coli,* enterococci, enterobacteria, *Listeria* spp. and sulfite-reducing clostridia. The methodology of microbiological analyses is presented in Table 1.

Table 1. Methodology of microbiological analyses of raw cow milk

Microorganisms	Media	Incubation conditions
Aerobic mesophylic bacteria	Plate Count Agar (Merck, Darmstadt, Germany)	30 °C, 72 hours
Psychrophilic bacteria	Plate Count Agar (Merck, Darmstadt, Germany)	6.5 °C, 10 days
Lactic acid bacteria	De Man, Rogosa, Sharpe Agar (Merck, Darmstadt, Germany)	30 °C, 48-72 hours, anaerobically
Staphylococci	Manitol Salt Phenol-red Agar (Merck, Darmstadt, Germany)	37 °C, 48 hours
Escherichia coli	Rapid *E. coli* 2 Agar (Bio-Rad, Marnes-la-Coquette, France)	44 °C, 24 hours
Enterococci	Compass *Enterococcus* agar (Biokar Diagnostics, France)	44 °C, 24 hours
Enterobacteria	Crystal-Violet Neutral-Red Bile Glucose Agar (Merck, Darmstadt, Germany)	37 °C, 24 hours
Listeria spp.	Listeria Selective Agar Base acc. Ottaviani and Agosti (Merck, Darmstadt, Germany)	37 °C, 24 - 48 hours
Sulfite-reducing clostridia	Iron Sulfite Agar (bioMérieux, Marcy l'Etoile, France)	37 °C, 24-48 hours, anaerobically

Identification of enterococci. Presumptive enterococci colonies (n = 43) were randomly selected from Compass *Enterococcus* agar (Biokar Diagnostics, France) and grown in Brain Hearth Infusion broth (BHI, bioMerieux, France) with 6.5% NaCl for 24 h at 37 °C. Cultures were streaked on Blood agar (Croatian Veterinary Institute), incubated in the same conditions and used in the API 20 Strep procedure, according to the manufacturer's instructions (BioMérieux, Marcy l'Etoile, France), and interpreted with Api*web*™V 1.2.1. software.

A sample for MALDI TOF MS analysis was prepared following the ethanol/formic acid extraction procedure recommended by the manufacturer (Bruker Daltonik, Bremen, Germany). A few colonies were suspended in 300 µL water (Sigma-Aldrich, St. Louis, USA), and 900 µL of absolute ethanol (Gram-mol, Zagreb, Croatia) was added and mixed with the cell suspension. After centrifugation at 13000 rpm for 2 min, the supernatant was discarded. The pellet was mixed with 10 µL 70% formic acid (v/v) (Sigma-Aldrich, USA) and an equal volume of acetonitrile (Sigma-Aldrich, USA) was added. The mixture was centrifuged at 13000 rpm for 2 min. 1 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) and air dried at room temperature. Mass spectra were automatically generated using 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 a Bruker bacterial test standard. Recorded mass spectra were processed with the MALDI Biotyper 3.0 software package (Bruker Daltonik, Germany), using standard settings. The MALDI Biotyper output is a log score value in the range 0-3.0, representing the probability of correct identification of the isolate, computed by comparison of the peak list for an unknown isolate with the reference spectrum in the database. The identification criteria used were as follows: a score of 2,300 to 3,000 indicated highly probable species level identification, a score of 2,000 to 2,299 indicated secure genus identification with probable species identification, a score 1,700 to 1,999 indicated probable identification to the genus level, and a score of <1,700 was considered unreliable.

Statistical analysis. Statistical analysis was performed using the commercial statistical software, STATISTICA version 12 (StatSoft.Inc., 2012). Comparison of bacterial counts between healthy cows and drug-treated cows was performed using the Mann-Whitney *U* test. The McNemar test was applied in describing the agreement of the two methods, API Strep and MALDI-TOF MS, in identification of enteroccoci. A P-value of <0.05 was considered significant.

Results and discussion

The results of the microbiological analyses of the milk samples are shown in Table 2.

Table 2. Bacterial counts (log_{10} cfu/mL) in raw milk according to udder health status

Microorganisms (log_{10}cfu/mL)	Healthy cows (n = 17)	Treated cows (n = 19)	Mann-Withney *U* P<0.05
Enterococci	<1 - 3.6	<1 - 5	0.692036
Enterobacteria	<1 - 3	<1 - 4.69	0.274298
Escherichia coli	<1 - 3.23	<1 - 3.3	0.861644
Staphylococci	<1 - 4.0	2 - 4	0.579215
Total viable count	3 - 3.77	3 - 5.39	0.011764
Psychrophilic bacteria	<2 - 3.95	<2 - 5.17	0.466116
Lactic acid bacteria	<2 - 3.65	<2 - 4.77	0.004800

Bacterial populations are presented in range values. Signifficantly higher lactic acid bacteria count and total viable count were observed in milk originated from drug-treated udders (P<0.05, Mann-Withney *U* test).

In general, higher average counts of monitored microbial populations were found in raw milk from previously drug-treated cows with cured mastitis. However, statistically significant differences (P<0.05) in bacterial counts in relation to the origin of the milk samples were found in total viable count and lactic acid bacteria count. Sulfite-reducing clostridia and *Listeria* spp. were absent in all tested samples. Having in mind that all the milk collected from drug-treated cows complied with current regulations (somatic cell count below 400.000/mL, total viable count below 100.000/mL, free of residues), it is important to consider some other microbiological factors which could have a negative impact on milk quality and safety. Thus, from our results it is clear that milk, originating from drug-treated cows with cured mastitis would have lower durability and processing value, primarily due to high lactic acid bacteria and psychrotrophic bacteria counts (SAMARŽIJA et al., 2012). Higher counts of fecal/environmental contaminants were also present, such as enterobacteria and enterococci. The sources of milk contamination may be numerous. In this respect VACHERYOU et al. (2011) identified 25 bacterial genera, both in the milk and throughout the dairy farm. However, technologically relevant genera, such as *Enterococcus, Lactobacillus, and Lactococcus,* were present in the milk but not in the farm environment. In general, enterococcal numbers in different foodstuffs can be very variable, even within the same batch. The same was true of our milk samples, since enterococci ranged, when present, from 1.3 to 5 log_{10}cfu/mL. The most common species isolated from raw milk are *Enterococcus faecalis* and *Enterococcus faecium* (QUIGLEY et al., 2013). Our results are in agreement with this finding, regardless of the method used, API 20 Strep or MALDI-TOF MS. WERNER et al. (2012) identified 64 *E. faecalis* and 37 *Enterococcus faecium* from untreated bovine mastitis samples by MALDI-TOF

Table 3. Biochemical profiles of enterococci isolated from milk determined by API 20 Strep

Parameter	*E. faecalis* (n = 24)	*E. faecium* (n = 5)	*Enteroococcus* spp. (n = 5)	Not identified (n = 8)	*Leuconostoc* spp. (n = 1)
Acetoin (Voges Proskauer)	24 (100)	5 (100)	5 (100)	8 (100)	1 (100)
Hipuric acid	0	0	0	0	0
Esculin hydrolase	22 (91.67)	5 (100)	5 (100)	6 (75)	0
Pyrolydonyl arylamidase	23 (95.83)	5 (100)	5 (100)	8 (100)	1 (100)
α-galactosidase	0	3 (60)	0	7 (87.5)	1 (100)
β-glucuronidase	0	0	0	0	0
β-galactosidase	0	3 (60)	0	0	0
Alkaline phosphatase	0	0	0	0	0
Leucine aminopeptidase	23 (95.83)	5 (100)	5 (100)	8 (100)	1 (100)
Arginin dihydrolase	24 (100)	5 (100)	5 (100)	8 (100)	1 (100)
Fermentation of					
D-ribose	24 (100)	5 (100)	5 (100)	8 (100)	1 (100)
L-arabinose	0	4 (80)	5 (100)	1 (12.5)	0
D-manitol	24 (100)	4 (80)	5 (100)	2 (25)	0
D-sorbitol	24 (100)	1 (20)	5 (100)	2 (25)	0
D-lactose	24 (100)	5 (100)	5 (100)	8 (100)	1 (100)
D-trehalose	24 (100)	5 (100)	5 (100)	8 (100)	0
inulin	0	3 (60)	0	0	0
D-raffinose	0	5 (100)	1 (20)	7 (87.5)	1 (100)
starch	24 (100)	5 (100)	5 (100)	2 (25)	0
Glycogen	1 (4.17)	3 (60)	3 (60)	1 (12.5)	0

The numbers indicate the positive strains in each parameter and in the brackets is the percentage of positives

MS, which is comparable with our results with respect to the proportions of these two species. Agreement of API 20 Strep and MALDI-TOF MS identification in our study was found for *Enterococcus faecalis* (n = 24). There was a statistical significant difference in enterococci identification between API Strep and MALDI-TOF MS (P = 0.008; Table 4).

Table 4. Agreement of API 20 Strep and MALDI-TOF MS identification of enterococci from raw milk

		MALDI-TOF MS		
		Enterococci; yes	Enterococci; no	Total
API 20 Strep	Enterococci; yes	34	0	34
	Enterococci; no	9	0	9
	Total	43	0	43

McNemar test: $\chi^2 = 7.1$; P = 0.008

Table 5. Non concordant results of API 20 Strep and MALDI-TOF MS identification of enterococci from raw milk

Strain	API 20 Strep	MALDI-TOF MS
B21	not identified	*E. faecium*
B22	not identified	*E. faecium*
B23	not identified	*E. faecium*
B24	not identified	*E. faecium*
B26	*Leuconostoc* spp.	*E. faecium*
B27	not identified	*E. faecalis*
B28	not identified	*E. faecium*
B29	*E. faecium*	*E. durans*
B102	*E. faecium*	*E. durans*
B103	not identified	*E. durans*
B142	*E. faecium*	*E. faecalis*
B144	*E. faecium*	*E. faecalis*
Z113	*Enterococcus* spp.	*E. durans*
Z114	*Enterococcus* spp.	*E. faecalis*
Z115	*Enterococcus* spp.	*E. faecalis*
Z116	*Enterococcus* spp.	*E. faecalis*
Z117	*Enterococcus* spp.	*E. durans*
Z146	not identified	*E. faecalis*

As presented in Table 5, the MALDI-TOF MS technique revealed additional identification of *E. faecalis, E. faecium* and *E. durans* strains, which had remained unidentified or identified only to the genus level (*Enterococcus* spp.) by the API test. In addition, four *E. faecium* strains identified by API 20 Strep were determined as *E. faecalis* and *E. durans* by MALDI-TOF MS. This could be in line with the results of WINSTON et al. (2004), who demonstrated that API 20 Strep may incorrectly speciate enterococci, i.e. *E. faecium*. A very low correlation was also reported between API 20 Strep and genetic methods in identifying food-related enterococci (GOMES et al., 2007).

In conclusion, the MALDI-TOF MS technique is a reliable method for identification of enterococci from milk samples, and could be a beneficial tool in quick diagnostics at both farm (mastitis) and retail level (e.g. raw milk vending machines).

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SAŽETAK

Konzumiranje sirovog mlijeka može predstavljati potencijalni rizik za potrošače uslijed prisutnosti patogenih bakterija i bakterija kvarenja. U ovom radu istražen je sastav mikroflore sirovog kravljeg mlijeka s posebnim naglaskom na prisutnost enterokoka. Određivan je ukupni broj bakterija, psihrofilnih bakterija, bakterija mliječne kiseline, stafilokoka, *Escherichia coli,* enterokoka, enterobakterija, *Listeria* spp. i sulfitreducirajućih klostridija primjenom standardnih mikrobioloških metoda. Mlijeko je potjecalo od zdravih krava (n = 17) i krava s liječenim mastitisom (n = 19). Kolonije enterokoka (n = 43) su nasumično odabrane sa selektivne podloge te podvrgnute fenotipskoj identifikaciji pomoću API 20 Strep, a potom MALDI-TOF masenom spektrometrijom. Statistički značajne razlike ($P<0,05$) s obzirom na zdravstveni status vimena zabilježene su u ukupnom broju bakterija i bakterija mliječne kiseline. Rezultati identifikacije izolata *Enterococcus faecalis* bili su u potpunom suglasju primjenom MALDI-TOF MS i API 20 Strep. MALDI-TOF MS tehnikom identificirani su izolati enterokoka koje API 20 Strep sustavom nije bilo moguće identificirati ili su bili identificirani do razine roda. Rezultati istraživanja pokazuju da se MALDI-TOF MS može smatrati pouzdanom metodom u rutinskoj identifikaciji enterokoka iz sirovog mlijeka.

Ključne riječi: mlijeko, enterokoki, MALDI-TOF MS