

Occurrence of enterococci in mastitic cow's milk and their antimicrobial resistance

Hanna Róžańska¹, Aleksandra Lewtak-Piłat²,
Maria Kubajka¹, Marcin Weiner¹

¹Department of Microbiology, ²Department of Hygiene of Food of Animal Origin,
National Veterinary Research Institute, 24-100 Puławy, Poland
bruna@piwet.pulawy.pl

Received: July 11, 2018

Accepted: February 21, 2019

Abstract

Introduction: The aim of the study was to evaluate the occurrence of enterococci in inflammatory secretions from mastitic bovine udders and to assess their antimicrobial resistance. **Material and Methods:** A total of 2,000 mastitic milk samples from cows were tested in 2014–2017. The isolation of enterococci was performed by precultivation in buffered peptone water, selective multiplication in a broth with sodium azide and cristal violet, and cultivation on Slanetz and Bartley agar. The identification of enterococci was carried out using Api rapid ID 32 strep kits. The antimicrobial susceptibility was evaluated using the MIC technique. **Results:** Enterococci were isolated from 426 samples (21.3%). *Enterococcus faecalis* was the predominant species (360 strains), followed by *E. faecium* (35 isolates), and small numbers of others. The highest level of resistance was observed to lincomycin, tetracycline, quinupristin/dalfopristin (Synercid), erythromycin, kanamycin, streptomycin, chloramphenicol, and tylosin. Single strains were resistant to vancomycin and ciprofloxacin. All isolates were sensitive to daptomycin. *E. faecalis* presented a higher level of resistance in comparison to *E. faecium*, except to nitrofurantoin. **Conclusion:** The results showed frequent occurrence of enterococci in mastitic cow's milk and confirmed the high rate of their antimicrobial resistance.

Keywords: cows, mastitis, *Enterococcus* spp., occurrence, antimicrobial resistance.

Introduction

Udder inflammations (mastitis) are the most frequent and cost-generating illness of dairy cows all over the world (1–3, 9, 17, 20, 21, 25, 26, 28, 29). Many different microorganisms can infect the mammary gland. Due to their different aetiology and epidemiology, contagious and environmental cases of mastitis are distinguished (1, 4, 9, 20, 26, 27). Streptococci are mainly responsible for contagious mastitis. Enterococci are one of the environmental causative agents of mastitis. These opportunistic bacteria are a part of normal physiological gut flora in humans and animals, but over last years they have become one of the main pathogens causing numerous infections in humans, mainly those hospital-acquired, such as bacteraemia and infections of the urinary tract, skin, soft tissue, abdomen and pelvis, and central nervous system. These infections are caused mainly by *E. faecalis* (about 80.0%) and *E. faecium* (10.0%–

15.0%) (3, 6, 14, 16, 19, 22, 31). The high tolerance of enterococci to disadvantageous conditions allows for their long survival in the environment, including in abattoirs. For this reason, potential infections of the mammary gland are easy and simple. Enterococci are characterised by a high level of resistance to many antibacterial substances, both by intrinsic and acquired mechanisms. Due to their ability to acquire and transfer resistance-determining genes to other bacteria, they are perceived as a good indicator of antimicrobial resistance in the environment (15, 20, 22). Another matter of concern is the possibility of the transmission of enterococci from the inflamed udder to humans. The growing consumption of raw, unpasteurised milk and products produced from this milk seems to indicate that there is a possibility of transfer of potentially pathogenic and antimicrobial-resistant enterococci to humans *via* the food chain. (1, 3, 6, 8, 13, 15, 22, 24, 28, 31, 32). There are no sufficient data from Poland about the occurrence of *Enterococcus* spp. in mastitic

cow's milk or about their susceptibility to antimicrobials. For this reason, this investigation was undertaken to evaluate the occurrence of enterococci in milk of cows with clinical and subclinical mastitis and to assess their antimicrobial resistance.

Material and Methods

In total, 2,000 milk samples were taken by physicians from individual cows suspected of mastitis. The samples were collected in different parts of Poland and were sent to our laboratory under temperature-controlled conditions. The samples were frozen until analysis. To isolate enterococci, 1 ml of the sample was incubated in buffered peptone water overnight at $37 \pm 1^\circ\text{C}$. Then, 100 μL of the culture was transferred into a broth with sodium azide and crystal violet (azide dextrose broth supplemented with 1.6% bromocresol purple and 1.3 mL/L crystal violet, Merck, Germany). After incubation (24 h at 37°C), one loop (10 μL) of the culture was spread on the surface of Slanetz and Bartley agar (Oxoid, UK) and incubated overnight at 37°C . The suspected colonies were identified using the API rapid ID 32 STREP kit (bioMérieux, France) following the manufacturer's instructions. The antimicrobial resistance of the confirmed *Enterococcus* isolates was evaluated using the minimum inhibitory concentration (MIC) technique (in Sensititre CMV3AGPF NARMS Plates, Trek Diagnostic Systems, UK) with decreasing levels of tigecycline (TGC), tetracycline (TET), chloramphenicol (CHL), daptomycin (DAP), streptomycin (STR), tylosin tartrate (TYLT), quinupristin/dalfopristin (SYN), linezolid (LZD), nitrofurantoin (NIT), penicillin (PEN), kanamycin (KAN), erythromycin (ERY), ciprofloxacin (CIP), vancomycin (VAN), lincomycin (LIN), and

gentamycin (GEN). All investigations were performed with a Sensititre Autoreader device (Trek, UK). The reference *E. faecalis* ATCC 29212 strain was used as a control. The results were interpreted automatically, according to CLSI standards. For comparison of the resistance to each substance possessed by *E. faecalis* and *E. faecium*, the Z test for two independent proportions was used. For trends evaluation, the mobile means method was applied.

Results

Enterococcus spp. bacteria were isolated from 426 samples (21.3%). In this number, 360 isolates were identified as *Enterococcus faecalis* and 35 as *E. faecium*. A small number of isolated specimens were identified as *E. hirae*, *E. casseliflavus*, *E. durans*, and *E. avium*. They were not included in the result analysis. The results of antimicrobial resistance of the isolated *Enterococcus* spp., *E. faecalis*, and *E. faecium* strains are summarised in Table 1 and Fig. 1. The highest *Enterococcus* spp. resistance was to lincomycin (82.16%), followed by tetracycline (61.5%), Synercid (60.8%), erythromycin (48.83%), kanamycin (47.42%), streptomycin (46.48%), chloramphenicol (44.83%), and tylosin (42.49%). The lowest rates of resistance were to vancomycin and ciprofloxacin (0.94 and 0.47%, respectively). All strains were sensitive to daptomycin. As shown in Table 1 and Fig. 1, significant differences in resistance between *E. faecalis* and *E. faecium* were observed, especially to chloramphenicol, erythromycin, nitrofurantoin, quinupristin/dalfopristin (Synercid), streptomycin, and tetracycline. In total, the isolates of *E. faecium* presented lower resistance than *E. faecalis*, except to nitrofurantoin.

Table 1. Antimicrobial resistance of enterococci isolated from mastitic cow's milk (n = 2,000)

Antimicrobials	Number/% of resistant strains		
	<i>Enterococcus</i> spp. (n = 426)	<i>Enterococcus faecalis</i> (n = 360)	<i>Enterococcus faecium</i> (n = 35)
Chloramphenicol (CHL)	191/44.84	173/49.43	7/20.0
Ciprofloxacin (CIP)	2/0.47	2/0.57	1/2.86
Daptomycin (DAP)	0	0	0
Erythromycin (ERY)	208/48.83	177/50.57	11/31.43
Gentamicin (GEN)	47/11.03	31/8.86	2/5.71
Kanamycin (KAN)	202/47.42	196/56.0	15/42.86
Linkomycin (LIN)	350/82.16	294/84.0	25/71.43
Linezolid (LZD)	15/3.52	14/4.0	1/2.86
Nitrofurantoin (NIT)	27/6.34	17/4.86	13/37.14
Penicillin (PEN)	11/2.58	12/3.43	2/5.71
Quinupristin/dalfopristin (SYN)	259/60.8	296/84.57	15/42.86
Streptomycin (STR)	198/46.48	167/47.71	10/28.57
Tetracycline (TET)	262/61.5	287/82.0	11/31.43
Tigecycline (TGC)	5/1.17	4/1.14	0
Tylosin (TYLT)	181/42.49	170/48.57	11/31.43
Vancomycin (VAN)	4/0.94	3/0.86	1/2.86

The antimicrobial resistance trends of *E. faecalis* isolated in 2014–2017 are shown in Table 2 and Fig. 2. Because of the low number of *E. faecium* isolates, the trends for this species were not analysed. The presented data indicate some differences between the years, especially for chloramphenicol, erythromycin,

lincomycin, streptomycin, tetracycline, and tylosin, for which a decrease in resistance was observed. On the basis of the results, 53 resistance profiles were established. In total, 193 *E. faecalis* strains (45.31%) were resistant to at least three substances from three different groups (multiresistant strains).

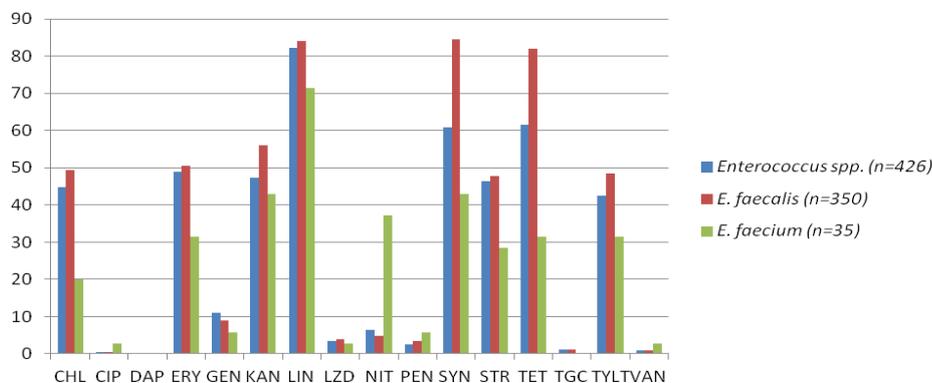


Fig. 1. Comparison of the antimicrobial resistance of enterococci isolated from mastitic milk samples. For antibiotic abbreviations see Table 1

Table 2. Trends in antimicrobial resistance of *Enterococcus faecalis* in 2014–2017

Antimicrobials	Number/% of resistant strains				
	Total (n = 360)	2014 (n = 127)	2015 (n = 64)	2016 (n = 90)	2017 (n = 79)
Chloramphenicol (CHL)	173/49.43	89/70.08	27/42.19	31/34.44	26/32.91
Ciprofloxacin (CIP)	2/0.57	0	0	1/1.11	1/1.27
Daptomycin (DAP)	0	0	0	0	0
Erythromycin (ERY)	177/50.57	92/72.44	29/45.31	29/32.22	27/34.18
Gentamicin (GEN)	31/8.86	4/3.15	8/12.5	10/11.11	9/11.39
Kanamycin (KAN)	196/56.0	90/70.87	35/54.69	37/41.11	34/43.04
Linkomycin (LIN)	294/84.0	116/91.34	44/68.75	71/78.89	63/79.75
Linezolid (LZD)	14/4.0	2/1.57	8/12.5	3/3.33	1/1.27
Nitrofurantoin (NIT)	17/4.86	2/1.57	8/12.5	4/4.44	3/3.8
Penicillin (PEN)	12/3.43	6/4.72	1/1.56	2/2.22	3/3.8
Qunupristin/dalfopristin (SYN)	296/84.57	106/83.46	42/65.63	81/90.0	67/84.81
Streptomycin (STR)	167/47.71	89/70.08	19/29.69	31/34.44	28/35.44
Tetracycline (TET)	287/82.0	111/87.4	42/65.63	71/78.89	63/79.75
Tigecycline (TGC)	4/1.14	2/1.57	1/1.56	1/1.11	0
Tylosin (TYLT)	170/48.57	82/64.57	27/42.19	29/32.22	32/40.51
Vancomycin (VAN)	3/0.86	2/1.57	0	1/1.11	0

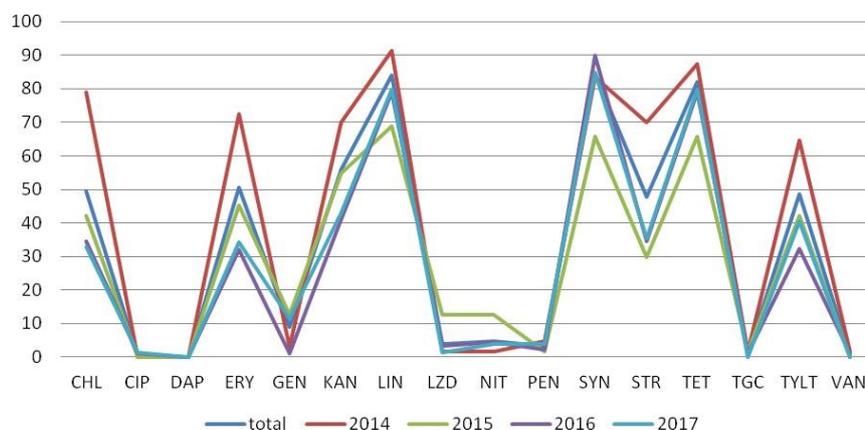


Fig. 2. Trends in antibacterial resistance of *E. faecalis* in 2014–2017. For antibiotic abbreviations see Table 2

Discussion

Opinions concerning the occurrence of enterococci in mastitic cow's milk vary. Some authors indicate a rare occurrence of enterococci in mastitic milk samples. Petersson-Wolfe *et al.* (28) showed 0.3% to 1.3% prevalence in milk samples from cows with subclinical mastitis in Sweden. Botrel *et al.* (2) isolated these bacteria in 2.4% of milk samples from cows with clinical mastitis and 3.1% in subclinical cases in France. Similar findings were reported by Gürler *et al.* (15) who isolated *Enterococcus* spp. in 3.26% of milk samples from subclinically mastitic cows in Turkey. Concurring results were described by other authors in Sudan (11), Slovakia (18), and Poland (24). In contrast, Kuyucouglu *et al.* (23) isolated enterococci from 10.9% of mastitic milk samples in Turkey. Our results are much more similar to those reported by three groups: Cameron *et al.* (5) who recovered enterococci from 15.25% of samples tested in Canada; Cervinkova *et al.* (7) who isolated these bacteria in 16.1% of samples in the Czech Republic; and Kateete *et al.* (21) who isolated enterococci in 19.5% of samples in Uganda. According to Hamzah *et al.* (17), enterococci were present in 60.0% of mastitic milk samples collected in Iraq. The cited data showed enterococci diversity in mastitic milk samples depending on geographical region and animal production specifics in different countries. In our experiments, *E. faecalis* was the predominant species, similar to the results presented by Cameron *et al.* (5), Kuyucouglu *et al.* (23), and Hamzah *et al.* (17). In studies performed by Kateete *et al.* (21), *E. faecium* was most often isolated, whereas Klimiene *et al.* (22) pointed to *E. durans* as the predominant strain in Lithuania.

Enterococci exploit several mechanisms of intrinsic and acquired resistance to antimicrobials. The intrinsic resistance concerns β -lactams, cephalosporins, clindamycin, and low concentrations of aminoglycosides, whereas the acquired one reduces susceptibility to tetracyclines, ciprofloxacin, erythromycin, linezolid, daptomycin, quinupristin/dalfopristin, and vancomycin (8, 15). This regularity, described by many authors, seems to be partially confirmed in our results. In general, almost all authors indicated a high level of antimicrobial resistance of enterococci; however, this level differs by species, substance, and country. The enterococci isolated from mastitic milk samples in Poland resisted these therapeutics to a greater extent than those from other countries.

According to the data presented in Table 1 and Fig.1, *E. faecalis* demonstrates a higher rate of resistance than *E. faecium*, except as it resists nitrofurantoin (4.86% and 37.14%, respectively). The data presented by other authors are not clear. According to Klimiene *et al.* (22), *E. faecium* showed higher resistance to β -lactams than *E. faecalis*. El Zubeir *et al.* (11) noted the wide range of resistance

of enterococci to antimicrobials, especially to enrofloxacin, kanamycin, gentamicin, lincomycin, and oxacillin. According to Erbas *et al.* (12), 1.1% of the isolates in Turkey showed resistance to vancomycin, 10.7% to chloramphenicol, 28.7% to erythromycin, and 81.1% to tetracycline. Fabianova *et al.* (13) noted 4.5% of strains resistant to vancomycin, 2.1% to teicoplanin, 13.0% to erythromycin, 39.1% to gentamicin, 45.6% to tetracycline, and 93.5% to ampicillin. There are no data to which to compare the trends in antimicrobial resistance of enterococci in the analysed period (2014–2017). In our opinion, the obtained results (Table 2, Fig. 2) should only be treated as tentative because the samples were not taken in the same places each year. The frequent occurrence of antimicrobial-resistant enterococci in mastitic milk samples indicates a potential possibility of transfer of these bacteria to humans, especially when consuming unpasteurised milk because these bacteria are also isolated from healthy cows prior to exhibition of mastitis symptoms (7). Similar markers of pathogenicity and resistance were detected in strains isolated from food of animal origin and from humans (6, 8, 10, 11, 13). Our results confirmed the frequent presence of the resistant enterococci in mastitic milk, which can be considered as a potential risk to humans.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: This scientific work was financially supported by the National Veterinary Research Institute in Puławy.

Animal Rights Statement: The authors declare that the experiments on animals were conducted in accordance with the local Ethical laws and regulations as regards care and use of laboratory animals.

References

1. Barlow J.: Mastitis therapy and antimicrobial susceptibility: a multispecies review with a focus on antibiotic treatment of mastitis in dairy cattle. *J Mammary Gland Biol Neoplasia* 2011, 16, 383–407.
2. Botrel M.-A., Haenni M., Morignat E., Sulpice Ph., Madec J.-Y., Calavas D.: Distribution and antimicrobial resistance of clinical and subclinical mastitis pathogens in dairy cows in Rhône-Alpes, France. *Foodborne Path Dis* 2010, 7, 479–485.
3. Bradley A.J.: Bovine mastitis: an evolving disease. *Vet J* 2002, 164, 116–128.
4. Burmańczuk A., Kowalski C., Roliński Z., Zań R., Krasucka D.: Activity of β -lactam antibiotics against certain microorganisms which cause mastitis in cows. *J Vet Res* 2016, 60, 267–271.
5. Cameron M., Saab M., Heider L., McClure J.T., Rodrigues-Lecompte J.C., Sanchez J.: Antimicrobial susceptibility patterns of environmental streptococci recovered from bovine milk samples in the Maritime province of Canada. *Front Vet Sci* 2016, 3, art. 79. Doi: 10.3389/fvets.2016.00079.

6. Cariolato D., Andrighetto C., Lombardi A.: Occurrence of virulence factors and antibiotic resistances in *Enterococcus faecalis* and *Enterococcus faecium* collected from dairy and human samples in North Italy. *Food Control*, 2008, 19, 886–892.
7. Cervinkova D., Vlkova H., Borodacova I., Makovcova J., Babak V., Lorencova A., Vrtkova I., Marosevic D., Jaglic Z.: Prevalence of mastitis pathogens in milk from clinically healthy cows. *Vet Med* 2013, 58, 567–575.
8. Chajęcka-Wierzchowska W., Zadernowska A., Łaniewska-Trokenheim Ł.: Antibiotic resistance of *Enterococcus* strains present in food. *Kosmos* 2017, 314, 1, 67–79.
9. Ebrahimi A., Nikookhah F., Nikpour S., Majiiian F., Gholami M.: Isolation of streptococci from milk samples of normal, acute, and subclinical mastitis cows and determination of their antibiotic susceptibility patterns. *Pak J Biol Sci* 2008, 11, 148–150.
10. Economou V., Gousia P.: Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infect Drug Resist* 2015, 8, 49–61.
11. El Zubeir I.E.M., Kutzer P., El Owni O.A.O.: Frequencies and antibiotic susceptibility patterns of bacteria causing mastitis among cows and their environment in Khartoum State. *Res J Microbiol* 2006, 1, 101–109.
12. Erbas G., Parin U., Turkyilmaz S., Ucan N., Oztruk M., Kaya O.: Distribution of antibiotic resistance genes in *Enterococcus* spp. isolated from mastitis bovine milk. *Acta Vet Beograd* 2016, 66, 336–346.
13. Fabianová J., Ducková V., Čročko M.: Presence of enterococci in cow milk and their antibiotic resistance. *Potravinárstvo* 2010, 4, 17–21.
14. Galal Abdel Hammeed K., Sender G., Korwin-Kossakowska A.: Public health hazard due to mastitis in dairy cows. *Anim Sci Pap Rec* 2006, 25, 2, 73–85.
15. Gürler H., Findik A., Gültiken N., Ay S.S., Çiftçi A., Koldaş E., Arslan S., Findik M.: Investigation on the etiology of subclinical mastitis in jersey and hybrid-jersey dairy cows. *Acta Vet Beograd*, 2015, 65, 358–370.
16. Hammerum A.M., Lester C.H., Heuer O.E.: Antimicrobial-resistant enterococci in animals and meat: a human health hazard? *Foodborne Path Dis* 2010, 7, 1137–1148.
17. Hamzah A.M., Kadim H.K.: Isolation and identification of *Enterococcus faecalis* from cow milk samples and vaginal swab from human. *Entomol Zool Sci* 2018, 6, 218–222.
18. Idriss S.H.E., Foltys V., Tančin V., Kirchnerová K., Tančinová D., Zaujec K.: Mastitis pathogens and their resistance against antimicrobial agents in dairy cows in Nitra, Slovakia. *Slovak J Anim Sci* 2014, 47, 33–38.
19. Jackson C.R., Lombard J.E., Dargatz D.A., Fedorka-Cray P.J.: Prevalence and antimicrobial resistance of enterococci isolated from US dairy cattle. *Lett Appl Microbiol* 2010, 52, 41–48.
20. Kagkli D.M., Vancanneyt M., Vandamme P., Hill C., Cogan T.M.: Contamination of milk by enterococci and coliforms from bovine faeces. *J Appl Microbiol* 2007, 103, 1393–1405.
21. Kateete D.P., Kabugo U., Baluku H., Nyakarahuka L., Kyobe S., Okee M., Najjuka Ch.F., Jaloba M.L.: Prevalence of antimicrobial susceptibility patterns of bacteria from milkmen and cows with clinical mastitis in and around Kampala, Uganda. *PLOS ONE*, 2013, 8, 5, e63413.
22. Klimienė I., Ružauskas M., Špakauskas V., Mockeliūnas R., Pereckienė A., Butrimaitė-Ambrozevičienė Č.: Prevalence of Gram-positive bacteria in cow mastitis and their susceptibility to beta-lactam antibiotics. *Vet Med. Zoot* 2011, 56, 65–72.
23. Kuyucouğlu Y.: Antibiotic resistances of enterococci isolated from bovine subclinical mastitis. *Eurasian J Vet Sci* 2011, 27, 231–234.
24. Malinowski E., Lassa H., Kłossowska A., Smulski S., Markiewicz H., Kaczmarowski M.: Etiological agents of dairy cows' mastitis in western part of Poland. *Pol J Vet Sci* 2006, 9, 191–194.
25. Oliver S.P., Murinda S.E.: Antimicrobial resistance of mastitis pathogens. *Vet Clin North Am Food Anim Pract* 2012, 28, 165–185.
26. Overesch G., Stephan R., Perreten V.: Antimicrobial susceptibility of gram-positive udder pathogens from bovine mastitis milk in Switzerland. *Schw Arch Tier* 2013, 155, 339–350.
27. Persson Y., Nyman A.K., Grönlund-Andersson U.: Etiology and antimicrobial susceptibility of udder pathogens from cases of subclinical mastitis in dairy cows in Sweden. *Acta Vet Scand* 2011, 53, 36 <http://www.actavetscand.com/content/53/1/36>.
28. Petersson-Wolfe C.S., Currin J.: Environmental streptococci and *Enterococcus* spp.: a practical summary for controlling mastitis. 2012, www.ext.vt.edu.
29. Rocha B., Mendonca D., Niza-Ribeiro J.: Trends in antibacterial resistance of major bovine mastitis pathogens in Portugal. *RCPV* 2014, 109, 79–88.
30. Rügsegger F., Ruf J., Tschour A., Sigrist Y., Rooskopf M., Hässig M.: Antimicrobial susceptibility of mastitis pathogens of dairy cows in Switzerland. *Schw Arch Tier* 2014, 156, 483–488.
31. Xiaohu W.U., Zhang Q., Ma Y., Zhang Y., Kan W., Zhao X.: Prevalence of virulence genes and resistance to antibiotics in pathogenic enterococci isolated from mastitic cows. *J Vet Med. Sci* 2016, 78, 1663–1683.
32. Zdolec N., Dobranić V., Butković I., Koturić A., Filipović I., Medvid V.: Antimicrobial susceptibility of milk bacteria from healthy and drug-treated cow udder. *Vet Archiv* 2016, 86, 163–172.